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ANDROGEN SIGNALING AXIS AS DRUG TARGET IN CASTRATION-RESISTANT PROSTATE CANCER:

Preclinical development of novel treatments and models

Riikka Oksala



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to my family

ABSTRACT

Riikka Oksala

Androgen signaling axis as a drug target in the castration-resistant prostate cancer: Preclinical development of novel treatments and models

University of Turku, Faculty of Medicine, Institute of Biomedicine, Physiology, Drug Research Doctoral Programme (DRPD)

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Prostate cancer is a major global challenge due to the increasing number of aging population and frequency of diagnosis. Already in 1941, Huggins and Hodges showed that metastatic prostate cancer is responsive to androgens. This important observation has led to new therapies, androgen-deprivation therapy being the standard of care for men with metastatic disease. During the past decade new treatments have been primarily targeted to the androgen signaling axis either by 1) inhibition of androgen production in the adrenal glands and in the cancerous tissue, or 2) blocking the binding of androgens to androgen receptor (AR) in cells and inhibiting AR nuclear translocation. Despite this remarkable progress, the disease is still incurable and there is urgent need for better, more effective treatment strategies.

This study is a part of the nonclinical development and characterization of novel therapies for castration-resistant prostate cancer (CRPC). Darolutamide (ODM-201), currently being in phase III studies, is a structurally different, novel, oral, high-affinity second-generation AR antagonist, which showed a minimal blood-brain barrier penetration in our nonclinical models, and therefore it may have a better safety profile compared to other second-generation antiandrogens. Based on our *in vitro* studies, darolutamide is a very potent AR antagonist and also showed antagonism in all studied AR mutations, including the AR(F877L) mutation where the clinical reference compound, enzalutamide, showed agonism. Another investigational compound, ODM-204, is a non-steroidal, orally active inhibitor of CYP17A1 enzyme and AR antagonist. We showed that the both compounds had significant tumor growth inhibition in the CRPC xenograft models. In addition, ODM-204 demonstrated potent inhibition in CYP17A1 enzyme both *in vitro* in cell based assay and isolated microsomes, and *in vivo* in rodent and monkey efficacy models. In the nonclinical pharmacokinetic studies both compounds showed favorable profiles, therefore supporting further studies in CRPC patients.

It is well known that in CRPC overexpressed AR allows multiple ligands to work as an agonist, including several steroids secreted from adrenal glands. As mice adrenal glands, in contrast to human, have not been shown to synthesize androgens, the relevance of mice xenograft models in studying CRPC has been questioned. As part of this study, we showed that mouse adrenals produce significant amounts of steroids that contribute to the AR-dependent growth of CRPC xenograft tumor model. This study therefore provides novel data from nonclinical development of new drug candidates for CRPC, and translational research tools and information for further drug development.

Keywords: castration-resistant prostate cancer, androgens, xenograft

TIIVISTELMÄ

Riikka Oksala

Androgeenisignaloinnin säätely lääkekehityskohteena kastroatioresistentissä eturauhassyövässä: uusien hoitojen ja mallien prekliininen kehitys

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia, Lääketutkimuksen tohtoriohjelma (DRPD)

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Eturauhassyöpä on maailmanlaajuinen ongelma johtuen väestön ikääntymisestä ja lisääntyvistä diagnooseista. Jo 1941 Huggins ja Hodges osoittivat, etäpesäkkeisen eturauhassyövän olevan riippuvainen mieshormoneista eli androgeeneista. Tämä merkittävä havainto on johtanut lukuisien uusien hoitojen kehittämiseen ja androgeenien tuotannon esto on ollut levinneen taudin keskeinen hoito jo pitkään. Viimeisen vuosikymmenen aikana on hoidot ovat kehittyneet merkittävästi. Ensisijaisesti ne ovat keskittyneet 1) androgeenien tuotannon estämiseen lisämunuaisissa ja syöpäkudoksessa, tai 2) androgeenien sitoutumisen estämiseen androgeenireseptoreihin ja rajoittamaan reseptorien kulkeutumista tumaan. Uusista lääkkeistä huolimatta, sairaus on yhä parantumaton ja uusia tehokkaampia hoitomuotoja tarvitaan.

Tämä tutkimus on osa uusien hoitojen prekliinistä kehitystä kastroatioresistenttiin eturauhassyöpään. Jo Faasi III kliinisiin tutkimuksiin edennyt darolutamide (ODM-201) on uudenlainen, suun kautta otettava korkea-affiniteettinen toisen polven antiandrogeeni, joka ei prekliinisissä malleissa ole läpäissyt veri-aivoestettä ja siksi se saattaa olla muita toisen polven antiandrogeenejä turvallisempi. *In vitro* tulostemme perusteella darolutamide on tehokas AR-antagonisti kaikissa tutkimissamme AR mutaatioissa, myös AR(F877L) mutaatioissa, jossa kliininen vertailuyhdiste entsalutamidi osoitti agonismia. ODM-204 on uusi kokeellinen, ei-steroidaalinen, oraallinen lääkeaine, joka estää CYP17A1-entsyymiä ja toimii AR-antagonistina. Tutkimuksissamme molemmilla yhdisteillä havaittiin merkittävää tuumorin kasvun estoa kastroatioresistentin eturauhassyövän eläinmalleissa. Lisäksi ODM-204 osoitti tehokasta CYP17A1-entsyymien toiminnan estoa sekä *in vitro* solu-mallissa, että eristetyissä mikrosomifraktioissa, ja *in vivo* tehomalleissa jyrksijöissä ja apinassa. Yhdisteiden lupaavat prekliiniset farmakokineettiset tulokset tukivat jatkotutkimusten tekemistä kastroatioresistenttiä eturauhassyöpää sairastavilla potilailla.

Kastroatioresistentissä eturauhassyövässä AR:n määrä kasvaa ja se voi aktivoitua useiden erilaisten yhdisteiden, mukaan lukien lisämunuaisessa tuotettujen steroidihormonien avulla. Ksenografitmallien soveltuvuus kyseisen syövän tutkimukseen onkin kyseenalaistettu, koska toisin kuin ihmisellä, hiiren lisämunuaisen ei ole osoitettu tuottavan androgeeneja. Kuitenkin osana tutkimustamme osoitimme hiiren lisämunuaisen tuottavan merkittäviä määriä steroidihormoneja, jotka edistävät eläinmallissa syövän kasvua. Tämä tutkimus tarjoaa uutta tietoa kastroatioresistentistä eturauhassyövästä ja sen hoitoon kehitettävistä lääkkeistä, sekä uusia prekliinisiä työkaluja lääkekehityksen tueksi.

Avainsanat: kastroatio-resistentti eturauhassyöpä, mieshormonit, ksenografi

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ABBREVIATIONS

5 α -DH-DOC	5 α -Dihydro-Deoxycorticosterone
11-DOC	11-Deoxycorticosterone
11-OHA4	11 β -Hydroxyandrostenedione
11KDHT	11-Keto-5 α -Dihydrotestosterone
11KT	11-Ketotestosterone
A4	Androstenedione
ACTH	Adrenocorticotrophic Hormone
ADT	Androgen Deprivation Therapy
ADX	Adrenalectomy
AR	Androgen Receptor
AREs	Androgen Responsible Elements
C	Cortisol
CTC	Circulating Tumor Cell
DOC	11-deoxycorticosterone
CRH	Corticotropin-Releasing Hormone
CRPC	Castration-Resistant Prostate Cancer
DBD	DNA Binding Domain of Androgen Receptor
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone Sulphate
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GR	Glucocorticoid Receptor
hCG	human Chorionic Gonadotropin
HSD	Hydroxysteroid Dehydrogenase
LBD	Ligand Binding Domain of Androgen Receptor
LH	Luteinizing Hormone
Lhr	Luteinizing hormone receptor
LLOQ	Lowest Level Of Quantitation
mCRPC	metastatic Castration-Resistant Prostate Cancer
NTD	N-terminal Domain of Androgen Receptor
ORX	Orchidectomy
OS	Overall Survival
P	Progesterone
PDX	Patient-derived Xenograft
PFS	Progression Free Survival
PSA	Prostate-specific Antigen
SD	Standard Deviation
T	Testosterone
TNM	Tumor Nodes Metastasis

LIST OF ORIGINAL PUBLICATIONS

The study is based on the following original publications, which are referred to in the thesis using Roman numerals I-III.

- I. Moilanen A-M., Riikonen R, **Oksala R**, Ravanti L, Aho E, Wohlfahrt G, Nykänen PS, Törmäkangas OP, Palvimo JJ and Kallio PJ. Discovery of ODM-201, a new generation androgen receptor inhibitor targeting resistance mechanisms to androgen signaling-directed prostate cancer therapies. *Sci. Rep.* (2015) 5, 12007
- II. **Oksala R**, Moilanen AM, Riikonen R, Rummakko P, Karjalainen A, Passiniemi M, Wohlfahrt G, Taavitsainen P, Malmström C, Ramela M, Metsänkylä HM, Huhtaniemi R, Kallio PJ, Mustonen MVJ. Discovery and Development of ODM-204: A Novel Nonsteroidal Compound for the Treatment of Castration-Resistant Prostate Cancer by blocking the Androgen Receptor and Inhibiting CYP17A1. *J. Steroid Biochem. Mol. Biol.* (2018) (in press).
- III. Huhtaniemi R*, **Oksala R***, Knuutila M, Mehmood A, Aho E, Laajala TD, Aittokallio T, Laiho A, Elo L, Ohlsson C, Kallio P, Mäkelä S, Mustonen MVJ, Sipilä P, Poutanen M. *Equal contribution. Mouse adrenals contribute to the androgen dependent growth of castration-resistant VCaP prostate cancer xenografts. *Submitted.*

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1 INTRODUCTION

Prostate cancer is the most common solid organ malignancy and the most common cancer in males in Europe (Mottet N et al., 2017). Worldwide, more than one million men per year receive the diagnosis (Siegel RL et al., 2017). The frequency of clinically diagnosed prostate cancer varies extensively and is highest in Northern and Western Europe (>200 per 100 000 men/year). However, the incidence of autopsy-detected cancers is almost similar in different parts of the world (Arnold M et al., 2015). Prostate cancer is more prevalent in older population, the median age of diagnosis being more than 65 years (Howlader N et al., 2014, SEER Cancer Fact Sheet, Cancer Stat Facts: Prostate Cancer, 2017). The early phases in local prostate cancer are often asymptomatic, because of tendency of this cancer type to grow slowly with-low-grade aggressiveness. The activation of androgen receptor (AR) and AR signaling axis with several mechanisms are accepted to be the hallmarks of oncogenesis in all the states of the prostate cancer.

After local treatments, androgen deprivation therapy (ADT) is the first systemic treatment of recurring prostate cancer. Regardless of the initial sensitivity and high biochemical response rate (80-90%), to this treatment (by measuring prostate-specific antigen (PSA) from plasma), the disease typically progresses to castration-resistant prostate cancer (CRPC), however, still remaining AR-driven. During the past decade three new second-generation therapies, abiraterone, enzalutamide, and apalutamide, targeted directly to the AR signaling axis, have been approved for the treatment of patients with metastatic CRPC. All treatments have represented breakthroughs in the field and provided significant benefits for the patients. Nevertheless, resistance also to these new agents will be developed over time. Since treatment of CRPC with both agents is moving to the earlier states of the disease, the resistance mechanisms concern an increasing number of patients.

During the past decade the understanding of AR activation in CRPC has increased, and several different resistance mechanisms have been recognized, including *de novo* production of androgens or activation of adrenal precursors in the tumors, AR amplification and overexpression, AR mutations, and development of constitutively active AR variants (Taplin ME et al., 1995, Visakorpi T et al., 1995, Koivisto P, 1997, Auchus RJ, 2004a, Guo Z et al., 2009, Labrie F et al., 2009, Chang K-H et al., 2011). However, additional basic research and more detailed understanding of these complex resistance mechanisms in CRPC are needed. It cannot be emphasized enough that a growing number of patients are still waiting for better therapies. Thus, it is imperative to develop novel preclinical models for demonstrating these resistance mechanisms, which will be useful tools in further drug development.

2 REVIEW OF LITERATURE

2.1 History of the prostate cancer and its diagnostics

The prostate is an exocrine gland, approximately 20 g of weight and 4×2.5 cm of dimensions, lying deep in man's pelvis, below the urinary bladder and in front of the rectum. The urethra runs through the center of the prostate gland, which is encapsulated by a fibroelastic layer, and subdivided into five lobes. As to the internal structure, the human prostate gland is divided into four subdivisions: the anterior nonglandular fibromuscular stroma, the periurethral portion, the peripheral, the transitional and the central zones (Aumuller G, 1983, McNeal JE et al., 1984). It consists of epithelial and stromal compartments, which are separated by the basement membrane, a compact structure of collagen fibers containing various extracellular matrix proteins produced by both epithelial (laminins) and stromal cells (collagens) (Bonkhoff H et al., 1991). Epithelium is composed of two layers: highly AR expressed luminal cells, which also produce prostatic secretions and a basal epithelial layer, which separates luminal layer from the stroma (Brawer MK et al., 1985, Nagle RB et al., 1987, van Leenders GJ et al., 2003). The stromal section consists of several different cell types, smooth muscle cells being the most predominant (Bartsch W et al., 1979), but also containing fibroblastic, neuronal, lymphatic, and vascular cell types (Marker PC et al., 2003). Approximately half of the stromal cells also express AR.

The disease states of the prostate cancer include clinically localized disease, which in significant number of cases after primary treatment is followed by biochemical relapse (rising of plasma PSA without evidence of metastases) hormone-sensitive prostate cancer (HSPC) with metastases or castration-resistant prostate cancer (CRPC), without radiographically detected metastases, meaning the recurrence of cancer, albeit castrate levels of testosterone (T), and finally the lethal form of the disease, metastatic CRPC (mCRPC). The disease states of the prostate cancer are illustrated in Figure 2, as presented by The Prostate Cancer Clinical Trials Working Group 3 (PCWG3) (Scher HI et al., 2016).

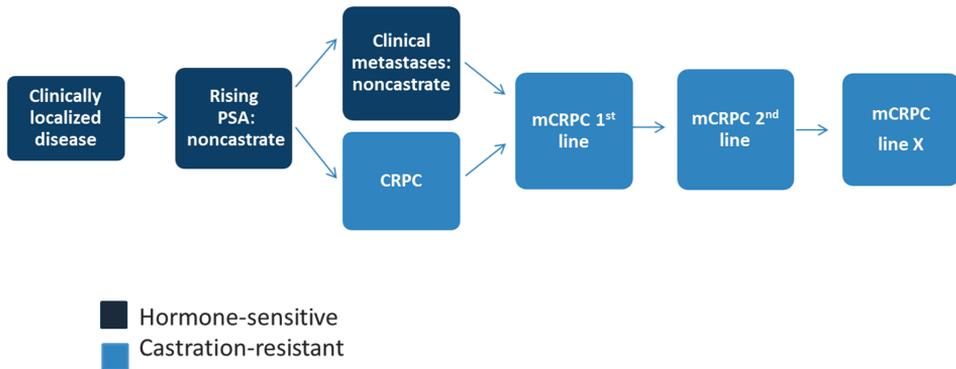


Figure 1: Disease states of the prostate cancer, based on the definitions of the Prostate Cancer Clinical Trials Working Group 3 (Scher HI et al., 2016). PSA= prostate-specific antigen, CRPC = castration-resistant prostate cancer, mCRPC = metastatic CRPC.

The diagnostic methods of prostate cancer include prostate-specific antigen (PSA) analysis from blood, digital rectal examination and systematic 12 core sextant prostate biopsies taken by transrectal ultrasound guidance (Andriole GL et al., 2009) or by magnetic resonance imaging (MRI) (Panebianco V et al., 2015). Measuring of serum PSA provides an easy, cost-efficient, screening tool to avoid unnecessary prostate biopsies. Nevertheless, it should be remembered that PSA is a continuous parameter: the higher the value, the more likely is the existence of prostate cancer. The cut-off value is considered 3.1 $\mu\text{g/l}$ for World Health Organization-calibrated PSA assays and 4 $\mu\text{g/l}$ in traditionally calibrated assays (Stephan C et al., 2009). Prostate cancer diagnosis involves staging of the cancer tissue by the Tumor Nodes Metastasis (TNM) staging system, which classifies cancer depending on its size and location, providing information about the tumor (T; which illustrates local cancer confined to the prostate), nearby lymph nodes (N; spans metastases to the closest lymph nodes through the lymphatic system), and distant organ metastases (M; describes the spread of metastases to distant sites through the lymph or blood circulation) (Edge SB et al., 2010). In addition, the histopathological examination is used to provide the Gleason prostate cancer score (van der Kwast TH et al., 2003), originally developed by Dr. Donald Gleason, an American pathologist, in the 1960s. This original grading system contains two grades in the form of numbers: a primary grade for the predominant histological pattern and a secondary grade for the next common pattern, both on a scale of 1 to 5 based on the microscopic architecture and appearance of the cells and then a total score (Gleason DF et al., 1974). The Gleason grading system was recently updated and renamed as Prognostic Gleason Grade Grouping System. In that classification Gleason score lower than 6 belongs to prognostic grade group I, Gleason score 3+4=7 (prognostic grade group II), Gleason score 4+3=7 (prognostic grade group III); Gleason score 4+4=8 (prognostic grade group IV); and Gleason score 9-10 (prognostic grade group V), reflecting prognosis more accurately to cancer biology than the Gleason system (Nelson JB, 2014, Epstein JI et al., 2016a, Epstein JI et al., 2016b,

Leslie SW et al., 2017). In addition, several other diagnostic and imaging tools, like multiparametric magnetic resonance imaging can be used to investigate the possibility of an anteriorly located prostate cancer in unclear cases (Arumainayagam N et al., 2013, Sonn GA et al., 2014).

2.2 Local prostate cancer

Although prostate cancer is typically diagnosed in males, there is increasing evidence that the initiation of the prostate cancer occurs already earlier in life (Packer JR et al., 2016). Early histopathological premalignant changes in the prostate are prostatic intraepithelial neoplasia (PIN) from low-grade to high-grade PIN and then to invasive carcinoma (Chrisofos M et al., 2007, Iczkowski KA et al., 2014). PIN changes have been proposed to rise to progressive morphologic abnormalities, starting from the normal epithelium with increased luminal cell hyperplasia being biochemically, genetically and phenotypically similar to prostate cancer and finally disturbing of basement membrane (Shen MM et al., 2010). Abundant data supports the concept that high-grade PIN is the main precursor lesion to invasive adenocarcinoma of the prostate (Bostwick DG et al., 1996, Montironi R et al., 2011, Merrimen JL et al., 2013, De Marzo AM et al., 2016). The majority of prostate adenocarcinomas are formed of luminal-type prostate epithelial cells that need AR signaling for survival. The critical role of AR in the normal prostate is well established, and the AR signaling axis has been proven to be critical for Prostate cancer in all subsequent phases of disease progression.

2.2.1 Treatment of local prostate cancer

Standardized local treatment options for early-stage prostate cancer are approved in the Clinical Guidelines (Cornford P et al., NIH, 2018). Generic options contain surgery (e.g. radical prostatectomy), radiation therapies including external beam radiation therapy or brachytherapy, chemotherapy, and androgen deprivation (Parker C et al., 2015, Mottet N et al., 2017). Also newer therapies such as cryotherapy and high-intensity focal ultrasound have been applied. Combination therapies, for instance brachytherapy combined with radiation therapy may be also given in locally advanced prostate cancer. Furthermore, active surveillance by enhanced monitoring with regular measurements of serum PSA, confirmatory prostate biopsies, and MRI are offered for patients with lower-risk disease to avoid potentially harmful treatments (Parker C, 2004). The decision of the treatment or treatment combinations are always based on careful estimation of patient and disease related factors, thus enabling individualized treatment options. Currently the 5-year survival rate for patients with localized disease or regional metastases is almost 100%

(American Cancer Society, Cancer Statistics 2017 Slide Presentation, 2017). Two independent studies have shown that most patients with low-risk disease can avoid treatment, with only 1% increased risk of death for prostate cancer during a 10-year follow-up (Klotz L et al., 2010, Selvadurai ED et al., 2013). Recently reported PRIAS study, with large amount of cases; over 5000 men across 18 countries, confirmed the safety of active surveillance as a treatment option for men with low-risk prostate cancer (Bokhorst LP et al., 2016). Also in Finland active surveillance is actively used in patients with a local, low-risk disease (Prostate Cancer: Current Care Guidelines Abstract, 2014).

2.3 Advanced states of prostate cancer

The development of recurring disease after local therapy, systemic androgen-deprivation therapy (ADT) has been the cornerstone of treatment for metastatic prostate cancer over seventy years, when Huggins and Hodges in 1941 first pioneered castration therapy as a treatment option of the metastatic prostate cancer (Huggins C et al., 1972, Huggins C et al., 2002). ADT includes surgical (bilateral orchidectomy) or medical castration using gonadotrophin hormone-releasing hormone (GnRH) agonists or antagonists, both of which reduce serum T to so-called 'castration level' (0.5 ng/ml). Today GnRH agonists are the first-choice in testosterone-lowering therapy (Jan de Jong I et al., 2007). Initially, ADT induces apoptosis of androgen-dependent prostate cancer epithelial cells and regression of androgen-dependent tumors, proving near-certain remission, both clinically and biochemically, leading into disease control and optimally, into several years of effective disease stabilization (Francini E et al., 2017). Nevertheless, the therapeutic effect of ADT is limited and approximately 2/3 of the patients progress to the lethal metastatic form of the disease within two years (Eisenberger MA et al., 1985, Attar RM et al., 2009, Cookson MS et al., 2013).

Progression to CRPC is typically identified by rising PSA levels, while T remains under castration levels (Vis AN et al., 2009, Hu R et al., 2012). Although the long history of prostate cancer research, it was only a decade ago, when the critical role of AR as a major oncogenic driver and the role of the residual hormones still present after ADT in CRPC were understood and suggested to be critical for the development and progression of CRPC (Knudsen KE et al., 2010, Nelson PS, 2012). First this was demonstrated in the xenograft models, where an elevation in post-castration AR levels was shown to be the major molecular change associated with CRPC (Chen CD et al., 2004), and further evidence to support this has accumulated during the recent years from preclinical and clinical data (Knuutila M et al., 2014, Network CGAR, 2015). The verification of the central role of AR in CRPC pathophysiology was confirmed by the significant clinical responses observed in CRPC patients treated with the new second-line hormonal

treatments abiraterone or enzalutamide, both recently approved by the FDA and EMA (de Bono JS et al., 2011, Scher HI et al., 2012).

2.3.1 Treatment options for advanced prostate cancer

Despite slow progression of the disease and several treatment alternatives with promising results, prostate cancer is the second leading cause of cancer-related deaths in men (Ferlay J et al., 2015), especially the metastatic castration-resistant prostate cancer (mCRPC) (Siegel RL et al., 2017). Also in the non-metastatic prostate cancer cases after local treatment, the risk of disease progression is relatively high; approximately 15-33% of the patients, depending on the Gleason score, can develop metastasis in two years (Smith MR et al., 2005, Hirst CJ et al., 2012). Overall, the data suggests that 10-20 % of all prostate cancer cases progress and develop CRPC within five years of follow-up, median survival being only 14 (range 9-30) months (Alemayehu B et al., 2010, Kirby M et al., 2011).

2.3.1.1 Androgen deprivation therapy with GnRH analogues

GnRH agonists affect by suppressing T to castration levels (Heidenreich A et al., 2011). Strong stimulation of luteinizing hormone (LH) and its release primarily induces a marked temporary elevation in T (Labrie F et al., 1980, Engel JB et al., 2007). This overstimulation of LH release leads within 7-10 days to desensitization and/or downregulation of pituitary receptors for GnRH. Furthermore this phenomenon leads to the downregulation of pituitary GnRH receptors and the suppression of LH production, consequently diminishing testosterone-to-castrate levels (Labrie F et al., 1980, Labrie F et al., 1985, Rick FG et al., 2015).

Leuprolide, a nonapeptide, was the first GnRH analogue developed to get an FDA approval in 1985. Goserelin was accepted four years later (Roach M, 3rd et al., 2007) and histrelin and triptorelin followed in the early 2000s (Deeks ED, 2010, Ploussard G et al., 2013). Results from clinical trials have shown GnRH agonists to be equal to surgical castration in terms of survival, clinical effects and time to disease progression (Seidenfeld J et al., 2000, Sharifi N et al., 2005) and no substantial differences between leuprolide and goserelin were observed (Gommersall LM et al., 2002). Treatment with GnRH agonists can be considered the first pathway-specific targeted therapy in prostate cancer. The GnRH antagonist's mechanism of action differs from agonists in that they directly bind competitively to the pituitary GnRH receptors and antagonizing receptors causing a significant direct reduction in circulating LH and FSH levels and further suppression in the synthesis of T (Belanger A et al., 1980, Labrie F et al., 1983, Schally AV, 2007), without any T flare observed with agonists (Labrie F et al., 1983). Currently, only one

GnRH antagonist, degarelix approved by the FDA in 2008, is in clinical use (Moul JW, 2014).

Several clinical studies comparing the efficacy of leuprolide against degarelix have been conducted. In the study performed by Klotz and colleagues, the results indicated that 81% of patients treated with leuprolide had an expected flare in T, whereas over the same time period more than 95% of the patients treated with degarelix experienced reduction in systemic T levels to ≤ 0.5 ng/mL in 3 days (Klotz L et al., 2008). Furthermore, numerous studies have shown medical castration with a GnRH agonist to be equivalent to bilateral orchidectomy (Maximum androgen blockade in advanced prostate cancer: an overview of the randomised trials. Prostate Cancer Trialists' Collaborative Group, 2000), which is rather rarely used in clinical practice nowadays. ADT has been a standard treatment for advanced prostate cancer for almost eighty years and is included also in the treatments with current second-generation therapies.

The significant benefits observed with GnRH analogues in localized prostate cancer presenting more than one-third reduction in the prostate cancer deaths (Peto R et al., 2003), there are several long-term safety and toxicity problems and the treatment is linked with significant morbidity (Nguyen PL et al., 2015). Castrate levels of T have been shown to decrease bone mineral density, atrophy of the skeletal muscle, and increase the frequency of heart failure and myocardial infarction (Cheung AS et al., 2014, Edelman S et al., 2014, Skolarus TA et al., 2014, Nguyen PL et al., 2015). ADT has also been connected to changes in the metabolism and body composition (Saylor PJ et al., 2013) and increased risk of dementia has been reported (Nead KT et al., 2017). In addition, several other symptoms including decreased sexual function and impotence, gynecomastia, hot flashes and fatigue have been observed. These all are factors, which decrease the quality of life (Nguyen PL et al., 2015). Clinical preparations for both agonists and antagonist are given as subcutaneous depot injections: 1-, 3-, 4- and 6-month formulations are available for leuprolide and for degarelix a one-month injection.

2.3.2 First-generation AR antagonists

The first-generation nonsteroidal antiandrogens, flutamide, bicalutamide, and nilutamide antagonize the actions of androgens at the receptor level by competing with T, DHT and the other androgens for ligand binding sites on the AR, and thereby, inhibiting tumor growth (Kolvenbag GJ et al., 1998). Today, they are mainly used in combination with GnRH agonists preventing clinical 'flare-up' of T to reach 'complete androgen blockade'. The study by Crawford and colleagues was the first in demonstrating the benefits of combination therapy with GnRH agonist Lupron (leuprolide) and antiandrogen flutamide in a large-scale randomized trial (Crawford ED et al., 1989). This study successfully

pointed out the benefits of using combined androgen blockade as a first-line treatment for advanced prostate cancer: the patients who received the combination regimen lived on average 7.3 months longer than those who received leuprolide plus placebo (Crawford ED et al., 1989). Similar efficacy observed with GnRH agonists was proven with bilateral orchidectomy as monotherapy or combination with flutamide (Eisenberger MA et al., 1998). With nilutamide, there are no comparative trials available with castration or with other antiandrogens, since it is currently not licensed for monotherapy. In preclinical models bicalutamide has shown 4-fold and clinically 10-fold greater affinity for the AR than flutamide and nilutamide (Furr BJ, 1996, Kolvenbag GJ et al., 1998) but nevertheless, all three non-steroidal anti-androgens work rather effectively. Bicalutamide has significantly longer half-life (7 days) compared with flutamide (6-8h) and nilutamide (2 days) permitting once daily dosing (McLeod DG, 1997, Mahler C et al., 1998).

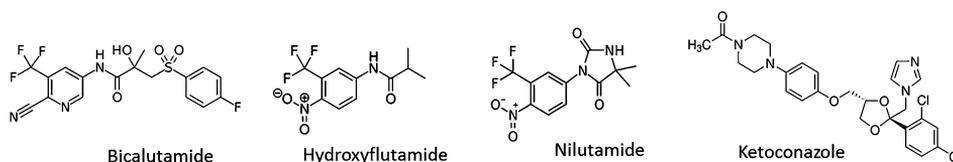


Figure 2: The structures of first-generation non-steroidal AR antagonists bicalutamide, hydroxyflutamide and nilutamide and unspecific androgen synthesis inhibitor ketoconazole. (Structures form PubChem-database).

2.3.3 *First-generation androgen biosynthesis inhibitors ketoconazole and 5-alpha reductase inhibitors*

Historically, an off-label use of ketoconazole, an imidazole derivative antifungal agent, has been reported to achieve response rates in CRPC ranging from 20% to 75% (Yap TA et al., 2008). The mechanism of action of ketoconazole is a consequence of a nonselective inhibition of several cytochrome P450 enzymes including CYP17A1. De Coster and colleagues confirmed that ketoconazole given at a dose of 400 mg with hydrocortisone three times daily decreased levels of T, androstenedione (A4) and dehydroepiandrosterone (DHEA) in patients with prostate cancer (De Coster R et al., 1986). Ketoconazole has also been shown to cause continuous responses and decline in PSA levels in hormone refractory prostate cancer and in mCRPC (Scholz M et al., 2005, Keizman D et al., 2012). CYP17A1 inhibition with ketoconazole has only partial response and a rise in adrenal androgens at the state of cancer progression in patients with antiandrogen withdrawal syndrome, a phenomenon in prostate cancer patients, whose cancer has started to grow again despite complete androgen blockade and who have responded by stopping the antiandrogen (Small EJ et al., 2004). Use of ketoconazole as a second-line hormonal therapy is rare because of weak tolerability; grade 3 or 4 toxicity

was reported in more than 20% of the patients, with primary adverse effects being fatigue, and hepatic, neurologic or respiratory issues (Small EJ et al., 2004).

Enzyme 5 α -reductase (SRD5A) converts T into DHT in prostate cells, where intracellular DHT concentration is approximately five times higher than the levels of T (Montgomery RB et al., 2008). There are three isoforms of SRD5s; in epithelial and stromal cells of the normal prostate both SRD5A1 and 2 isoforms are present, while SRD5A2 is the dominant isozyme expressed in the stromal cells (Russell DW et al., 1994, Silver RI et al., 1994). In addition, SRD5A1 is present in the skin, scalp, liver and brain (Thigpen AE et al., 1993) and isoform SRD5A3 is demonstrated to be expressed only in prostate cancer and pancreas (Uemura M et al., 2008). Currently, two drugs that inhibit SRD5A activity are available; finasteride, a selective SRD5A2 inhibitor and dutasteride which inhibits both SRD5A1 and 2 (Thomas LN et al., 2008). The role of SRD5A inhibitors as a treatment option in prostate cancer appears to be limited, and the results are controversial suggesting that reduction of DHT is not enough for the treatment of CRPC.

Taplin et al. demonstrated in their phase II trial with asymptomatic CRPC patients that the combination treatment with ketoconazole, hydrocortisone and dutasteride decreased the PSA level more than 50% in 56% of the patients, showing benefit compared to studies with ketoconazole alone (Taplin ME et al., 2009).

2.4 Current treatment options for CRPC

2.4.1 Enzalutamide

The first second-generation antiandrogen, enzalutamide is an oral nonsteroidal small-molecule, derivative from diarylthiohydantoin (Jung ME et al., 2010), developed by Medivation and Astellas. Enzalutamide with high affinity (over 5-fold better than bicalutamide) competitively inhibits androgen binding to AR without any agonism, which has been seen within the presence of bicalutamide (Tran C et al., 2009). In addition to this, it inhibits nuclear translocation of the AR, DNA binding, and coactivator recruitment (Tran C et al., 2009, Guerrero J et al., 2013). The efficacy of enzalutamide *in vivo* was proven in an orchidectomized mouse LNCaP xenograft model mimicking CRPC (Tran C et al., 2009). Enzalutamide has also an active major metabolite (Scher HI et al., 2010). As early as in the first-in-man dose escalation study, enzalutamide showed antitumor activity with all tested doses (30-600 mg/day) in patients with and without previous chemotherapy, and the mean PSA response rate was higher than 50%. The decrease of PSA was dose-dependent up to 150 mg/day and no additional benefits with higher doses were found, and thus the dose for phase III trial was selected to be 160 mg/day (Scher HI et al., 2010).

Two phase III, randomized, double-blinded and placebo-controlled trials called AFFIRM and PREVAIL have been carried out with enzalutamide. The first one was designed for metastatic CRPC patients whose disease progressed after docetaxel (Scher HI et al., 2012) and the second one was carried out with patients after progression from ADT but treated with chemotherapy (Beer TM et al., 2014b). In both studies, the primary endpoint overall survival (OS) rate, favored enzalutamide treatment and significantly improved median OS at 4.8 and 2.2 months compared to placebo, respectively (Scher HI et al., 2012, Beer TM et al., 2014b). In both studies enzalutamide showed successful results also at other co-primary endpoints, like PSA response rate, and progression-free survival. Enzalutamide was the first approved second-generation antiandrogen (Scher HI et al., 2012, Beer TM et al., 2014b) and is recommended for the patients with mCRPC before and after chemotherapy (Cookson MS et al., 2013, Cookson MS et al., 2015).

Generally, enzalutamide has been well tolerated. The most common adverse effects reported are fatigue, diarrhea, hot flushes, headache and pain in the musculoskeletal system. Also hypertension has been observed. In the AFFIRM trial a clear increase in the incidence of seizures in the enzalutamide group were noted and caution should be used when using enzalutamide in patients with a history of seizures. The absorption of enzalutamide was shown to be fast: maximum concentration was achieved in 1 h, half-life being approximately 1 week (Scher HI et al., 2010). Enzalutamide is highly, over 97%, bound, to plasma proteins, primarily albumin. The metabolism of enzalutamide occurs primarily via cytochrome P450 CYP3A4 and CYP2C8 enzymes (Gibbons JA et al., 2015b). Clinical trials have further shown that after oral dosing, enzalutamide induces CYP2C9 and CYP2C19 expression, and significantly reduces plasma exposures of warfarin (56%) and omeprazole (70%), well-known substrates of CYP2C9 and CYP2C19 (Gibbons JA et al., 2015a). Importantly, enzalutamide can induce CYP3A4 activity, which has been first proven by decreased plasma levels of midazolam (Gibbons JA et al., 2015a). A recent analysis confirmed that enzalutamide is a stronger inducer of CYP3A4 than the positive control rifampicin (Weiss J et al., 2017). Since CYP3A4 is involved in the elimination of about 50% of all marketed drugs (Zhou SF, 2008), the influence for numerous pharmacokinetic drug–drug interactions should be taken into account.

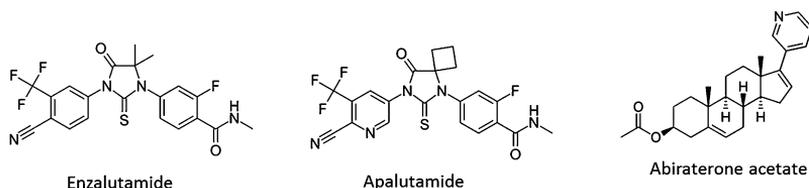


Figure 3: The structures of second-generation androgen signaling axis inhibitors enzalutamide, apalutamide and abiraterone. (Structures from PubChem-database).

2.4.2 Apalutamide

Apalutamide is a small molecule with high structural similarity to enzalutamide developed by Janssen. Like enzalutamide, also apalutamide binds directly to the ligand-binding domain of the AR, inhibits *in vivo* tumor growth in a CRPC xenograft mouse model and also inhibits several androgen-mediated gene transcriptions in AR-overexpressing prostate cancer cellular *in vitro* models, including PSA and TMPRSS2 (Clegg NJ et al., 2012). Compared to enzalutamide, apalutamide has equal *in vitro* activity, but better activity *in vivo* in the CRPC xenograft mouse model and also in intact male dogs, in which apalutamide induced castrate-like histopathological changes in androgen-dependent reproductive organs (Clegg NJ et al., 2012). Similarly to enzalutamide, apalutamide has an active metabolite, N-desmethyl apalutamide.

In the first-in-man dose escalation study apalutamide was administrated with repeated once-daily-dosing (30–480 mg). Dose-proportional increases both in the maximum plasma concentration and area under the plasma concentration-time curve were observed (Rathkopf DE et al., 2013). In the same study several pharmacodynamics efficacy markers were also measured. Decline in plasma PSA levels at 12 weeks was significant, 46.7% of patients had a $\geq 50\%$ decline in PSA as compared with baseline. In addition, positron emission tomography/computed tomography imaging was conducted to monitor [^{18}F] fluoro- α -dihydrotestosterone binding to AR in tumors before and during treatment; also in this marker apalutamide showed efficacy by reducing FDHT uptake across dose levels (Rathkopf DE et al., 2013). In a phase II study, apalutamide showed good PSA response ($> 50\%$ decline in PSA from baseline after 3 months) in 91% of nonmetastatic treatment-naïve cases, in 88% of metastatic treatment-naïve cases, and in 24% of metastatic post-abiraterone cases (Smith MR et al., 2016). Very recently published phase III data showed promising results, with median metastasis-free survival of 40.5 months compared to 16.2 months in the placebo group (Smith MR et al., 2018). The effect was observed in all subgroups, including patients in all age groups, with short PSA doubling time and with both local and regional nodal disease.

In general, apalutamide was well tolerated compared to placebo, and the majority of adverse events were grade 1 or 2. Higher rates of hypertension, rash, fatigue, arthralgia, weight loss, falls and fractures were observed, and also diarrhea, dizziness and hypothyroidism occurred (Smith MR et al., 2018). The most common reason for the discontinuation of the treatment was progression of the disease (Smith MR et al., 2018). Interestingly, also seizures in 0.2% of the patients were observed, although apalutamide has been reported to exhibit low affinity for gamma-aminobutyric acid type A receptor ($\text{IC}_{50} = 3.0 \mu\text{M}$) and the brain levels were 4-fold lower than with enzalutamide, thus suggesting a low seizurogenic potential (Clegg NJ et al., 2012).

Mean oral bioavailability of apalutamide is 100% and the median time to achieve maximum plasma concentration was 2 h. Apalutamide is highly bound to plasma proteins (96%) at steady state, with mean apparent volume of distribution 276 l and food has no clinically relevant effects on exposure. At steady state, apalutamide has a mean half-life of 3–4 days, and is mainly eliminated by CYP2C8 and CYP3A4 metabolism (FDA, 2018). In *in vitro* studies apalutamide has been classified moderate to strong inducer of CYP3A4 and CYP2B6, and moderate inducer to several other CYP enzymes and may also induce UDP-glucuronosyl transferase (FDA, 2018). Therefore concomitant administration of apalutamide with medications that are metabolized by CYP3A4, CYP2C9 or CYP2C19 may decrease exposures of these drugs and cause a loss of activity of these medications. In addition, drugs which are sensitive substrates of CYP3A4, CYP2C9, CYP2C19, UDP-glucuronosyl transferase may result in a loss of activity (FDA, 2018). In February 2018 apalutamide received its approval in the USA for the treatment of non-metastatic CRPC.

2.4.3 *Abiraterone acetate*

Abiraterone acetate is a steroidal, orally administered small-molecule derivative from pregnenolone, developed by Cougar Biology and Janssen Biotech. It is the prodrug of abiraterone, a potent, selective and irreversible inhibitor of the CYP17A1, (Barrie SE et al., 1994, Potter GA et al., 1995, Rowlands MG et al., 1995). Cytochrome P45017A1 is expressed in testicles, adrenal cortex and prostate, being a key enzyme in cortisol synthesis in the adrenal cortex via its 17 α -hydroxylase activity and playing a central role in androgen biosynthesis with its 17,20-lyase activity catalyzing the conversion of 17-hydroxypregnenolone to the main adrenal androgens, DHEA (Auchus RJ, 2001, Auchus RJ, 2004b) and A4 (Attard G et al., 2008). In addition, the expression of CYP17A1 in CRPC cells has been demonstrated in several studies (Cai C et al., 2011a, Mostaghel EA et al., 2011, Efstathiou E et al., 2012).

Abiraterone acetate efficiently inhibits both 17 α -hydroxylase and 17,20-lyase reactions *in vitro* with IC₅₀ values of 2.9 and 4 nM, respectively (Potter GA et al., 1995). The first-in-man studies both with castrated and non-castrated men abiraterone demonstrated to reduce serum T levels as expected. However, in non-castrated men, the suppression of T was only transient and in castrated men abiraterone additionally decreased castrate serum T levels (O'Donnell A et al., 2004). Combined with prednisone, abiraterone has shown to reduce serum DHEA nearly 75% and DHEA-S, A4 and T to almost undetectable levels (Attard G et al., 2008, Ryan CJ et al., 2010). In addition, to suppress androgen levels, abiraterone decreases PSA levels, and has shown clinical responses in a single agent phase I/II studies (Attard G et al., 2009, Reid AH et al., 2010). Due to the strong CYP17A1 inhibition in abiraterone acetate, an increase in adrenocorticotrophic hormone

(ACTH) secretion resulted in a rise in secretion of mineralocorticoids, 11-deoxycorticosterone (DOC) and corticosterone. Increased mineralocorticoid secretion is associated with side effects, including hypertension, hypokalemia, and fluid overload, detected already in a phase I study (Attard G et al., 2008).

Later on, phase I and II trials established that mineralocorticoid-related symptoms can be largely avoided by concomitant treatment with low-dose of prednisone (5 mg/twice a day) (Attard G et al., 2012), which has been included to the dosing schema in phase III trials for abiraterone. Abiraterone acetate was compared to placebo plus prednisone both in chemotherapy-naïve (COU-AA-302) and in post-docetaxel settings (COU-AA-301), and both analyses showed a significantly longer median OS, 4.4 months (Ryan CJ et al., 2015) and 4 months (de Bono JS et al., 2011), respectively. In addition to this primary endpoint, all secondary endpoints, including several study-specific parameters like serum PSA level, radiographic progression-free survival and pain score favored abiraterone treatment in both studies. In spite of intensive research and development, abiraterone acetate is the only FDA-approved CYP17A1 inhibitor for men with mCRPC.

Based on preclinical studies, the combination, abiraterone acetate, and dutasteride decreased intratumoral T and DHT levels but did not block their production totally (Pham S et al., 2014). Very recently, also in a clinical phase II trial, the combination of abiraterone acetate and dutasteride in CRPC patients has been reported to increase androgen synthesis inhibition (McKay RR et al., 2017). The investigators indicate that persistent activation of the AR axis still continues to be a major driver of the disease progression, albeit they did not directly compare the efficacy with abiraterone acetate as a monotherapy but instead, they considered that further multi-layered approaches to AR inhibition in CRPC might provide a benefit for the patients (McKay RR et al., 2017).

Recently two phase III studies (LATITUDE and STAMPEDE) suggested a new therapeutic use to abiraterone together with ADT for patients with HSPC and also for non-metastatic patients (Fizazi K et al., 2017, James ND et al., 2017). Both results showed significant impact, including increased OS and PFS, on the treatment outcomes of advanced prostate cancer with ADT and abiraterone used concurrently, thus strongly suggesting a new standard of care to use abiraterone together with ADT (Fizazi K et al., 2017, James ND et al., 2017). These results may also change the selection for right patients and the right timing to abiraterone treatment, and furthermore, to identify the mechanisms of resistance during the use of abiraterone (Small EJ, 2017). In addition, some of the patents of abiraterone will soon expire and generic forms of the drug will become available. Potentially, in clinical practice this will increase the use of abiraterone and thereby increase the number of patients developing resistance to the drug in the future.

Abiraterone resistance has been linked to numerous AR-dependent resistance mechanisms, which will be reviewed in detail in section 2.7, and also several AR

independent mechanisms such as glucocorticoid receptor activation, immune-mediated resistance and a neuroendocrine variant of the disease have been described (Kantoff PW et al., 2010, Arora VK et al., 2013, Svensson C et al., 2014, Carver BS, 2016). Interestingly, decreased but still detectable levels of DHEA-S have been recently reported from several clinical trials in patients treated with abiraterone acetate (Taplin ME et al., 2014, Tamae D et al., 2015, McKay RR et al., 2017). This may indicate an incomplete inhibition of CYP17A1 with abiraterone acetate (McKay RR et al., 2017), but also current methods are more sensitive to detect low levels of hormones than earlier. Moreover, a trend for increased serum pregnenolone and progesterone (P) (hormones upstream of CYP17A1 enzyme) levels has been observed in the patients treated with abiraterone (Taplin ME et al., 2014, Snaterse G et al., 2017). Both hormones may, by themselves or as precursors, facilitate agonistic ligands for AR activation (Gregory CW et al., 2001, Taplin M-E et al., 2004).

The labeled daily dose for abiraterone is 1000 mg to be taken on fasted state to minimize variability in the absorption (Todd M et al., 2012). Maximal plasma drug concentrations have been achieved within 2-4 hours, terminal half-life being circa 12 hours (Attard G et al., 2008, Ryan CJ et al., 2010). Notably, abiraterone has shown significant food effect, over 10-fold increased drug exposures were observed after a high-fat meal compared to fasted state (NCT01424930). This may cause a potential safety risk for the patients, if they accidentally take the drug with food. Abiraterone has shown to be highly bound to plasma proteins, including albumin, and it is predominantly excreted to feces (Acharya M et al., 2013). Overall, the drug is well tolerated and occurrence of the significant adverse effects did not differ from the control group. However, it has several common side effects like fatigue, back pain, nausea and constipation, at the range of roughly 25%. In addition to mineralocorticoid excess and changes in electrolytes, hepatic transaminase abnormalities were detected in approximately 6 % of the patients (Kluetz PG et al., 2013). The incidence of adverse cardiac effects was not significantly different compared to placebo, but the frequency of cardiac failure was higher in abiraterone-treated patients (Procopio G et al., 2015). Abiraterone is a strong inhibitor of CYP1A2 and CYP2D6 drug metabolizing enzymes, and co-administration of known substrates for these enzymes like beta-blockers, serotonin reuptake inhibitors as well as codeine and tramadol (Chi KN et al., 2013) should be prescribed with caution as their plasma exposures may significantly increase. Abiraterone itself is a CYP3A4 substrate (Bernard A et al., 2015) and concomitant treatments with CYP3A4 inducers like enzalutamide (Gibbons JA et al., 2015a) or inhibitors like bicalutamide and ketoconazole should be prescribed with caution.

2.4.4 Chemotherapy

Docetaxel has been the first-line cytotoxic treatment for CRPC and actually it was the only life-prolonging therapy available from its approval in 2004 through 2010, proven with two randomized clinical trials (Petrylak DP et al., 2004, Tannock IF et al., 2004). The second-generation taxane, cabazitaxel has also been proven to work as second-line chemotherapy for CRPC (de Bono JS et al., 2010), and shown not only to be as effective as docetaxel but also to show efficacy in tumors resistant to docetaxel (Galsky MD et al., 2010, Sartor O et al., 2011). Both docetaxel and cabazitaxel are semisynthetic taxanes, with a mechanism of action based on their capability to stabilize microtubules involved in the mitotic spindle, leading to inhibition of mitosis and finally cell death (Jordan MA et al., 2004). Preclinical findings suggested that taxanes could also have a direct influence on AR activation (Gan L et al., 2009). AR nuclear translocation from docetaxel-treated and untreated tumors was studied by immunohistochemistry and the data showed significantly reduced AR nuclear translocation in the docetaxel-treated patients (Zhu ML et al., 2010). This may indicate that docetaxel treated tumors responded also via the androgen-dependent signaling pathway (Darshan MS et al., 2011, Fitzpatrick JM et al., 2014). In contrast to docetaxel, cabazitaxel may promote AR nuclear accumulation, although it diminishes the total AR level (Martin SK et al., 2015). However, taxane treatments often fail due to resistance. With docetaxel the mechanism has been proposed to be related to the increased expression of P-glycoprotein-1 by preventing sufficient accumulation of anticancer drugs within the cells, and thereby, avoiding their cytotoxic or pro-apoptotic effects (Rowinsky EK et al., 1998, Galsky MD et al., 2010). For cabazitaxel, several resistance mechanisms have been described, including multidrug resistance via activation of transporters such as P-glycoprotein and ABCB1 (Malofeeva EV et al., 2012), modifications in microtubule composition having an elevated class III β -tubulin and reduced BRCA1 expression (Duran GE et al., 2015). In addition, alterations in the epithelial-to-mesenchymal transition have been observed, linking the increased amount of mesenchymal markers to cabazitaxel resistance (Duran GE et al., 2015).

2.4.5 Radium-223

Nearly all mCRPC patients have radiologic signs of bone metastases, which is one of the main causes of death, disability, and reduced quality of life (Tannock I et al., 1989b, Parker CC et al., 2013b). Radium-223 dichloride (radium-223) is a selectively bone-targeted alpha emitter, specifically targeted to bone metastases by inhibiting increased bone turnover. In the metastatic sites, it emits high-energy alpha particles of short range (<100 μm) (Bruland OS et al., 2006). As a bone-seeking calcium mimetic, it is bound selectively into newly formed bone, especially within the microenvironment of metastatic

sites (Henriksen G et al., 2002, Henriksen G et al., 2003). The medicine is administered as an intravenous injection at four-week intervals, totally six times. In early phase I/II studies in patients with bone metastases, a promising safety profile has been established for radium-223, with reduction of pain and improvement in disease-related biomarkers, like bone alkaline phosphatase and PSA (Nilsson S et al., 2007, Nilsson S et al., 2013, Parker CC et al., 2013a, Parker CC et al., 2013b). In the ALSYMPICA trial, Radium-223 showed significantly longer median OS (3.6 months) compared to placebo in men with CRPC (Parker C et al., 2013). The limitations of the use of Radium-223 based on the facts that its efficacy is based on a single phase III trial. It has not been compared to other therapies in randomized trials, even though the results in ALSYMPICA trial are considered clinically significant. In addition, the intravenous dosing route and unclear definition in the right timing of Radium-223 may decrease the favor of using the drug. Finally, the costs of a six-dose treatment is relatively high (~28 000 €/patient).

2.4.6 Immunotherapy

Understanding of the critical role of the immune system in cancer development and progression has led to several new treatment approaches. Cancer cells can adopt numerous active immune escape strategies to protect them from detection and destruction by the immune system (Carosella ED et al., 2015) and the activation of inhibitory immune checkpoint pathways is a central mechanism for the immune resistance of tumor cells (Pardoll DM, 2012). Current immuno-therapy strategies are designed to boost or reactivate antitumor immunity in the cancer microenvironment mainly by restoring T cell-mediated antitumor immunity by targeting therapies with cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1), and their corresponding ligands B7-1/B7-2 and PD-L1, respectively (Modena A et al., 2016). Despite high interest and several studies performed with immune checkpoint inhibitors, to date they have not shown significant survival improvement in CRPC as single agents (Nuhn P et al., 2018). However, there are numerous clinical trials ongoing with immune checkpoint inhibitors combined to AR-targeted therapies, such as enzalutamide or abiraterone and also with cytotoxic agents like docetaxel (Modena A et al., 2016).

Sipuleucel-T (Provenge[®]) is vaccine therapy, the first biological and personalized treatment for prostate cancer with a new mechanism of action. It is designed to stimulate a T cell response against prostatic acid phosphatase (PAP), an antigen, which has shown to be rather abundant specifically in prostate cancer cells (Plosker GL, 2011). The therapy is prepared individually for each patient by harvesting their dendritic cells (antigen-presenting cells) in a technique named leukapheresis. The harvested cells are taken at the manufacturing plant and assorted with a proprietary fusion protein (PA2024) containing the PAP antigen (Plosker GL, 2011). *Ex vivo* activated blood product is then re-infused

back to the patient to induce an immunological response against cancer cells carrying the PAP antigen. The FDA approved the therapy based on the data from the IMPACT trial showing significantly improved median survival 4.1 months compared to placebo (Kantoff PW et al., 2010). Sipuleucel-T therapy is approved in the US, but not currently in Europe (Heidenreich A et al., 2014), most probably because of the high pricing (USD 93 000 for three infusions) and the complex manufacturing process (Ozdemir BC et al., 2017).

2.5 AR and androgen signaling

AR and the androgen signaling pathway play a vital role in male sexual differentiation. It has a significant role in the development and function of male reproductive organs such as prostate and thus, in the etiology of prostate cancer (Roy AK et al., 1999, Buchanan G et al., 2001b, Heinlein CA et al., 2004). In addition, AR also plays a role in non-reproductive organs, such as muscle, bone, hair follicles, and brain (Lee DK et al., 2003). To understand the key role of AR and the steroidogenesis in CRPC, the regulation of steroid production, the adrenal and gonadal steroid biosynthesis, and AR structure and biology in normal physiology are summarized in the next paragraphs.

2.5.1 Role of hypothalamus and pituitary in the regulation of steroid production

Steroid hormones are small lipophilic molecules that circulate in the blood stream controlling many physiological functions including the balance of energy, metabolism, and electrolytes, sex differentiation as well as responses to stress. All steroid hormones are derivatives from 27-carbon cholesterol, and a specific enzymatic machinery systematically oxidizes this carbon structure to 21-carbon steroids (containing progestins, glucocorticoids, and mineralocorticoids), further to 19-carbon steroids (androgens) and finally to 18-carbon steroids (estrogens) (Selye H et al., 1946). The main structures of steroid hormones and their precursors were identified in the 1930s, producing the basic understanding of the main steroid biosynthesis (Kendall EC et al., 1937, Steiger M et al., 1938) and in the 1980s it was recognized that in all steroidogenic tissues, similar steroidogenic reactions and enzymes are catalyzing the formation of hormones (Miller WL, 1988).

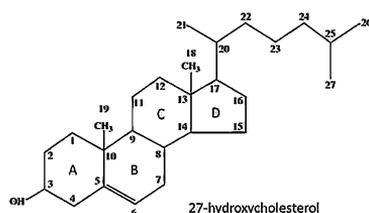


Figure 4: 27-hydroxycholesterol structure presented with IUPAC-approved ring lettering and atom numbering.

The main androgen in the circulation is T, and its production is closely regulated by the hypothalamic–pituitary–gonadal axis. The increase of T in the circulation utilizes negative feedback on the hypothalamus and pituitary. Gonadotropin-releasing hormone (GnRH) neurons release GnRH in a pulsatile pattern from the arcuate nucleus of the hypothalamus every 90–120 min, stimulating the gonadotropic cells in the anterior pituitary to produce LH and FSH to the circulation and then LH stimulates the Leydig cells in the testis to produce T. Similarly to testicles, adrenal steroid biosynthesis is regulated by a negative feedback system, in which corticotropin-releasing hormone (CRH) secreted from the hypothalamus activates adrenocorticotropin hormone (ACTH) release from the pituitary, and ACTH then stimulates the zona fasciculata cells to produce cortisol (Veldhuis JD et al., 2013) and drives DHEA-S production from zona reticularis cells. Cortisol, but not DHEA-S, utilizes negative feedback on CRH and ACTH.

2.5.2 Androgen biosynthesis in males

The plasma concentrations of steroids only partially reflect to the biological activity of those hormones (Labrie F, 1991a). Steroid synthesis includes multiple reactions by which cholesterol is converted to biologically active steroid hormones, catalyzed by a series of enzymes of cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD)-families. Most steps in the steroid biosynthesis are irreversible, typically catalyzed by CYPs (R. Nelson D, 1998). The terminal steps and the peripheral conversions are commonly mediated by HSDs and are possibly reversible, although every HSD-enzyme has a strong directional preference in the intact cells (Cooper WC et al., 2007, Mizrahi D et al., 2009). These enzymes are mainly located in the membrane of endoplasmic reticulum and mitochondria to catalyze redox reactions using electrons transported from the membrane (Thomas JL et al., 1989, Lachance Y et al., 1990), but also located in the peroxisomes and partly being cytoplasmic (Wang T et al., 2010, Miller WL, 2013). Schematic chart of human steroidogenesis is presented in Figure 5.

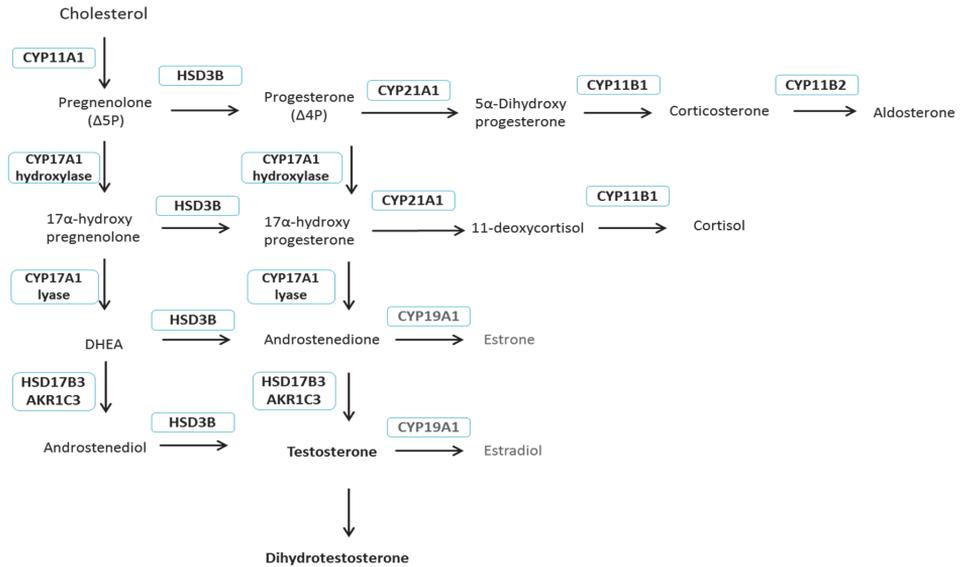


Figure 5: Human steroidogenesis.

The first, rate-limiting and hormonally regulated enzymatic reaction in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone by the side-chain cleavage enzyme (P450_{scc}, named later as CYP11A1). The enzyme converts 27-hydroxycholesterol to 21-pregnenolone; “the mother hormone” of the whole steroid biosynthesis. The reaction takes place in the inner membrane of mitochondria (Black SM et al., 1994, Miller WL, 1998). Three different sequential biochemical steps, hydroxylation at C-22 of cholesterol, followed by hydroxylation at C-20, and finally the cleavage of the C-20-C22 bond (Miller WL et al., 2011) are part of this remarkably slow enzymatic reaction, at the maximum rate of roughly seven turnovers per minute (Tuckey RC et al., 1993, Auchus ML et al., 2012). The transport of cholesterol from the outer to inner mitochondrial membranes, to the site for CYP11A1 catalysis, is provided by steroidogenic acute regulatory protein (StAR) (Clark BJ et al., 1994, Chang TY et al., 2006). However, despite extensive studies; the mechanism of StAR’s action is not yet completely understood (Bose HS et al., 2002, Miller WL, 2007).

Pregnenolone is directly metabolized via the microsomal CYP17A1 enzyme to form 17 α -hydroxypregnenolone. Typically for cytochrome P450 enzymes, residing in the smooth endoplasmic reticulum, CYP17A1 receives electrons from NADPH via the flavoprotein P450-oxidoreductase, both the 17-hydroxylase and 17,20-lyase reactions consume one molecule of each molecular oxygen and NADPH. Additionally, it has been shown that the reaction rates of the 17 α -hydroxylation of pregnenolone and P are roughly equal (Lee-Robichaud P et al., 1995, Auchus RJ et al., 1998). Next the 17,20-lyase reaction catalyzes the formation of the DHEA from 17 α -hydroxypregnenolone and formation of A4 from

17 α -hydroxyprogesterone favoring the production of DHEA (Lee-Robichaud P et al., 1995). This reaction utilizes also small hemoprotein cytochrome b₅, which stimulates the 17,20-lyase activity of CYP17A1 almost 10-fold by an allosteric mechanism (Katagiri M et al., 1995, Lee-Robichaud P et al., 1995, Auchus RJ et al., 1998). In addition, during human P metabolism via CYP17A1 in adrenals, formation of 16 α -hydroxyprogesterone has been observed (Swart P et al., 1993). Although the clinical relevance of 16 α -hydroxyprogesterone remains still unclear, the elevated 16 α -hydroxyprogesterone levels in prostate cancer and several other diseases may contribute to the hormone dependency of these clinical conditions by inducing both AR and progesterone receptor-activated target genes (van Rooyen D et al., 2017). Finally the majority of the DHEA is sulfonated by sulfotransferase SULT2A1 in zona reticularis to form DHEA-sulphate (DHEA-S), one of the major C₁₉ steroids secreted by adrenal gland that circulates in low micromolar concentrations in the bloodstream. Also, small amounts of A4, T, and other 19-carbon steroids are directly formed by the adrenals (Nakamura Y et al., 2009).

In Leydig cells, the pregnenolone is converted by CYP17A1 (P450c17) to 17-OH-pregnenolone and consequently to DHEA. Human Leydig cells richly express 3 β -hydroxysteroid dehydrogenase 2 (3 β HSD2) and 17 β -hydroxysteroid dehydrogenase 3 (17 β -HSD3), which direct primarily the downstream metabolism of DHEA to T (Sharifi N et al., 2012). 3 β HSD enzyme catalyzes two reactions in the steroid biosynthesis: 1) the hydroxyl group conversion to a keto group on C-3 and 2) the isomerization of the double bond from the B ring (Δ 5 steroids; carbon-carbon double bond between C-5 and C-6), to the A ring (Δ 4 steroids) (Thomas JL et al., 1989, Lachance Y et al., 1990, Lorence MC et al., 1990b). 3 β HSD is one of the central enzymes in the steroid biosynthesis producing P from pregnenolone, 17-hydroxyprogesterone from 17-hydroxy-pregnenolone, androstenedione from DHEA and T from A4, respectively (Lee TC et al., 1999). Based on the markedly higher K_m value for the 3 β HSD than for the 17-hydroxylation reaction, the Δ 5 pathway is more favorable in testicles (Auchus RJ et al., 1998). In human, two enzymatically and biochemically very similar 3 β HSD isoforms have been established: 3 β HSD1, catalyzing 3 β HSD activity in the placenta, breast, liver and brain (Lachance Y et al., 1990, Lorence MC et al., 1990a, Simard J et al., 2005) and 3 β HSD2, which is the principal isoform in the gonads and adrenals (Lachance Y et al., 1991, Rheume E et al., 1991).

2.5.3 *Structure and function of androgen receptor*

AR, (NR3C4; nuclear receptor subfamily 3, group C, gene 4), is a member of the steroid receptor superfamily of nuclear receptors and is a ligand-dependent transcription factor, which controls the expression of specific genes (Lubahn DB et al., 1988a, Mangelsdorf DJ et al., 1995). AR regulates, among other things, the growth, differentiation,

angiogenesis, and metabolism of the normal prostate and also in human prostate cancer. The human AR gene has been mapped to the X chromosome, at the locus Xq11-Xq12, encoding for a protein with a molecular mass 110 kDa and consisting of 920 amino acids (Migeon BR et al., 1981, Lubahn DB et al., 1988b, Trapman J et al., 1988, Brown CJ et al., 1989, van Laar JH et al., 1989, McEwan IJ et al., 2000). The AR gene contains eight exons interrupted by introns of varying lengths and show structural similarity to the other class I steroid hormone receptors containing eight exons (Kuiper GG et al., 1989, Lubahn DB et al., 1989). The AR protein consists of three major functional domains: (1) the N-terminal domain (NTD), followed by (2) the DNA binding domain (DBD), and (3) the C-terminal ligand binding domain (LBD), which is connected to the DBD by a flexible hinge region (Mangelsdorf DJ et al., 1995).

NTD is encoded by exon 1 and is critical for engaging the cellular transcription complex, harboring two transactivating regions, termed transcription activation units 1 (TAU1) and 5 (TAU5), both essential for the AR activation (Jenster G et al., 1995). It also binds to the COOH-terminal LBD (Doesburg P et al., 1997). This hormone-dependent interaction between the NTD and LBD might have a role in stabilization of the AR dimer and in stabilization of the ligand receptor complex by reducing the rate of ligand dissociation and receptor degradation (Zhou ZX et al., 1995, Centenera MM et al., 2008). In addition, it has been described that the NTD is capable of modulating DNA binding of AR by interacting with coactivators like the P160 family transcription factors TTB and IIF (McEwan IJ et al., 1997, Bevan CL et al., 1999). This interaction may lead to a lower binding affinity for both selective and non-selective response elements (Brodie J et al., 2005).

DBD consists of a highly preserved core, nearly identical in both AR and glucocorticoid receptors (GR) (Shaffer PL et al., 2004), making up of two zinc-nucleated components encoded by exons 2 and 3, directing the binding of AR to specific DNA sequences (Luisi BF et al., 1991). The nuclear localization signal (NLS), encoded in exon 4, is responsible for nuclear import of the receptor and locates at the junction between the DBD and the hinge region (Jenster G et al., 1993, Zhou ZX et al., 1995).

The hallmark of AR-targeting therapies is the LBD, which is encoded by exons 5-8 (Gelman EP, 2002, Heinlein CA et al., 2004, Claessens F et al., 2008). Its 3D-structure and folding is typical for nuclear receptors (Huang P et al., 2010), albeit the ligand binding pocket is rather flexible and may accommodate ligands with different structures. There are 18 amino acid residues interacting more or less directly with the bound ligand, with a few specific hydrogen bonds and hydrophobic relations defining hormone selectivity (Matias PM et al., 2000). In the absence of these ligands AR is isolated in the cytoplasm bound to chaperone proteins (e.g. Heat shock protein 90), where it is inactive and in a conformation that possesses high affinity for ligand binding. When binding to the ligands in the cytoplasm, the AR separates from chaperone proteins, translocate into the nucleus

and subsequently undergoes homodimerization prior to binding to androgen responsible elements (AREs) in cis-regulatory regions of androgen-dependent target genes (van Royen ME et al., 2012).

KLK3, one of the best characterized AR target gene is encoding a serine protease named prostate-specific antigen (PSA) (Lilja H, 1995). PSA can be detected only in low plasma or serum levels from healthy males, while elevated PSA levels can be measured in prostate cancer, but due to its limited cancer specificity, it might be elevated also in other diseases like prostatitis and benign prostate hyperplasia (BPH). As a diagnostic tool, the detection of PSA from plasma or serum has had a great influence on prostate cancer detection and efficacy of the used treatments (Ryan CJ et al., 2006, Beekman KW et al., 2008).

In prostate epithelial cells AR regulates the expression of NKX3.1 and FOX family transcription factors as well as e.g. IGF1R, UBE2C, UGT2B15, KLK3, TMPRSS2, FKBP5, among others, controlling cell growth, differentiation and function in prostate (Chmelar R et al., 2007, Takayama K et al., 2013). Over 50% of prostate cancer cases were recognized to have erythroblastosis virus E26 oncogene homolog (ERG) over-expression (Tomlins SA et al., 2005). In the majority of tumors, overexpression of ERG is driven by fusion of the ERG gene with transmembrane protease, serine 2 (TMPRSS2), which is a prostate-specific enzyme under the androgen regulation (Yu J et al., 2010). Although multiple studies have been conducted, the functional role of this fusion is not fully understood (Barbieri CE et al., 2015). Additionally, other initiators such as activation of the PI3K/Akt pathway may be required in the presence of fusions to fully induce malignant transformation (Carver BS et al., 2009, King JC et al., 2009).

2.5.4 Sustained androgen receptor signaling in CRPC

Several clinically relevant resistance mechanisms, specific for AR reactivation, have been identified in CRPC with currently available technologies. Unfortunately, most CRPC patients treated with the next-generation AR-targeting therapies will ultimately develop resistance and the progression of the disease. Still, even at this stage, CRPC seems to be driven largely by the AR and its signaling axis. The main resistance mechanisms for CRPC are illustrated in Figure 6.

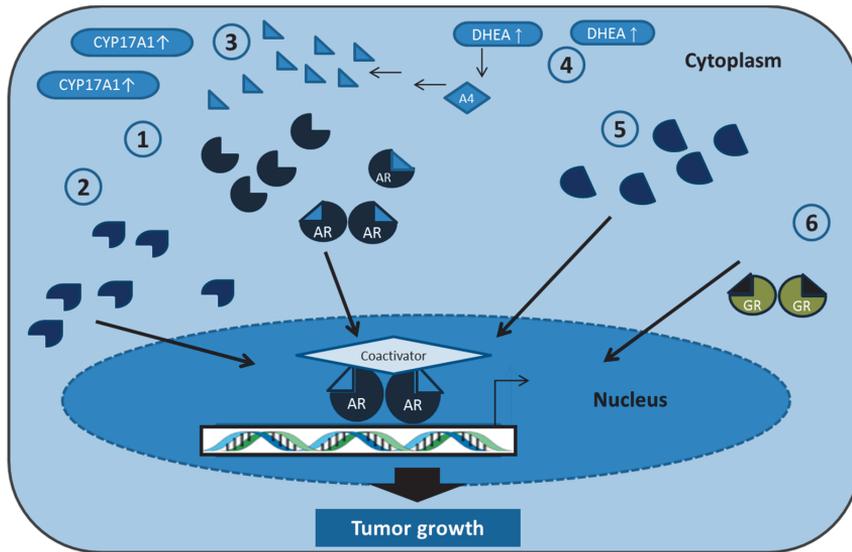


Figure 6: Schematic representation of nuclear receptor mediated resistance mechanisms in CRPC. 1. AR amplification and overexpression, 2. AR point mutations, 3. Increased/altered steroid biosynthesis, 4. Increased amount of adrenal precursors, 5. Constitutively active AR-variants and 6. Activation of GR.

2.5.4.1 AR amplification and overexpression

AR gene amplification, the most frequent genetic alteration in prostate cancer, is observed in up to 50% of CRPC patients (Koivisto P, 1997, Linja MJ et al., 2004, Robinson D et al., 2015), whereas untreated tumors very rarely contain AR gene amplification (Bubendorf L et al., 1999, Network CGAR, 2015). It has been hypothesized that AR gene amplification, with resultant expression of increased AR protein levels, is involved in the development of hormone-resistant prostate cancer (Visakorpi T et al., 1995, Miyoshi Y et al., 2000, Haapala K et al., 2007). Elevated AR expression increases basal AR levels and thereby sensitizes tumor cells to lower levels of androgens, facilitating tumor cell growth when the supply of androgens is limited, for example, as a consequence of hormone-deprivation therapy (Visakorpi T et al., 1995, Chen CD et al., 2004, Haapala K et al., 2007, Waltering KK et al., 2009). The mechanisms leading to AR overexpression without gene amplification are still partly unclear. Increased transcription rates, or stabilization of the mRNA or protein have also been suggested for the reasons, but an instant response to castration and rapid reduction in androgen levels are most probably behind the overexpression because androgens normally suppress AR transcription in prostate epithelial cells (Shan LX et al., 1990). Recently, AR amplification has been detected also from circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA)

isolated from CRPC patients treated with abiraterone and enzalutamide (Azad AA et al., 2015a, Romanel A et al., 2015).

VCaP prostate cancer cell line has AR amplification (Korenchuk S et al., 2001), being a valid model for studying the effects of antiandrogens *in vitro*, and similarly enzalutamide-resistant LNCaP cells express higher levels of AR compared to naïve LNCaP cells (Yamamoto Y et al., 2015). *In vivo*, two different LuCaP PDX-models have been shown to express high AR-levels, which are further increased after the treatment with abiraterone (Mostaghel EA et al., 2011). In addition, *in vivo* xenografted VCaP tumors have increased AR levels, especially in CRPC state (Yu Z et al., 2014b, Knuuttila M et al., 2018), providing excellent tools for nonclinical research of CRPC.

2.5.4.2 AR mutations in CRPC

AR mutations are very rare in early stages of prostate cancer. However, approximately 20% of CRPC patients carry AR mutations, especially when treated with ADT, indicating an adaptation to ADT by altered AR action (Taplin ME et al., 1995, Taplin M-E et al., 2003, Eisermann K et al., 2013). Over 150 different AR mutations have been identified in prostate cancer patients, wherein the LBD is a mutational hotspot and a minority occurs in exon 1 (Gottlieb B et al., 2012). In the LBD, mutations map to amino acid residues 670–678, 701–730, and 874–919 (Buchanan G et al., 2001a), with the key AR mutations being AR(L702H), AR(W742C), AR(H875Y), and AR(T878A) (Barbieri CE et al., 2012, Gottlieb B et al., 2012, Grasso CS et al., 2012, Robinson D et al., 2015). Mutations in the LBD affect both ligand affinity and co-regulator recruitment, causing decreased specificity of AR to androgens and also to activation with other steroids (Feldman BJ et al., 2001). Consequently, anti-androgens activate mutated AR, which may be part of the mechanisms responsible for progression to CRPC (Eisermann K et al., 2013).

The first identified AR mutation was also established in the early 1990s from LNCaP human prostate cancer cell line (Veldscholte J et al., 1990b), and soon after it was verified to be present in tumors with flutamide-treated CRPC patients (Suzuki H et al., 1993, Taplin ME et al., 1999). The AR with AR(T878A) mutation can be activated by the first-generation anti-androgens like hydroxyflutamide and cyproterone acetate, but also by P, glucocorticoids and estrogens (Berrevoets CA et al., 1993, Boudadi K et al., 2016). This mutation has been detected most often after abiraterone acetate treatment (Cai C et al., 2011b, Chen EJ et al., 2015), perhaps due to the efficient CYP17A1 inhibition and increased tissue P levels, which may favor cancer to drive this mutation (Watson PA et al., 2015). These findings may indicate that P or other steroids upstream of CYP17A1, can alter tumor cells for specifically this AR(T878A) mutation and further support that

this might be one mechanism for acquired resistance to abiraterone. In LNCaP cells this mutation occurs naturally, providing a tool for experimental settings.

The second mutation identified, AR(L702H), was also found in hormone refractory prostate cancer patients (Suzuki H et al., 1993, Watanabe M et al., 1997). It was further proven that tumors having this mutation are dependent on endogenous glucocorticoids such as cortisol (Zhao XY et al., 2000). Also exogenous glucocorticoids like dexamethasone have been reported to activate this AR mutation (Krishnan AV et al., 2002, van de Wijngaart DJ et al., 2010). Functional studies with a panel of structurally related steroids have shown that the steroidal 17 α -hydroxyl group plays a crucial role in the activation of AR(L702H) (van de Wijngaart DJ et al., 2010). This mutation, in combination with T878A, is present in MDA prostate cancer cells, thus providing a valuable preclinical model (Navone NM et al., 1997, Zhao XY et al., 1999).

The third mutation, AR(W742C) was identified from xenograft that developed resistance to bicalutamide (Hara T et al., 2003). It also modifies hydroxyflutamide activity from antagonism to agonist (Watson PA et al., 2015). However, its presence in clinical samples is relatively rare, although bicalutamide has been extensively used world-wide for almost two decades (Grasso CS et al., 2012, Robinson D et al., 2015). The growth of KUCaP xenografts carrying the AR(W742C) mutation is accelerated by treatment with bicalutamide and flutamide (Yoshida T et al., 2005). The fourth mutation, AR(H875Y), has been shown to be activated by DHEA, estradiol, P, and hydroxyflutamide and reported to produce a greater transcriptional response than the wild-type AR (Taplin ME et al., 1995, Tan J-a et al., 1997, Steketee K et al., 2002). It has also been shown to be present in the CWR22Rc1 cell line (Sramkoski RM et al., 1999) which is derived from a primary metastasized prostate cancer (Pretlow TG et al., 1993) and therefore, it might be a relevant mutation also in CRPC.

The most recently found AR mutation, AR(F877L), was identified *in vitro* in LNCaP cells after treatment with second-generation antiandrogen enzalutamide (Balbas MD et al., 2013, Korpál M et al., 2013). By using circulating cell-free DNA, F877L was further identified in some progressive-state CRPC patients treated with apalutamide (Joseph JD et al., 2013) or enzalutamide (Azad AA et al., 2015a). This mutation alters the antagonistic activity of enzalutamide and could potentially allow agonistic activity for the drug (Eisermann K et al., 2013). However, the clinical significance of this mutation needs further validation and wider studies (Rodríguez-Vida A et al., 2015).

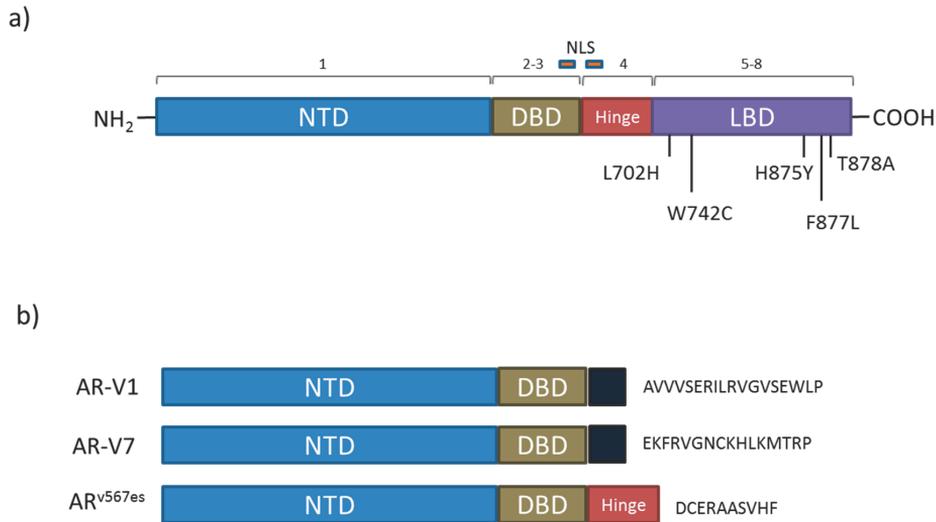


Figure 7: The protein structure of androgen receptor (AR), mutations associated to CRPC and AR splice variants. a) AR is 920 amino acids long, consisting of four functional domains, which are encoded by eight exons. The nuclear localization signal (NLS) marked within exons 3 and 4. The key mutations described to be relevant in CRPC. b) The protein structures of truncated AR splice variants presented with the in-frame of variant specific amino acids derived from the alternative splicing events.

2.5.4.3 Androgen Receptor Splice Variants

It has been estimated that 90% of human genes go through alternative splicing (Wang GS et al., 2007, Pan Q et al., 2008), and these events have also been described in prostate cancer (Tepper CG et al., 2002, Dehm SM et al., 2011, Haile S et al., 2011). Initially these variants were thought to be a product of calpain-mediated cleavage of full-length protein (Libertini SJ et al., 2007), not a change in mRNA splicing. Subsequently, numerous AR splice variants (AR-Vs) in mRNA and protein level have been recognized (Guo Z et al., 2009). During the progression of CRPC, as a result of ADT, increased expression of both full length AR (Shan LX et al., 1990) and also the levels of AR-Vs have been detected (Watson PA et al., 2010, Chan SC et al., 2014). Recently, also resistance during abiraterone acetate or enzalutamide treatments has been suggested to induce AR mRNA splicing (Antonarakis ES et al., 2014, Ware KE et al., 2014). AR-Vs are expressed also in the normal prostate (Hornberg E et al., 2011), but the expression levels in CRPC samples have been higher (Guo Z et al., 2009).

To date, three different AR splice variants (AR-V1, AR-V7 and AR^{v567es}) in human have been identified (Guo Z et al., 2009, Hu R et al., 2009, Watson PA et al., 2010, Hu R et al., 2011, Hu R et al., 2012). AR-V1 encodes a splice variant consisting of exons 1–3,

ending in a cryptic exon (CE1) which was joined with exon 3. AR-V7 (old name AR3) encodes a protein with exons 1–3 and a c-terminal cryptic exon called (CE3) located within intron 3. This is an AR splice variant having an antibody, which allows detection from clinical samples. The third clinically relevant variant AR^{v567es} encodes a protein comprised of exons 1–4, and because of a frame-shift due to loss of exons 5–7, exon 8 has a stop codon generated after the first 10 amino acids causing a shortened exon 8 (Nyquist MD et al., 2013).

A common structural feature of these variants is the total or partial elimination of the LBD still having the NH₂-terminal and DNA-binding domain (Arora VK et al., 2013, Scher HI et al., 2016). Although no ligand binding occurs, the truncated proteins have been suggested to remain constitutively active, and thus, capable of acting as transcription factors (Hu R et al., 2009, Sun S et al., 2010, Ware KE et al., 2014). In addition, these incomplete AR structures have been proposed to lead to general resistance to current therapeutics such as AR antagonists, since their mode of action is based on the binding to the LBD (Zhang X et al., 2011). However, only two AR-Vs have a complete NLS, AR^{v567es} and murine mAR-V4 retain both exons 3 and 4, and display such properties, which have been linked to constitutive androgen-independent nuclear translocation (Watson PA et al., 2010, Hu R et al., 2011). Antonarakis and colleagues showed the link between detectable levels of AR-V7 mRNA (isolated from circulating tumor cells) and the resistance to enzalutamide and abiraterone treatments (Antonarakis ES et al., 2014). AR-V7 was more common in patients treated with enzalutamide but having no advantage compared to patients responding to enzalutamide (Efstathiou E et al., 2015). In addition, in the same study, the amount of cytoplasmic AR correlated to decreased PSA levels and increased T levels, suggesting that enzalutamide suppresses the AR signaling but could also induce the adaptive feedback (Efstathiou E et al., 2015). Significant progression and critical advances have been made during the past decade since the first observation of AR-Vs. However, further research is still needed to explain the role of AR-Vs in mCRPC (Luo J et al., 2017). The main unresolved question is, whether AR-Vs drive therapeutic resistance or are they more like passengers acting as biomarkers.

AR-Vs have been studied widely in different nonclinical models. In VCaP xenograft tumors rapid increase of full length AR and AR-V7 at protein level was shown after castration (Watson PA et al., 2010, Knuutila M et al., 2014). The highest AR-V levels have been presented to remain in the 22rv1 cells, which are resistant to enzalutamide (Dehm SM et al., 2008). However, the growth is only weakly driven by androgens (Li Y et al., 2013) and might not represent the clinical state, although a lot of AR-V7 is exhibited (Ma Y et al., 2016). Some studies have presented that by knocking down the AR-V7 in prostate cancer cell lines sensitivity to enzalutamide has been increased (Nadiminty N et al., 2013, Liu C et al., 2014).

2.5.4.4 Expression of AR co-factors

A series of co-regulator complexes increase (co-activators) or suppress (co-repressors) transcriptional activity of AR and more than 300 different co-regulators have already been recognized (Heemers HV et al., 2007, Wang GS et al., 2007, DePriest AD et al., 2016). The general characteristic is shared between many AR co-regulators modulating other proteins in the complex. They can affect through phosphorylation, methylation, acetylation or ubiquitylation, but also as molecular chaperones, recruiters of transcriptional machinery and RNA splicing regulators (Wolf IM et al., 2008). Co-regulators can alter transcriptional activity through a variety of mechanisms like AR stabilization, inducing homodimerization and nuclear translocation of AR, and by altered chromatin structure and DNA occupancy, through enrollment of general transcription factors, and by priming and assembling of the transcription preinitiation complex (Heemers HV et al., 2007, Shiota M et al., 2011).

The first identified and most known of these coregulatory proteins belong to the p160 coactivator family, including steroid receptor coactivator 1 (SRC1), transcription intermediary factor 2 (TIF2) and steroid receptor coactivator 3 (SRC3). Expression levels of the SRC family members are increased in prostate cancer, and especially high SRC1 and TIF2 levels are reported in CRPC (Heemers HV et al., 2007, Taylor BS et al., 2010, Hodgson MC et al., 2012). They specifically bind to the AR NTD, influencing transactivation through their direct histone acetyltransferase activity, as well as through indirect recruitment of secondary coactivators such as p300 to induce chromatin remodeling (Chakravarti D et al., 1996), and probably leading to increased sensitivity of AR to weak agonists such as A4 and DHEA (Taylor BS et al., 2010). ARA70 and ARA55 may also affect ligand specificity of the receptor. It has been shown that ARA70 overexpression may improve AR activation in response to weak AR agonists or enable AR antagonists to act as agonists (Yeh S et al., 1999). Furthermore, ARA55 has been reported to bind in a hormone-dependent manner through its C-terminal LIM domains which results in increased AR activity and altered specificity of receptor binding to alternative ligands to bind to AR (Fujimoto N et al., 1999, Kasai M et al., 2003).

2.5.4.5 The role of glucocorticoid receptor in CRPC

Glucocorticoid receptor (GR) belongs to the same nuclear receptor family as AR, both having highly conserved DBD domain with 83% identity, and the similarities of NTD and LBD are 15% and 98%, respectively (Claessens F et al., 2014). All class I steroid receptors including also progesterone (PR) and mineralocorticoid receptors (MR) have high similarity (Mangelsdorf DJ et al., 1995). In treatment naïve prostate cancer, GR levels have been reported to be low or missing (Yemelyanov A et al., 2012, Isikbay M et

al., 2014), but on the CRPC state GR has been identified both in the models of enzalutamide resistance and in the bone metastases of patients treated with enzalutamide (Arora VK et al., 2013). The increased GR was associated with poor clinical response to enzalutamide, and in the absence of androgens GR may also interact with AREs altering the expression of numerous, but not all, AR target genes (Arora VK et al., 2013). In the preclinical setting the use of GR antagonist has restored tumors' sensitivity to enzalutamide (Arora VK et al., 2013). However contradictory to AR, GR is vital for life and clinical use of GR antagonist is not an option. These findings and their clinical significance need still further analyses from CRPC clinical specimens (Sharifi N, 2014).

Synthetic glucocorticoids, with the antineoplastic effects, are widely used in patients with CRPC as a part of systemic therapies like cytotoxic chemotherapy with taxanes. They are used to manage adverse effects e.g. pain, nausea, vomiting, hypersensitivity and fluid retention (Tannock I et al., 1989a, de Bono JS et al., 2010, Schwartz JR, 2012) and are also used with abiraterone as glucocorticoid replacement therapy (de Bono JS et al., 2011). Glucocorticoids can be also used for palliative purposes alone or as a combination with mitoxantrone (Kantoff PW et al., 1999, Berry W et al., 2002, Tannock IF et al., 2004). Glucocorticoids have also many beneficial effects in CRPC, such as anti-inflammatory effects and suppression of cell proliferation and angiogenesis (Nishimura K et al., 2001, Yano A et al., 2006). In numerous clinical trials the concomitant glucocorticoid use with ADT or abiraterone or also as single-agent treatment leads to suppression of ACTH, resulting in reduced levels of circulating adrenal androgenic precursors, such as A4 or DHEA (Geller J et al., 1985, Storlie JA et al., 1995, de Bono JS et al., 2011, Holder SL et al., 2015, Venkitaraman R et al., 2015).

The effects of glucocorticoids on prostate cancer could be also contradictory. Increased expression of heat shock proteins, which facilitate binding of DHT to AR, and thereby enhance AR-mediated transcriptional activity, has been observed (Azad AA et al., 2015b). In addition, glucocorticoids, especially dexamethasone has been shown to suppress secretion of IL-6, which can promote the progression of CRPC towards a hormonally independent disease (Yan TZ et al., 2008, Richards J et al., 2012).

2.5.4.6 Altered steroidogenesis in CRPC

DHT was conducted already in 1978 from the CRPC tissue (Geller J et al., 1978), but the method used allowed cross-reactivity with other hormones causing some uncertainties (Sharifi N, 2013). Two decades later, similar findings were described in several studies both in local prostate cancer and CRPC (Nishiyama T et al., 2004, Titus MA et al., 2005, Montgomery RB et al., 2008). DHT concentrations around 1 nM are enough for AR activation and induction of the AR-dependent gene expression (Deslypere JP et al., 1992).

In advanced prostate cancer, where GnRH analogues have been standard of care for more than three decades, testicular T production is inhibited almost completely (~97%) (Labrie F et al., 2009). However, an observation in the same study demonstrated intraprostatic DHT levels to be decreased only by roughly 60% (Labrie F et al., 2009), showing extratesticular and also intracrine androgen production (Labrie F, 1991b, Labrie F, 2003).

Already in 1985 Labrie and colleagues showed that DHEA, a weak androgen precursor, was produced in the adrenal cortex, within the zona reticularis (Labrie F et al., 1985). Later also other adrenal androgens, like A4, DHEA-S the sulphate ester of DHEA, and 11-beta-hydroxyandrostenedione (11-OHA4) have been confirmed to be secreted from the adrenal gland (Endoh A et al., 1996, Labrie F, 2011, Rege J et al., 2013). Androgen synthesis in the adrenal cortex takes place via the classical $\Delta 5$ pathway (Miller WL et al., 2011, Turcu AF et al., 2015), and the 17,20-lyase activity of CYP17A1, catalyzed with the small hemoprotein cytochrome b₅, produces C-19 androgen DHEA (Auchus RJ et al., 1998). DHEA can be further converted to A4 by 3 β HSD2 enzyme or can be converted to DHEA-S, present at the micromolar (μ M) concentration in the bloodstream of adult men. Both DHEA and A4 can be further converted to active androgens, such as T and DHT, in CRPC tissue via 3 β HSD and SRD5A isoenzymes, both of which have been shown to be present in cancerous tissues, thus suggesting that the tumors may acquire metabolic capacity to increase the local concentration of biologically active androgens (Holzbeierlein J et al., 2004, Mohler JL et al., 2004a, Montgomery RB et al., 2008).

Recently in CRPC a gain-of-function mutation was identified in one of the key steroidogenesis enzymes, namely 3 β HSD1 (Evaul K et al., 2010). This arises from a single nucleotide change at position 1245 (A to C), substituting an asparagine for threonine at amino acid position 367. The significance of this alteration is that the mutated enzyme increases resistance to ubiquitin-mediated decline, leading to intracellular accumulation of the protein, and to increased capacity of this enzyme to drive conversion of DHEA to A4, thereby allowing more efficient DHT synthesis in tumors (Chang KH et al., 2013). Furthermore, it has also been suggested that ADT may select for this mutation particularly in CRPC tumors in patients, who are heterozygous for the variant, losing of heterozygosity or obtaining a second variant allele through a somatic mutation (Chang KH et al., 2013). This would lead to stable enzyme expression, thus providing another adaptive mechanism through which tumors possibly recur under androgen deprivation. Thereby, the gain-of-function 3 β HSD1 (1245C) SNP is associated with quick resistance and poorer survival after ADT in patients with prostate cancer (Hearn JWD et al., 2016).

To date, the sources of residual androgens in the CRPC tumors are not completely elucidated. Several organs including adrenal glands, fat, skin and kidney can uptake and metabolize weak circulating adrenal androgens and support conversion towards more active androgens (Labrie F, 2011, Schiffer L et al., 2017). Three generally accepted synthetic pathways for increased levels of signaling androgens in CRPC tumors have been

described: the classical (canonical) *de novo* pathway from cholesterol in adrenal glands, peripheral tissues and tumors, the backdoor pathway, and the 5 α -dione pathway in tumors. All three pathways are illustrated in Figure 8.

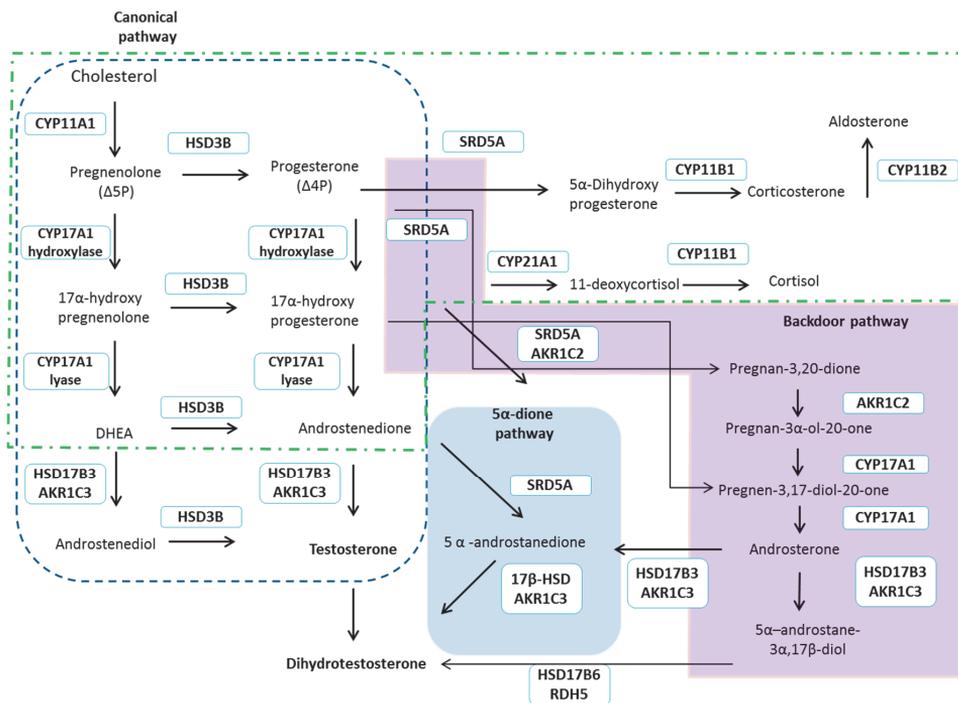


Figure 8: Classical and nonclassical pathways of androgen biosynthesis including adrenal steroid biosynthesis. The backdoor pathway marked in light pink and the 5 α -dione pathway in blue. The classical pathway, which is known to occur in the testicles, is marked with blue dash line and in the adrenal glands with green dash line.

As described in section 2.5.2, in the classical pathway of androgen biosynthesis pregnenolone and P (C-21 steroids) produced from the cholesterol (C-27 steroid) by CYP11A1, are first converted to DHEA and A4 (C-19 steroids) via sequential hydroxylase and lyase reactions, both catalyzed by CYP17A1 enzyme. Then precursors are further metabolized to T via HSD17 β 3, shown to be mainly testis-specific, or by the aldo-keto reductase family 1 member C3 (AKR1C3) enzyme, which has been described to catalyze this step within other tissues (Nakamura Y et al., 2009). Finally, the peripheral conversion of T to DHT is carried out by SRD5A1 or SRD5A2 in the target tissues (Andersson S et al., 1990, Fung KM et al., 2006, Mostaghel EA et al., 2008). Both CYP11A1 and CYP17A1 have been reported to be present also in CRPC tissue (Locke JA et al., 2008). Additionally, the transcripts encoding these proteins are up-regulated in CRPC unlike in treatment-naïve prostate cancer (Montgomery RB et al., 2008).

Efstathiou and colleagues have also shown the presence of CYP17A1 in a subset of mCRPC tumor-infiltrated bone marrow samples (Efstathiou E et al., 2012). Contradictory findings from metastatic tumor specimen have been also published, showing only low abundances of transcripts encoding CYP17A1 and 3 β HSD1, both essential for *de novo* steroidogenesis, (Hofland J et al., 2010).

CRPC tissue has been shown to express more SRD5A1 than SRD5A2 (Titus MA et al., 2005, Stanbrough M et al., 2006a, Thomas LN et al., 2008). It has also been shown that SRD5A1 catalyzes the hepatic 5 α -reduction, in contrast to the adrenal and gonadal steroid biosynthesis, where SRD5A2 is found to be dominant (Andersson S et al., 1990, Thigpen AE et al., 1993). These differences in the expression levels of SRD5A1 and 2 and a new isoform SRD5A3, whose role in androgen metabolism is not fully understood, may cause alterations in the DHT formation (Mitsiades N et al., 2012). The enzymatic machinery has been proven to occur also in the CRPC tissue. Eight enzymatic steps are required for the production of DHT from cholesterol via the classical pathway. The numerous adrenal precursors in serum serve as the major substrate pool of androgen production in CRPC. Kumagai and colleagues showed *in vitro* that three androgen dependent cell lines, LNCaP, VCaP and PC346C, convert adrenal androgen precursors DHEA, androstenedione and A4 for more active androgens, which activate AR-regulated cell growth, concluding that *de novo* steroidogenesis contributes much less to cell growth than adrenal androgen precursors do in these cell lines (Kumagai J et al., 2013a). However, the classical pathway in CRPC cannot be definitively ruled out.

In the backdoor pathway, originally detected in Tammar Wallabies, the formation of 5 α -androstane-3 α ,17 β -diol offers an alternative route via Δ 5-pathway for DHT synthesis, without involving T as an intermediate (Auchus RJ, 2004a). In this pathway, the C-21 precursors, P and more actively 17 α -hydroxyprogesterone are converted to pregnan-3,20-dione and pregnen-3,17-diol-20-one by SRD5A. The next step is catalyzed by AKR1C2 forming pregnan-3,20-dione to pregnan-3 α -ol-20-one, and forming 17 α -hydroxyprogesterone to pregnen-3,17-diol-20-one, respectively. Further conversion is catalyzed by CYP17A1 to androsterone (Auchus RJ, 2004a, Fiandalo MV et al., 2014), which is then metabolized to 5 α -androstane-3 α ,17 β -diol by 17 β HSD3 and AKR1C3 similar to the classical pathway (Penning TM et al., 2000). The last step, conversion to DHT, is catalyzed by retinol dehydrogenase 5 (Auchus RJ, 2004a, Locke JA et al., 2008), HSD17 β 6, and HSD17 β 10 (Bauman DR et al., 2006, Penning TM et al., 2007).

This route is shown in castrated LNCaP xenografts, where ¹⁴C-labelled acetic acid has been used as starting material. The synthesis of P, pregnen-3,17-diol-20-one and DHT were detected, albeit in relatively low abundancies (Leon CG et al., 2010), while no T production was observed. The results from another *in vivo* study with LNCaP xenografts indicate that tumors might use this backdoor pathway more actively when animals are

treated with androgen synthesis inhibitors like ketoconazole and finasteride (Locke JA et al., 2009).

The third alternative pathway named 5 α -dione pathway, first described in 2011 by Chang and colleagues, suggesting intratumoral DHT production without using T as a substrate. In this route A4 is primarily converted to 5 α -androstanedione via SRD5A1 and then further reduced to DHT catalyzed by HSD17 β 3 (Chang K-H et al., 2011, Campbell TJ et al., 2012). The main evidence of this pathway in CRPC is produced in preclinical models with six different prostate cancer cell lines, and the reaction was confirmed by studying fresh metastatic CRPC tissues from two patients (Chang K-H et al., 2011).

2.5.5 Newly identified derivatives and intermediates of adrenal steroid biosynthesis activating AR

Besides classical intermediates and end products of adrenal hormones, including gluco-, and mineralocorticoids, several other precursors have been recently identified. These include 11-deoxycorticosterone (11-DOC), immediate precursor of 11-corticosterone and 11 β -hydroxyandrostenedione (11-OHA4), both suggested to be precursors for AR-activating ligands or directly activating AR and thus playing a role in the progress of CRPC (Uemura M et al., 2010, Bloem LM et al., 2013, Storbeck KH et al., 2013, Swart AC et al., 2013). Clinical relevance was provided in studies, where low amounts of 5 α -11-DOC, a direct metabolite of 11-DOC formed via SRD5A, were detected in CRPC tissue (Mohler JL et al., 2004b). 11-OHA4 has been recognized in the adrenal androgen synthesis pathway (Schloms L et al., 2012) and confirmed to be an abundant adrenal C-19 steroid in the circulation (Yokokawa A et al., 2009, Rege J et al., 2013). This novel pathway was identified also in the androgen-dependent prostate cancer cell lines producing novel androgens such as 11-ketotestosterone (11-KT) and 11-keto-5 α dihydrotestosterone (11-KDHT) *in vitro* (Swart AC et al., 2013). Both of these compounds show potent AR agonism in the AR-dependent prostate cancer cells *in vitro*, presenting androgenic potencies equal to those of T and DHT, respectively (Pretorius E et al., 2016). In addition, their effects on known AR-regulated genes and protein expression are similar to those of T and DHT. However, their metabolism appears to be significantly slower than that of T and DHT, which may indicate substantial relevance in CRPC (Pretorius E et al., 2016). Small abundances of 11KT and 11KDHT have been further demonstrated in prostate samples from CRPC patients (du Toit T et al., 2018). A schematic chart of 19-C ketosteroid metabolism from 11OHA4 in CRPC is presented in Figure 9.

In addition to new AR axis inhibitors, “bipolar” androgen therapy with supraphysiological levels of T has been shown to prompt apoptosis in CRPC cells, where AR is overexpressed (D'Antonio JM et al., 2009). Rapid cycles of high T and near-castrate levels of T were used in the therapy, showing decreased AR expression and delay in resistance (Denmeade SR et al., 2010). Several other similar but relatively small trials have shown declined PSA and restored sensitivity to ADT (Schweizer MT et al., 2015, Teply BA et al., 2017). Currently, a larger randomized phase II study (TRANSFORMER, NCT02286921) is ongoing comparing bipolar androgen therapy with enzalutamide in asymptomatic men with mCRPC patients with previous failure on abiraterone acetate therapy (Dellis A et al., 2016, Mohammad OS et al., 2017).

3 AIMS OF THE STUDY

CRPC is an incurable disease, albeit great progress has been made in research during the past decade. Earlier detection of the disease and a growing number of available new treatment options have increased the positive signs and improvement in patient outcome. Although promising responses to novel treatments have been seen, in most cases resistance will eventually develop. Usually this is detected biochemically by a rise in serum PSA, which may again indicate a resurgence of AR activation.

Preclinical models, mostly mouse xenografts, have been widely used by studying the mechanisms of CRPC resistance and also in CRPC drug development. However, the species differences between human and mouse, especially in the adrenal androgen production has been assumed to affect uncertainties in the results and needs further validation.

The studies included in this thesis have been conducted as part of nonclinical discovery programs aiming to develop novel therapies for CRPC targeted towards the AR signaling axis.

The specific aims were:

- To test novel discovery drug candidates acting on AR-signaling axis for treatment of CRPC in multiple nonclinical *in vitro* and *in vivo* models.
- To compare the nonclinical efficacy of these novel drug candidates to the clinical reference compounds.
- To attempt to identify the benefits and challenges of these novel drug candidates compared to the clinical reference compounds.
- To characterize the mouse adrenal gland steroid hormone biosynthesis and steroid production in a CRPC xenograft model.
- To evaluate the significance of mouse adrenal steroid hormones to the growth of CRPC VCaP tumors in the xenograft model.

4 MATERIALS AND METHODS

4.1 General experimental design

In studies I and II the nonclinical development, including the evaluation of unique pharmacological profiles of novel, AR signaling agents darolutamide (ODM-201) (AR inhibitor) and ODM-204 (AR and CYP17A1 dual inhibitor) were carried out in *in vitro* and *in vivo*; both of the compounds are aimed to treat castration-resistant prostate cancer. The influence of mouse adrenal steroid hormones in the growth of androgen-dependent VCaP CRPC xenograft tumors was evaluated in study III. The value of preclinical mouse models in studying CRPC has to date been questioned due to the assumption that, in contrast to human, mouse adrenals do not produce steroids activating the AR.

All three studies were proof-of-principle studies, and part of the studies aim to produce new, better treatment options for CRPC. Furthermore our novel findings show that adrenal steroid production has an effect on VCaP CRPC xenograft tumor growth providing new nonclinical tools for CRPC research.

4.2 Screening cascades used for efficient compound optimization (I,II)

To ensure efficient optimization of new compounds with multiple parameters to improve in antiandrogen and combined antiandrogen and CYP17A1 inhibitor projects several assays were applied. The screening cascade used is presented in Table 1. It includes several steps, and only the best compounds progressed to *in vivo* studies. The systematic use of the screening funnel ensured appropriate progression of both ODM-201 and ODM-204 drug discovery projects and minimized the use of laboratory animals.

Table 1: Screening cascade applied for optimizing novel compounds.

The first screening cascade	
For antiandrogenic properties	CYP17A1 inhibition
Competitive AR binding assay AR antagonism in AR-HEK293 cells	Inhibition of 17,20-lyase in H295 cells
The second screening cascade	
Inhibition of androgen-dependent VCaP and LNCaP cell proliferation	Inhibition of 17 α -hydroxylation in ex vivo testicular microsomal assay
The third screening cascade	
Testing compounds against key AR mutations in vitro In vivo pharmacokinetics after single dose	Inhibition of hCG induced T production in rats after single dose of test compound
In vivo efficacy models	
Effect on test compounds to the castration-resistant growth of VCaP xenografted ORX mice	Effect of test compound after 14-day of dosing in intact rats In vivo pharmacokinetics and pharmacodynamics of test compound after single dose in intact male monkeys Effect on test compounds to the growth of VCaP xenografted intact mice

4.3 *In vitro* models

4.3.1 *Competitive AR binding assay (I, II)*

AR binding affinities of the compounds were studied in cytosolic fractions obtained from ventral prostates of castrated rats by a competition binding assay as previously described (Schilling K et al., 1984). Prostates were minced, and homogenized in buffer containing protease inhibitors (Roche). Supernatants were separated by centrifugation (220 000g, 45 min) and eluted with a dextran-coated charcoal solution to remove endogenous steroids. The dissociation constant of the [³H]-mibolerone was determined by a saturation binding method as previously described (Isomaa V et al., 1982). For the determination of Ki values, prostate cytosol preparations and 1nM [³H]-mibolerone were incubated overnight with increasing concentrations of test compounds. Then steroids were separated by treatment with dextran-coated charcoal suspension and bound radioactivity was

determined by using a microbeta counter (1450 MicroBeta Trilux, Liquid Scintillation & Luminescence Counter, Wallac). All procedures were carried out at 0–4 °C.

4.3.2 Cell lines (I, II)

Table 2: Cell lines used in I-III: All cells were grown in a humidified incubator with 5% CO₂ at 37 °C and cell culture reagents were purchased from Gibco.

Cell line	Origin	Additional information	Medium used	Study used
LNCaP	ECACC		RPMI-1640	II
VCaP	ATCC		RPMI-1640	I-III
DU-145	ATCC		DMEM	I, II
H295R	ATCC		DMEM+F12 Nutrient mixture (1:1)	I, II
H1851	ATCC		DMEM+F12 Nutrient mixture (1:1)	I, II
U2-OS	University of Helsinki	Used mutant AR vectors: pSG5-hAR-F877L pSG5-hAR-T878A pSG5-hAR-W742L Reporter gene construct: pGV5-basic-GRE-hiv-luc	DMEM	I, II
LN-AR-C	University of Tampere	Overexpressing of AR	RPMI-1640	I
HEK293	ATCC		DMEM	
AR-HEK293		Used gene constructs: Full length human AR (pSG5-hAR) androgen-responsive reporter (pcDNA3.1/GRE2-TK-Luc).	DMEM	I, II
HS-HEK293		A cell clone of AR-HEK293 having > 5-fold overexpression of AR	DMEM	I, II

All media were supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES and GlutaMAX (2 mM for AR-HEK293, HS-HEK293, and U2-OS) or (4 mM for VCaP, LNCaP, DU-145, LN-AR-C, and H1581). For maintaining the selection, the medium was supplemented with geneticin (50 mg/ml, LN-AR-C cells) or with hygromycin and geneticin (both 50 mg/ml, HS-HEK cells). For *in vitro* assays, corresponding phenol red-free media supplemented with steroid-depleted FBS were used.

4.3.3 AR antagonism (I, II)

Functional activity and potency of antiandrogens to activate hAR were determined in AR-HEK293 cells. The cells were incubated overnight in steroid-free assay, at 37 °C with 5% CO₂ with test compounds and 0.45 nM of T. After lysing of cells, activity was measured using a luciferase assay system (Promega Corporation), according to manufacturer's instructions with a Centro LB 960 microplate luminometer (Berthold Technologies).

4.3.4 AR nuclear translocation (I, II)

In the studies I and II, AR overexpressing HS-HEK293 cells were plated on microplates (BD) in medium. After 2-day incubation, cells were treated with test compounds together with 0.3 nM T for 4 (II) or 5 (I) hours, fixed and washed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 (Sigma), and with 3% BSA to block unspecific staining. For a high-content screening reader (Cellomics ArrayScan HCS VTI reader, Thermo), cells were incubated with Alexa Fluor® 488 conjugated AR antibody (details of used antibodies described in Table 6), washed and DNA was labeled with DAPI (Sigma, 1 µg/ml). Images were analyzed with a NucTrans. V3 assay algorithm (Thermo). In study I, AR nuclear translocation was also studied with a confocal microscope (LSM780, Zeiss). For that cells (HS-HEK293 or LN-AR-C) were plated on coverslips, incubated 48 h, treated with test compounds in a combination with T for 4 h, immunolabeled with AR antibody, washed, and treated with a secondary antibody labelled with Alexa Fluor® 546 and imaged. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories).

4.3.5 LNCaP and VCaP proliferation assays (I, II)

LNCaP or VCaP cells were treated with a submaximal concentration of previously optimized concentration of mibolerone and increasing concentrations of test compounds.

After 4-day incubation with the compounds, the cell viability was measured using a WST-1 cell proliferation assay (Roche), according to manufacturer's instructions. In addition, to rule out non-AR –mediated toxicity, AR-negative DU-145 and H1581 cells were treated with increasing concentrations of test compounds, and cell viability was measured as described above.

4.3.6 Mutant AR studies (I)

Expression vectors encoding AR mutants AR(F877L), AR(T878A), or AR(W742L) were transiently transfected with an androgen-responsive reporter gene construct to human U2-OS osteosarcoma cells using Lipofectamin TM2000 (Invitrogen). The mutant AR expression vectors were constructed as previously described (Adeyemo O et al., 1993). Assays were run in a 96-well format, with 1:20 of receptor and reporter construct DNAs diluted in Opti-MEM® and applied into each well. Increasing concentrations of the test compounds, with or without reference agonists T (0.6 nM) or DHT (10 nM) to induce reporter gene activation, were dosed to cells and incubated overnight. Luciferase activity was measured according to manufacturer's instructions with a microplate luminometer (Centro LB 960, Berthold Technologies).

4.3.7 In vitro inhibition of CYP17A1 (II)

Formation of 17 α -hydroxyprogesterone from P was tested using human, monkey, and rat testicular microsomes. Human testicular tissues were collected at the Turku University Hospital (Finland) from male patients with testicular tumors or prostate cancer (Clinical study protocol 3102001), monkey tissue was collected from sexually mature male monkeys at Covance Inc. (Münster, Germany) and rat testicular tissue from sexually mature rats at Orion Pharma (Turku, Finland). Samples were snap-frozen in liquid nitrogen and stored at –70 °C. To prepare the microsomes testicular tissues were homogenized 1:3 or 1:4 in cold 0.1 M phosphate buffer (pH 7.4, containing 5 mM MgCl₂) with homogenizing probes (Omni International) or using Potter-S homogenizer. Microsomal fractions were isolated by differential ultra-centrifugation at +4 °C and pellets were re-suspended in homogenization buffer further measuring protein concentrations with a protein assay kit (human and monkey samples) with protein assay dye reagent concentrate and rat samples with Pierce micro BCA protein assay kit according to manufacturer's instructions.

For the CYP17A1 inhibition studies, microsomal pools were prepared for each species by combining equal amounts of protein from each individual sample. P (0.5 μ M for human and monkey, 1.5 μ M for rat, final incubation concentrations), 0.1 mM phosphate

buffer (pH 7.4) containing 5 mM MgCl₂, and microsomes (0.05 mg/ml of final incubation volume) were preincubated (4 min) in the absence and presence of the increasing concentrations of compounds. Enzymatic reactions were started by NADPH and terminated after 15 minutes by acetonitrile. The samples were analyzed using liquid chromatography - tandem mass spectrometry (LC/MS-MS) to determine the level of 17 α -hydroxyprogesterone.

Conversion of 17 α -[21-³H] hydroxypregnenolone into DHEA and [³H]-labeled acetic acid was studied with the human adrenal cortex cell line H295R. Cells were seeded and incubated overnight at +37° C. Increasing concentrations of test compounds were added on cells and immediately thereafter, [³H]-labeled 17- α -hydroxypregnenolone was added in a final concentration of 3 nM, and incubated overnight at +37 °C, 5% CO₂. The activity of test compounds was measured from the supernatants, first extracting all steroids from the reaction mixture with dextran-coated charcoal suspension, then adding two volumes of scintillation liquid and by determining the formed [³H]-labeled acetic acid with a microplate counter on the next day.

4.4 *In vivo* models (I-III)

All animal studies were conducted in accordance with EU legislation (2010), and approved by the national Institutional Animal Care and Use Committees (Finnish and German) and the Animal Experiment Boards, and they fully meet the requirements as defined in the U.S. National Institutes of Health guidelines on animal experimentation. The following license numbers were used in the studies: ESAVI/2010/ 04566/Ym-23, ESAVI/7472 /04.10.03/2012 and ESAVI/1993 /04.10.03 /2011.

Table 3: Animals used in I-III. All used animals were males and their ages at the beginning of the study are given below.

Study	Species	Strain	Age
I	Mouse	BALB/c nude	7 weeks
		Hsd:Athymic Nude- Foxn1nu	7 weeks
		BALB/c	8-9 weeks
		Balb/cOlaHsd	8-9 weeks
II	Rat	HsdRCC HanWist	8 weeks
		Sprague Dawley	6 weeks
	Mouse	Hsd:Athymic Nude-Foxn1nu	7 weeks
	Monkey	Macaca fascicularis (Mauritius)	5-9 years
III	Mouse	Hsd:Athymic Nude-Foxn1nu	5-6 weeks

The mice were housed individually or grouped in ventilated IVC-cages under controlled conditions of light (12h light /12h dark), temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55\% \pm 15\%$) in specific pathogen-free conditions at the animal facility of Orion Corporation, Orion Pharma (**I**, **II** and **III**) or individually housed cages in the animal facility of the University of Turku (**I**) also with similar controlled temperature, humidity and light-dark cycles. The mice were given irradiated soy-free natural-ingredient feed and filtered, UV treated, tap water was available *ad libitum*. In order to maintain sodium balance, adrenalectomized mice (**III**) had unlimited access to 0.9% Sodium Chloride (NaCl, Saline), instead of water.

Rats (**II**) were housed in IVC-cages, with aspen chips as housing material, maximum 3 animals/cage, in a temperature- ($22 \pm 2^\circ\text{C}$) and humidity-controlled ($55\% \pm 15\%$) room with a 12h:12h light/dark cycle at the animal facility of Orion Corporation, Orion Pharma. Water and standard rodent diet were available *ad libitum*.

Male monkeys (**II**) were housed in a climate controlled room with a minimum of 8 air changes/hour. The temperature and relative humidity ranges were 19-25 °C and 40-70%, respectively. Artificial lighting was controlled automatically to give a cycle of 12h:12h light/dark cycle at the animal facilities of Covance, Münster, Germany. All animals were offered a certified lab diet for primates (LabDiet 50/48) twice daily. In addition, the animals regularly received fresh fruit and vegetables as food supplement. Tap water was provided *ad libitum*.

4.4.1 Xenograft models (I-III)

BALB/c and Athymic Nude male mice were used in all three studies (**I-III**). Animals were subcutaneously injected with VCaP cells in the mixture of medium and Matrigel.

The mice (I-III) were allocated to treatment groups based on tumor volume and/or PSA values aiming to obtain similar mean values in the groups, except for the surgical intervention study (ORX, ORX+ADX), where group allocation was carried out by using a specific algorithm (Laajala TD, et al. 2016), with matched baseline PSA concentration, tumor volume, PSA change from previous week, and animal weight as stratification criteria. Development of the tumors was monitored twice weekly by caliper measurements (I-III) and/or by measuring the serum concentration of PSA regularly from saphenous vein with a previously published method (Lovgren T et al., 1996). The volume of the tumor was calculated according to the formula $W^2 \times L/2$ (mm^3), where W is the shorter and L the longer diameter of the tumor. The tumors were grown for 7-9 weeks, until the mean volume of the tumors reached approximately 200-300 mm^3 , and the mean serum PSA value was ~ 20 $\mu\text{g/l}$. The mice included intact animals, SHAM-operated,

castrated (ORX) or castrated and adrenalectomized (ADX) mice. At the end of the experiments the mice were sacrificed, and serum, tumors and possibly additional tissues were collected for further analyses. Samples for steroid measurements and for RNA isolation were stored at -80°C after initial freezing in liquid nitrogen.

4.4.2 Inhibition of CYP17A1 in vivo in rat (II)

The effect of single oral doses of ODM-204 (3, 10, 30, or 100 mg/kg), abiraterone and galeterone both 30 mg/kg on the T production was studied in intact male rats (RCC Wistar). The test compounds were orally administered and one hour later followed by intramuscular (i.m.) injection of the human chorionic gonadotropin (hCG, Chorulon, 100 U/kg). Two hours after the hCG dosing, blood samples were taken from tail vein, and serum was separated by centrifugation. Serum T concentrations were determined by ELISA (Demeditec Diagnostics, Germany), according to manufacturer's instructions.

In the repeated-dosing study male rats (Sprague Dawley, n=9/group) were treated with a single dose of GnRH agonist leuprolide acetate (Enanton[®], 0.525 mg/animal), and 7 days later oral administration with ODM-204, 10, 30, or 50 mg/kg/day was initiated and continued for 14 days. After the treatment serum T level and weights of androgen-sensitive tissues were measured. To compare the effects of surgical castration and chemical castration induced by the GnRH agonist, a group of rats was castrated. The effects of treatments on the weights of prostate, seminal vesicles, testis, adrenal glands and liver were determined. In addition, the effects of treatments on pituitary gonadal axis and steroid synthesis pathways were evaluated by analyzing T, LH, cortisol, estradiol, P, and ACTH concentrations in serum. T radioimmunoassay and LH immunofluorometric assay were performed using previously described methods (Huhtaniemi I et al., 1985, Haavisto AM et al., 1993). ACTH was measured as a part of the MAP rat pituitary magnetic bead panels (Milliplex) and steroid/thyroid hormone magnetic bead panels (Milliplex) were used for cortisol, estradiol, and P.

4.4.3 Inhibition of CYP17A1 in vivo in monkey (II)

Sixteen purpose-bred cynomolgus monkeys (*Macaca fascicularis*) of Mauritian origin were included in the study. Animals of the same sex were pair- or single (if mature males are incompatible) housed in stainless steel cages. Maturity of the males was proven by the presence of sperm in the ejaculate. The animals were divided into four groups and blood samples for hormonal and ODM-204 samples for analytics were collected 1, 2, 5, 8, 10, 12 and 24 h after single once or twice daily drug administration (vehicle, 10 mg/kg qd or bid 8 hours apart and 30 mg/kg in 0.5% w/v methyl cellulose suspension). The

samples were collected from the femoral vein and centrifuged to obtain plasma that was kept frozen at $-20\text{ }^{\circ}\text{C} \pm 4\text{ }^{\circ}\text{C}$ prior to analysis.

4.4.4 Darolutamide and ODM-204 pharmacokinetic studies in mouse and monkey (I, II)

In study **I**, pharmacokinetic analyses were carried out by studying the penetration of darolutamide, ORM-15341, enzalutamide and apalutamide to the brain of nude male mice. The compounds were dosed orally for 7 days with 25, 50, or 100 mg/kg/bid of darolutamide or with 20 mg/kg/qd of enzalutamide or with a single oral dose of apalutamide (10 mg/kg). Control mice received vehicle formulation. Blood samples were collected by cardiac puncture under CO₂ anesthesia and plasma was separated by centrifugation. Brain samples from each group and time point were pooled and homogenized before the analysis. Concentrations of test items in mouse plasma and brain were determined by (LC-MS/MS) method with 1.00 ng/ml as the lower limit of quantification (LLOQ) in plasma for darolutamide, ORM-15341 and enzalutamide, 0.250 ng/ml for apalutamide and in brain 4.00 ng/g for darolutamide, 10.00 ng/g for ORM-15341, 5.00 ng/g for enzalutamide and 10.0 ng/g for apalutamide. Plasma and brain concentration vs. time were evaluated by noncompartmental analysis using WinNonlin® Professional v. 5.2 software (Pharsight Corporation). Brain/plasma ratios were calculated based on AUC₀₋₂₄ values for plasma and brain.

In study **II**, the quantitative analyses of ODM-204 in monkey plasma samples were prepared by liquid-liquid extraction and analyzed with a triple quadrupole mass spectrometer (Waters Acquity UPLC-TQD). The calibration range of the method was 0.5–3000 ng/ml and the LLOQ was 0.5 ng/ml.

4.5 Characterization of samples (I-III)

4.5.1 Hormonal measurements from media, serum and tissues (I-III)

Various steroids and hormones were measured in the present study. The analyses from media, microsomes, serum and tissues were conducted using multiple different methods, including several immunological and mass-spectrometric methods. In Table 4 the sensitivities of the used assays are shown for the different methods.

Table 4: The characteristics of used methodologies for hormonal analysis I-III

Hormone	RIA (T) / Delfia (LH) (Huhtaniemi et al. 1985)	ELISA (rat/mouse) (Demeditec Diagnostics, Germany)	Immuno- assay (Beckman n Coulter)	Milliplex Cat#RPTM AG-86K / Cat#STTH MAG-21K	ULPC- MS/MS / Orion Pharma	GC- MS/MS (Nilsson et al. 2015)
	Study used	I	II	II	II, III	II
Concentration limits	Assay range / (ng/ml)	Sensitivity / (ng/ml)	Sensitivity / (ng/ml)	Sensitivity / (ng/ml)	Calibration range / (ng/ml)	LLOQ / (ng/ml)
T	0.01 - 0.30	0.1-25	0.1	-	0.05-100	0.008
DHT	-	-	-	-	-	0.0025
DHEA	-	-	20	-	1-500	-
A4	-	-	-	-	0.05-100	0.012
P	-	-	0.10	-	-	0.074
Cortisol	-	-	4	-	-	-
ACTH	-	-	-	0.0032-10	-	-
LH	0.040 - 25000	-	-	0.0032-10	-	-

In study I, mice serum T was determined from 25- μ l aliquots of serum, which were extracted twice with 2 ml of diethyl ether and evaporated under nitrogen to dryness. The residues were reconstituted in PBS and measured using a standard radioimmunoassay as described previously (Huhtaniemi I et al., 1985).

In study II, H295R cells were exposed using different concentrations of test compounds and formation of T, DHEA, and A4 was determined by ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS). The accuracies of medium calibration samples were within $\pm 20\%$ of nominal values and precision $< 20\%$ at each concentration level. Quality control medium samples intra- and inter-batch precision and accuracy of the mean value at each concentration level was $< 20\%$ and $\pm 20\%$, respectively. The sample preparation was carried out by using liquid-liquid extraction. An aliquot of acidified medium was extracted using ethyl acetate:hexane (20:80, v:v). The analytes were separated with gradient elution in an Acquity UPLC[®] BEH C18 column (100 x 2.1, 1.7 μ m) and quantified by electrospray ionization equipment followed by selected reaction monitoring by the UPLC-MS/MS. An internal standardization based on peak area ratios was used for quantification of T, DHEA, and A4. Both rat serum T samples from the hCG-stimulation assay and monkey hormones (T, DHEA and cortisol from individual plasma samples taken at 5 and 10 h time points after first dose of ODM-204, and P and LH from pooled plasma samples (5 and 10 h time points) after ODM-204 dosing were studied by immunoassays according to manufacturer's instructions.

In study III, tumors and adrenals were homogenized in sterile water using a Tissuelyzer LT homogenizer (Qiagen, Venlo, The Netherlands), and intratumoral or -adrenal and serum concentrations of P, A4, T and DHT were measured using a previously described method applying GC-MS/MS (Nilsson ME et al., 2015). To compare relative changes in concentrations of studied hormones in plasma and tissues after interventions 1 g of tumor were compared to 1 ml of serum.

4.5.2 Serum PSA measurements (III)

In addition to tumor palpations, tumor growth was studied by analyzing serum PSA levels. Blood samples (100 µl) were collected from saphenous vein from VCaP xenografted mice every 10-day intervals, and PSA was measured with a time-resolved fluorometer (Wallac, PerkinElmer Analytical Life Sciences, Turku, Finland) as described in detail previously (Lovgren T et al., 1996).

4.5.3 Gene expression profiling (III)

4.5.3.1 RNA extraction

VCaP tumors and adrenals were homogenized using a Tissuelyzer LT homogenizer with Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA for RT-qPCR was extracted from the samples using TRIsure™ reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The RNA was handled with DNase I (Invitrogen amplification grade; Life Technologies, Carlsbad, CA, USA) and reverse transcribed by M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA) and oligo(dT) primers (Promega, Madison, WI).

4.5.3.2 RT-qPCR analyses (III)

The expression of steroidogenic enzymes and several receptors of interest were studied by RT-qPCR (presented in **Table 5**). The data were normalized to human ribosomal protein L19 (*RPL19*), and the amount of mRNA expressed was quantified using the Pfaffl method (Pfaffl MW, 2001). RT-qPCR reactions were performed using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and a 2× DyNAmo SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA). Measured genes and the primer sequences used in RT-qPCR are listed in **Table 5**.

Table 5: Primer sequences of RT-qPCR

Human Gene	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'
<i>AKR1C2</i>	CCTAAAAGTAAAGCTCTAGAGGCCGT	GAAAATGAATAAGATAGAGGTCAACATAG
<i>AKR1C3</i>	GCCAGGTGAGGAACCTTCAC	CAATTTACTCCGGTTGAAAATACG
<i>AR-FL</i>	CTTACACGTGGACGACCAGA	GCTGTACATCCGGGACTTGT
<i>AR-V1</i>	CCATCTTGTGCTCTTCGGAAATGTTATGAAGC	CTGTTGTGGATGAGCAGCTGAGAGTCT
<i>AR-V7</i>	CCATCTTGTGCTCTTCGGAAATGTTATGAAGC	TTTGAATGAGGCAAGTCAGCCCTTCT
<i>FKBP5</i>	AAAAGGCCACCTAGCTTTTTGC	CCCCCTGGTGAACCATAATACA
<i>HSD17B3</i>	CTGAAGCTCAACACCAAGGTCA	CTGCTCCTCTGGTCTCTTCAG
<i>KLK2</i>	CTGCCATTGCCTAAAGAAGAA	GGCTTTGATGCTTCAGAAGGCT
<i>KLK3</i>	CCAAGTTCATGCTGTGTGCT	GGTGTCTTGATCCACTTCC
<i>KLK4</i>	GGCACTGGTCATGGAAAACGA	TCAAGACTGTGACGGCCAGC
<i>NOV</i>	ACCGTCAATGTGAGATGCTG	TCTTGAAGTGCAGGTGGATG
<i>PMEPA1</i>	TGCCGTCCATCCTGGTT	AGACAGTGACAAGGCTAGAGAAAAGC
<i>L19</i>	AGGCACATGGGCATAGGTAA	CCATGAGAATCCGCTTGTTT
<i>SRD5A1</i>	CCTGTTGAATGCTTCATGACTTG	TAAGGCAAAGCAATGCCAGATG
<i>SRD5A2</i>	CTCTCTAAGGAAGGGGCCGAAC	GACAATGCATCCGCAAAACATA
<i>ST6GalNAc1</i>	AGGCACAGACCCAGGAAG	TGAAGCCATAAGCACTCACC
<i>SYTL2</i>	TCTGCCTTGAGAAAACAACAGTT	GCCAGTGGGTGGCACTAAAA
Mouse Gene	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'
<i>Ar</i>	GTCTCCGGAATGTTATGAA	AAGCTGCCTCTCTCCAAG
<i>Akr1c6</i>	CAGACAGTGCCTAAGTGATG	CGGATGGCTAGTCTACTTCTCT
<i>Cyp11a1</i>	AGATCCCTCCCTGGCGACAATG	CGCATGAGAAGAGTATCGACGCATC
<i>Cyp17a1</i>	CAAGCCAAGATGAATGCAGA	AGGATTGTGCACCAGGAAAAG
<i>Hsd3b1</i>	CAGGAGCAGGAGGTTTGTG	GTGGCCATTCAGGACGAT
<i>Hsd3b2</i>	CAGTTGTGGTGCAAGAGGA	CCTGGGAATGACACCTGTGA
<i>Hsd17b3</i>	CACGGGGATAAAGACCAGGT	GATCGCAGGAAAGAGCTTGG
<i>Hsd17b6</i>	TTTGGAGGATTCTACAGTTGCTC	TCACCCCGAAATCTTGAACCT
<i>L19</i>	GGACAGAGTCTTGATGATCTC	CTGAAGGTCAAAGGGAATGTG
<i>Lhcgr</i>	GCCCTGAGCCCTGCGACTGC	AAAGCGTTCCTGGTATGGTGGTT
<i>Mc2r</i>	TCTGACATCATGTTGGGCAGTCT	TGGTGATGTAACGGTCAAGCT
<i>Srd5a1</i>	TGAGCCAGTTTGGCGTGAT	CTCCACGAGCTCCCCAAAAT
<i>Srd5a2</i>	CACAGACATGCGGTTTAGCG	AACAAGCCACCTTGTGGGAT
<i>Srd5a3</i>	CTGGCTTAGTGCTCTGCTCA	CACAACGTGAATGGCTGCAT
<i>Ugt1a1</i>	GCAGAGTGGTTTATCCCCCT	AGGCGTTGACATAGGCTTCAA

The subset of genes, whose mRNA expression was analyzed, were selected among the AR interacting proteins or AR-regulated genes, steroidogenic enzymes and genes involved in the gonad or adrenal steroid synthesis or being involved in the hypothalamic negative feedback system (Kero J et al., 2000, Knuutila M et al., 2014, Knuutila M et al., 2018).

4.5.4 Protein analysis (III)

4.5.4.1 Sample preparation (III)

Tumor and adrenal gland samples were homogenized using TissueLyzer LT and stainless steel beads (Qiagen, Hilden, Germany) in RIPA-lysis buffer containing the following: 150 mmol/l Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l SDS, 100 mmol/l sodium orthovanadate (Sigma-Aldrich, St.Louis, MO), and cOmplete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). The samples were centrifuged (8000 g for 10 min, +4 °C), and total protein concentrations were measured with a bicinchoninic acid protein assay (Pierce, Rockford, IL).

4.5.4.2 Immunoblotting (III)

The tumor or adrenal samples (30 µg/sample) were loaded onto 4–20% Mini-PROTEAN® TGX™ Precast Protein SDS-PAGE gels (Bio-Rad) and separated under reducing conditions, followed by transfer onto blotting membrane (Amersham Hybond P 0.45 PVDF, GE Healthcare Life Sciences) and probed with antibodies (listed in Table 6). After washing, the membranes were probed with secondary HRP-linked antibodies. Visualization was carried out by using Cy5 and Cy3 detection with a Typhoon laser scanner (GE Healthcare Life Sciences) and imaged with an ImageQuant LAS 4000 camera system (GE Healthcare). ImageJ software version 1.51K (NIH, Bethesda, MD) was used for quantification of the membranes' band intensity.

Table 6: Used antibodies (I-III)

Antibody	Manufacturer	Name	Dilution (application)
Androgen receptor (AR)			
I)	Santa Cruz	N20	1:50 or 1:75 (HCS),
II)	Santa Cruz	sc-816	1:300 (CM)
III)	Abcam	N20	1:75 (HCS)
			1:2000 (IB)
CYP11A1	Santa Cruz	C-16	1:200 (IB)
CYP17A1	Santa Cruz	N-18	1:200 (IB)
GAPDH	HyTest		1:2500 (IB)
Anti-Rabbit IgG, HRP-linked	Cell Signaling		1:5000 (IB)
Rabbit anti-Goat IgG	ThermoFisher		1:5000 (IB)
	Scientific		
IgG DyLight 488 goat anti-rabbit	Abcam		1:200 (HCS)
IgG Alexa Fluor® 546 goat anti-rabbit	LifeTechnologies		1:800 (CM)

HCS = high-content screening, CM = confocal microscope, IB= Immunoblotting

4.6 Chemicals (I-III)

T was purchased from Fluka, mibolerone and [³H] mibolerone from Perkin Elmer, DHT from Sigma, DHEA and androstenedione from Sigma-Aldrich. Leuprolide acetate (GnRH-agonist) was bought from Takeda Pharmaceutical Company Ltd and bicalutamide was extracted from tablets, AstraZeneca. Darolutamide, ORM-15341, ODM-204, abiraterone acetate, galeterone, apalutamide and enzalutamide were synthesized by Orion Pharma.

4.7 Statistical analysis (I-III)

In study **I**, *in vitro* data was analyzed with GraphPad Prism 5 software (version 5.02) to obtain the K_i and IC_{50} values. For the VCaP xenograft experiment, the mean tumor volumes were calculated for each treatment group. The repeated measures ANOVA (RMANOVA) analysis was used as a statistical method to analyze tumor volume changes over the treatment time. With regard to the serum T levels, the differences between groups were analyzed using the Wilcoxon rank sum test.

In study **II**, the data was analyzed with GraphPad Prism 7 to obtain K_i and IC_{50} values. *In vivo* murine data were analyzed with SPSS 19.0 Statistical Package (SPSS Inc., Chicago, IL, USA). The data were reported as the mean \pm SEM for each group. In rat and mouse efficacy studies one-way-analysis of variance (ANOVA) was used to evaluate statistically significant differences ($p < 0.05$) between treatment groups. If significant differences were found, Dunnett's post hoc t-test was carried out. In the monkey PK/PD study, plasma hormone results were stated as percentages of the mean pre-treatment values (mean of values 72, 48, 24h before dosing), because of known high individual variation in the plasma hormones and because the circadian rhythm and possible stress-mediated variation cannot be excluded.

In study **III** the statistical tests were chosen depending on the results of the preliminary Shapiro-Wilk tests of data normality. Non-parametric Mann-Whitney, Kruskal-Wallis and Dunn's multiple comparison tests were applied in RT-qPCR comparisons on single gene level, and to test the differences in the steroid concentrations in intact, ORX and ORX+ADX mice, in comparison to vehicle and corticosterone treated animals. These univariate statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). The longitudinal analysis of PSA profiles between the interventions was performed using the mixed-effects models that infer differences in population growth slope co-efficients over the whole time period of the intervention (Laajala TD et al., 2016), where individual prognostic baseline variables were accounted for by incorporating baseline animal matching (Laajala TD, et al. 2016b).

The R statistical software (version 3.2.1) (R Development Core Team. R 2015) together with the preclinical analysis R-package hamlet (version 0.9.5) (Laajala TD, 2016c) were utilized in the longitudinal analyses.

5 RESULTS

5.1 Development of darolutamide and ODM-204

Darolutamide is a synthetic compound discovered by screening of compounds which inhibited AR transactivation in AR-HEK293 cells with continuous medicinal chemistry optimization. Darolutamide and its pharmacologically active main metabolite (ORM-15341) are novel and structurally distinct from any other known antiandrogens.

For ODM-204, combined antiandrogen and CYP17A1 inhibition were optimized. Several series of antiandrogens were evaluated using structural models of AR and CYP17A1. The goal was to incorporate a typical cytochrome heme binding moiety, presumably imidazole or pyridine derivative, into the structures without losing the antiandrogen activity. Five of these compound series were selected for further evaluation, and a set of such imidazole and pyridine derivatives were synthesized. Finally, the series to which ODM-204 belongs was chosen for optimization, because it exhibited the most balanced inhibition of both targets. The structures of darolutamide, ORM-15341 and ODM-204 are presented in Figure 10.

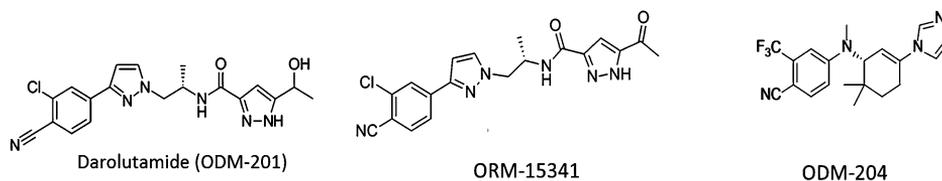


Figure 10: The chemical structures of darolutamide, ORM-15341 and ODM-204, novel second-generation androgen signaling axis inhibitors.

5.2 Characterization of antiandrogenic properties of darolutamide and ODM-204 (I, II)

All the novel compounds developed, namely darolutamide, ORM-15341 and ODM-204, showed clearly lower inhibition constant (K_i) values in AR binding assay than enzalutamide and apalutamide used as the clinical reference compounds. Furthermore, in transactivation assays in AR-HEK293 cells stably expressing full-length human AR and an androgen-responsive luciferase reporter gene, darolutamide, ORM-15341 and ODM-204 showed significantly more potent activity with full antagonism than the tested second-generation antiandrogens. Key properties of all studied compounds are presented in Table 7 and (I: Fig 1C and 1D, Fig 2, Fig 3; II: Fig 3A, 3B, 3C, Fig 5A and 5B).

Table 7: The key characteristics of the studied androgen signaling axis inhibitors (I-II)

Compound	AR binding affinity (K_i/nM)	AR antagonism (IC₅₀/nM)	Proliferation inhibition in VCaP (IC₅₀/nM)	Proliferation inhibition in LNCaP (IC₅₀/nM)
Darolutamide	11	26	230	-
ORM-15341	8	38	170	-
ODM-204	55	80	260	200
Enzalutamide	86	219	400 (I) 315 (II)	880
Apalutamide	93	200	420	-
Abiraterone	>30 000	nt	9000	1300
Galeterone	780	nt	2000	2600

- = data not shown

The antiproliferative properties of darolutamide, ORM-15341 and ODM-204 were studied in the VCaP cells. ODM-204 was also further studied in the LNCaP cells. Additional proliferation tests in LNCaP cells were performed for ODM-204, because of its potent antagonistic activity against AR(T878A) mutation (see chapter 5.3.1). When grown with a submaximal concentration of mibolerone (a synthetic androgen), darolutamide and ORM-15341 suppressed androgen-induced cell proliferation more efficiently than enzalutamide or apalutamide (Table 7, I:Fig. 4A). ODM-204 inhibited VCaP growth equally to enzalutamide (Table 7, II:Fig. 5A). Moreover, ODM-204 suppressed androgen-induced proliferation of LNCaP cells more efficiently than enzalutamide, abiraterone or galeterone (Table 7, II: Fig 5B). Neither darolutamide nor ODM-204 had an effect on the viability of the AR-negative cell lines (DU-145 or H1581) tested (I:Supplement Fig.S3, II:Supplement 2), confirming the antiproliferative properties being specific to AR-dependent cells.

The influence of antiandrogens on the subcellular localization of AR was studied in AR overexpressing HS-HEK293 cells by immunocytochemical labeling with an anti-AR antibody. In the absence of androgen AR was predominantly cytoplasmic, and exposure to T markedly increased the nuclear-cytoplasmic ratio of AR immunofluorescence intensity, indicating the movement of AR from the cytoplasm to the nucleus. In the presence of bicalutamide, AR was largely nuclear demonstrating that bicalutamide failed to block the T-induced nuclear translocation of AR. In contrast, in the presence of

darolutamide, ORM-15341, ODM-204, enzalutamide, or apalutamide, AR was predominantly cytoplasmic, showing the capability of these second-generation antiandrogens to inhibit the androgen-induced nuclear translocation of overexpressed AR (I: Fig. 3 and II: Fig.3 C).

5.2.1 Characterization of darolutamide, ORM-15341 and ODM-204 against mutant ARs (I, II)

Several common AR mutations in CRPC have been suggested to drive resistance to current antiandrogen therapies. The effects of the novel antiandrogens on key mutants in CRPC; AR(F877L), AR(W742L), and AR(T878A) were studied in transactivation assays in human U2-OS osteosarcoma cells, transiently transfected with expression vectors encoding the corresponding mutant ARs and an androgen-responsive luciferase reporter gene. The AR(F877L) substitution in AR switched enzalutamide and apalutamide from antagonists to agonists. Of the tested second-generation antiandrogens, only darolutamide and its main metabolite ORM-15341 functioned as full antagonists for all tested mutant ARs (Table 8, I:Fig 2). The dual inhibitor, ODM-204, showed similar potent antagonism in the studied mutations and in addition, it inhibited the activation of clinically well validated relevant AR(W742L) and AR(T787A) mutations with low IC₅₀ values. However, ODM-204 showed slight agonism in the AR(F877L) mutation at high concentrations > 2 μM (Table 8).

Table 8: Inhibition (IC₅₀-values) of wtAR and mutant AR(F877L), AR(W742L) and AR(T878A) by second-generation antiandrogens (I-II).

	wtAR IC ₅₀ (nM)	AR(F877L) IC ₅₀ (nM)	AR(W742L) IC ₅₀ (nM)	AR(T878A) IC ₅₀ (nM)
Darolutamide	65	66	1500	1782
ORM-15341	25	51	1160	700
ODM-204	80	6*	277	95
Enzalutamide	155	Agonist	>10 000	296
Apalutamide	168	Agonist	>10 000	1130

*) agonism at high concentrations > 2 μM

5.2.2 Characterization of ODM-204 to inhibit CYP17A1 *in vitro* and *in vivo* (II)

CYP17A1 possesses two sequential catalytic activities in the steroid biosynthesis; 17 α -hydroxylase activity and 17,20-lyase activity. The inhibitory effect of ODM-204 and two reference CYP17A1 inhibitors (galeterone and abiraterone acetate) on the hydroxylation-reaction required for the formation of 17 α -hydroxyprogesterone from P, were analyzed using rat, monkey and human testicular microsomes. The second CYP17A1-mediated reaction, the conversion of 17 α -hydroxypregnenolone to DHEA, was measured by acetic acid release assay in the H295R human adrenocortical carcinoma cells. In addition to quantifying the IC₅₀ values for specific reactions, the dose-dependent inhibition of T, DHEA and A4 production was analyzed by measuring the steroid concentrations by LC-MS/MS. In all assays ODM-204 and galeterone showed equally potent inhibition with nanomolar IC₅₀ values for both 17 α -hydroxylation and 17,20-lyase reactions. Clear species specificity was observed both with ODM-204 and galeterone (Table 9, II: Fig 2), where the IC₅₀ for the rat CYP17A1 was markedly higher than those measured for the human and monkey enzymes. Abiraterone presented significantly more potent inhibition in both reactions in all species compared with ODM-204 or galeterone (Table 9, II: Fig 2).

Table 9: The inhibition of CYP17A1 mediated 17 α -hydroxylase and 17,20-lyase reactions with ODM-204, galeterone and abiraterone (II)

Compound	Cells	Testicular microsomes		
	NCI-H295R IC50 (nM)	Human IC50 (nM)	Monkey IC50 (nM)	Rat IC50 (nM)
ODM-204	40	22	11	92
Galeterone	200	9	4	212
Abiraterone	0.4	1.3	3	2.1

Serum T concentrations in intact male hCG-induced rats were measured to study how efficiently orally dosed ODM-204 inhibit CYP17A1 *in vivo*. The data showed that serum T levels were significantly (***) $p < 0.001$ decreased by all tested doses of ODM-204, similar to that observed with the reference compounds, abiraterone and galeterone (II: Fig 4). In the 14-day intact rat assay, the data with ODM-204 in combination with leuprolide acetate clearly demonstrated that ODM-204 potentiated the efficacy of chemical castration, evidenced by further decrease in serum T levels and reducing the weight of androgen-sensitive organs in male rats (II: Fig. 4) more than that observed with leuprolide alone.

In order to ensure and to better predict the potential efficacy of ODM-204 in human and CPRC patients *in vivo*, its pharmacodynamics was studied in mature male cynomolgus monkeys. In these studies T levels reduced remarkably already after a single oral dose of ODM-204 with all studied doses (10-30 mg/kg). A decrease in DHEA was observed with the doses 10 mg/kg twice daily and 30 mg/kg/day, remaining lower than the control throughout the 24 h study period (II: Fig 6 A, B). Correspondingly, the levels of cortisol decreased, although the reduction was more distinct at the 10 h time point (II: Fig 6 C). As expected, the concentration of both LH and P increased dose-dependently and as a function of time (II: Fig 6D, E). Inter-animal variation of LH was relatively large, but mirrored with measured T concentrations (II: Fig 6A, D).

5.2.3 Characterization of pharmacokinetic properties of darolutamide and ODM-204 (I and II)

Pharmacokinetic (PK) analyses carried out in mice for darolutamide revealed that with the dose of 50 mg/kg, bid, the systemic exposure (AUC₀₋₂₄) for enzalutamide was 2.5 times higher than that for darolutamide. Moreover, enzalutamide exhibited a long plasma half-life (18.3 hours) while the half-life of darolutamide in mice was moderate (1.6 hours). This supports once daily dosing for enzalutamide and the use of higher and more frequent dosing for darolutamide (I: Supplementary Table S1).

The data from monkey study indicated that ODM-204 was well tolerated, the exposures were dose-dependent and showed good drug absorption and similar metabolism at all used dose levels (II: Fig 6F). The half-life for ODM-204 after oral dosing in monkeys was 6.5 h, suggesting twice daily dosing.

5.3 *In vivo* efficacy of darolutamide and ODM-204 or surgical interventions in the prostate cancer VCaP xenograft models

5.3.1 The characterization of ODM-204 in the xenografted VCaP tumors in intact male mice (II)

The effects of ODM-204 and abiraterone acetate on the volume of the subcutaneous VCaP tumors grown in intact mice were observed by bi-weekly tumor palpations. Intact VCaP xenografted mice were selected for the study to see the effect of CYP17A1 inhibition in the testicles to the tumor growth. The effect of adrenal steroids were not studied due to the assumption that mouse adrenals lack of CYP17A1 enzyme and do not produce any

androgens (van Weerden WM et al., 1992). During the 3-week study period ODM-204 significantly inhibited ($p < 0.001$) tumor growth compared to vehicle-treated animals. In a similar experimental setting, abiraterone acetate failed to show any efficacy, when systemic concentrations observed in clinical specimens were used (II: Fig 5C, D). No signs of treatment-related toxicity were observed, and the body weights of the mice did not differ from the vehicle-treated control animals during the ODM-204 treatment (II: Supplement 3).

5.3.2 Characterization of darolutamide, ODM-204 and surgical interventions in the xenografted VCaP CRPC tumors (I, II and III)

The *in vivo* efficacy of three antiandrogens (darolutamide, ODM-204 and enzalutamide) and CYP17A1 inhibitor abiraterone, and adrenalectomy (ADX) were studied in subcutaneous CRPC VCaP xenografts. In study III, in addition to palpations, serum PSA values were also monitored every tenth day. Darolutamide, with both studied doses 50 mg/kg qd and bid, showed statistically significant tumor growth inhibition compared to vehicle control (I: Fig 4B). Also in the same study darolutamide presented significantly better tumor growth inhibition compared to enzalutamide, which was dosed 20 mg/kg qd. In a similar experimental setting, ODM-204 administered with prednisone (2 mg/kg qd) provides significant CRPC tumor growth inhibition. However, tumor growth inhibition was weaker than that observed in the intact VCaP model. Abiraterone did not show any activity in the CRPC VCaP setting (II: Fig 5E, F).

The effects of surgical interventions (ORX and ORX+ADX) to the VCaP tumor growth and serum PSA concentrations were investigated. After the operations, the tumors started re-growth both in the ORX and in the ORX+ADX mice (III: Fig. 1A). In the ORX mice the tumors grew faster and re-growth occurred earlier than in the ORX+ADX mice, and at the end of the study the tumor volumes in the ORX+ADX mice were significantly smaller ($p < 0.001$) compared with the ORX group. In line with tumor volumes, serum PSA analyses showed a 6-fold decline after the ORX, while the ORX+ADX resulted in a 22-fold decrease in the serum PSA within two weeks. The serum PSA started to rise in the ORX group, reaching the pre-castration levels at about 7 weeks after castration. Also in the ORX+ADX group PSA levels began slowly to increase but did not reach the pre-castration levels within the study period (III: Fig. 1B) and significantly ($p < 0.05$) lower PSA concentrations were observed at the end of the study compared to the ORX mice. The data thus indicates significant contribution of the adrenal hormones to the CRPC VCaP xenograft growth.

In the ORX+ADX mice carrying the castration-resistant VCaP tumors, the effect of physiological dose of corticosterone (3.0 mg/kg qd) to the tumor growth was studied.

Glucocorticoid replacement did not affect the tumor growth in the ORX+ADX mice. As a consequence significantly slower growth rate was observed both with and without the glucocorticoid treatment compared to the ORX group (III: Fig. 1C).

5.4 Characterization of the role of mouse adrenals on the VCaP xenograft growth

5.4.1 Post-operational physiological changes (III)

The weight of the adrenal glands of the nude mice carrying VCaP xenografts increased significantly ($p < 0.01$) after the ORX (III, Fig. 1D). After combination of ORX+ADX, lack of glucocorticoids significantly increased the ACTH levels via regulation of negative feedback and the activation of HPA-axis compared to the ORX group (III: Fig. 1E).

5.4.2 Expression of adrenal steroidogenic enzymes and receptors in mice after ORX (III)

Certain key steroidogenic enzymes and other proteins related to the steroid biosynthesis were selected for the RT-qPCR analysis. The enzymes studied covered both the early steps of steroid synthesis having influence on glucocorticoid and androgen production as well as those specifically involved in the androgen production. ORX significantly increased the mRNA expression of the *Cyp11a1* ($p < 0.01$), *Cyp17a1* ($p < 0.05$), *Hsd3b1* ($p < 0.01$) and *Hsd3b2* ($p < 0.001$) and *Akr1c6* (homolog for human *Akr1c4*) ($p < 0.01$) enzymes. The increases were between 1.6-12.1 -fold after ORX, and the highest induction was detected in *Cyp17a1* expression (III: Fig. 2A). Statistically significant up-regulation of luteinizing hormone (*Lhcgr*, 29-fold), the melanocortin 2 receptor (*Mc2r*, 2-fold), and *AR* (2-fold) was also observed after ORX (III: Fig. 2C). In addition, a close correlation between the expression of *Cyp17a1* and *Lhcgr* mRNA was observed, $p < 0.001$ (III: Fig 2E).

5.4.3 Characterization of intra-adrenal, intra-tumor and serum levels of steroids in the mice carrying VCaP xenografts (III)

The intra-adrenal, intra-tumor and serum steroid concentrations from intact, ORX and ORX+ADX male mice were analyzed by a sensitive GC-MS/MS method at the end of the study, and the results are summarized in Table 10. Compared to the intact group, after

castration, only low circulating levels for all studied steroids were detected, and the concentrations were near LLOQ or were even undetectable after the ORX+ADX intervention (Table 10, III: Fig. 4A). All main classical androgens (A4, T and DHT) and P, being the precursor for the androgens, were detected in high (> 1 ng/g tissue) concentrations in the adrenals of both the intact and ORX mice. ORX had only a weak effect to the intra-adrenal hormone levels; the mean levels of P and DHT were unchanged, A4 had a non-significant tendency to increase and T decreased as predicted (Table 10, III: Fig. 3A). Due to the reduction in circulating hormones, the adrenal to serum ratio of all analyzed steroids increased after ORX (III: Fig 3B).

In the tumors, ORX decreased statistically significantly the P, A4 and T levels, and the levels of DHT stayed unchangeable. The concentrations of all measured hormones decreased significantly after ORX+ADX both in the serum and in the tumors, and the highest changes were observed in the P levels, a 13-fold decrease in the serum and a 21-fold decline in the tumor values, respectively (Table 10 and III: Fig 4B). Although ORX+ADX decreased also DHT levels to one tenth in the tumors compared to ORX, the concentrations were still roughly one magnitude higher than other measured androgens.

Table 10: The mean \pm SD values of measured hormones (P, A4, T and DHT) in the adrenal gland, tumor and serum (III).

Analyzed Hormone	Intact / Average \pm SD			ORX / Average \pm SD		
	AG (ng/g)	Tumor(ng/g)	Serum (ng/ml)	AG (ng/g)	Tumor (ng/g)	Serum (ng/ml)
P	4370 \pm 4274	3.51 \pm 5.078	0.80 \pm 1.634	5270 \pm 3727	1.1 \pm 1.65	0.24 \pm 0.266
A4	1.71 \pm 1.236	0.65 \pm 0.627	0.05 \pm 0.073	2.8 \pm 1.69	0.1 \pm 0.08	0.01 \pm 0.003
T	3.0 \pm 5.02	0.90 \pm 0.445	2.17 \pm 3.526	0.6 \pm 0.32	0.5 \pm 0.28	0.01 \pm 0.012
DHT	1.5 \pm 0.86	3.90 \pm 1.434	0.04 \pm 0.063	1.1 \pm 0.63	4.6 \pm 3.03	0.01 \pm 0.007
Analyzed Hormone	ORX+ADX / Average \pm SD					
		Tumor (ng/g)	Serum (ng/ml)			
P		0.052 \pm 0.0806	0.002 \pm 0.0273			
A4		0.063 \pm 0.1721	0.005 \pm 0.0027			
T		0.080 \pm 0.0552	0.0004 \pm 0.00086			
DHT		0.532 \pm 0.3749	0.001 \pm 0.0006			

AG= adrenal gland

5.4.4 The expression of AR and its variants, steroidogenic enzymes and AR-dependent genes in the VCaP tumors (III)

In the VCaP tumors, the expression of mRNAs for full length AR and for the two measured AR splice variants (*AR-V7* and *AR V-1*) were significantly induced after ORX (III: Fig 5A). The ORX+ADX significantly promoted the expression of the mRNAs more, although no induction was observed in the protein level of full length *AR* (III: Fig 5B). In addition to *AR*, the expression and changes in mRNA of several steroidogenic enzymes (*AKRIC2*, *AKRIC3*, *HSD17B3*, *SRD5A1* and *SRD5A2*) were detected by RT-qPCR. Notably, in the tumors only minor changes in the expression levels between the treatment groups were observed: ORX induced a significant increase in the *AKRIC3* and a similar effect was seen in *SRD5A1* levels after the ORX+ADX. In addition, the expression levels of a set of genes identified to be regulated by antiandrogens were analyzed. Of these genes, up-regulation of *SYLT2* and *NOV*, and down-regulation in the *FKBP5* and *ST6GAINAc1* genes were observed.

6 DISCUSSION

CRPC is a heterogeneous disease progressing with multiple resistance mechanisms. An increasing amount of data from both preclinical and clinical studies shows the central role for the AR-pathway in the recurrence of CRPC and has led to the development of new therapies. Darolutamide and ODM-204 are novel non-steroidal AR inhibitors for the treatment of CRPC, developed by Orion Corporation, Orion Pharma. This thesis work has been part of the biological evaluation of the compounds, in which the pharmacological activity *in vitro* and *in vivo* was studied in different models, in order to support the developmental work of both compounds.

6.1 Structural characterization of the novel androgen receptor blockers darolutamide and ODM-204

Androgens such as DHT and antiandrogens bind to the ligand-binding pocket of the AR leading to activation of the receptor or preventing its ligand-dependent activation. To date, the binding mechanism for the agonists has been recognized, while for the antagonists the mechanism still remains largely unclear. The conformational changes in the receptor, induced by second-generation antagonists, inhibit entry of AR to the cell nucleus. This further prevents the receptor from binding to DNA response elements and consequently its activity to recruit coactivators. Recently, structural simulations with enzalutamide indicated that helix 12 (H12) has a role also in antagonist binding, in addition to its significant role in the action of agonists by allowing specific conformation for activation of AR. This helix lies like a cover on top of the AR LBD while the C-ring of enzalutamide locates near to H12 reducing the distance between enzalutamide and H12, consequently preventing H12 from closing and altering the coactivator binding site and blocking transcription (Liu H et al., 2017).

Until now, all currently available first- and second-generation antiandrogens have similar structural motif in their A-ring, containing an anilide substituted with a trifluoromethyl group in meta-position and a nitro or cyano group in para-position; in nilutamide, enzalutamide, and apalutamide the amide of the anilide is a part of (thio)hydantoin structure (Helsen C et al., 2014). Structural similarities of current drugs allow them to penetrate to the brain, which may lead unwanted off-target toxicity or safety problems to the patients and limitations in their clinical use. Importantly, this has been taken into account already in the early structural design of darolutamide, and in the selection of the scaffold series under optimization.

ODM-204, a novel nonsteroidal dual-action compound, has a functional A-ring sharing the structural similarity with the first- and second-generation antiandrogens, flutamide and enzalutamide. This is a trifluoromethyl group in meta-position and a cyano group in para-position (Helsen C et al., 2014). The novelty and uniqueness of ODM-204 comes from the successful combination of the potent AR antagonism with effective CYP17A1 inhibition built in the C-ring of the molecule using typical cytochrome heme binding moiety with imidazole derivatives (Njar VC et al., 1999, Kaku T et al., 2011). Combination of CYP17A1 inhibition with AR antagonism is an interesting target combination. Accordingly there are several phase III clinical combination trials with abiraterone and enzalutamide, including ALLIANCE (abiraterone + enzalutamide in 1st line treatment of mCRPC, NCT 01949337) and STAMPEDE arm J (abiraterone + enzalutamide in mCRPC, NCT00268476) ongoing (Attard G et al., 2014). Furthermore a trial with combination of abiraterone and apalutamide (NCT03360721) has been initiated. The PLATO trial (combining abiraterone + enzalutamide after progression from enzalutamide, NCT01995513), was reported to be negative for its primary endpoint progression-free survival, possibly due to patient population carrying a high disease burden, and potential cross-resistance between enzalutamide and abiraterone (Yamada Y et al., 2016).

Treatment with the combination of enzalutamide and abiraterone may cause drug-drug interactions (Del Re M et al., 2017), since abiraterone has been shown to be a CYP3A4 substrate (Bernard A et al., 2015) and enzalutamide a CYP3A4 inducer (Gibbons JA et al., 2015a). Thus, enzalutamide decreases the plasma exposure of abiraterone. In addition, the different dosing instructions for abiraterone to be taken to empty stomach, and for enzalutamide to be taken with food, may cause challenges in the already relatively old, “multitreated” patient population. These inconveniences can be avoided with single agent medication such ODM-204, which has dual inhibitory properties.

6.2 Darolutamide and ODM-204 are potent second-generation antiandrogens

In preclinical models, darolutamide efficiently blocks AR-signaling by binding to AR with high affinity and impairing nuclear translocation of the overexpressed AR. Compared to enzalutamide, used as the clinical reference compound, darolutamide showed better activity than enzalutamide. The dual inhibitor ODM-204 showed equal properties both in the AR binding and nuclear translocation assays to those of enzalutamide. Comparing with the first-generation antiandrogen bicalutamide, both of the developed second-generation androgens, darolutamide and ODM-204, as well as enzalutamide used as reference compound, were clearly different inhibiting AR nuclear translocation in cellular assays in contrast to bicalutamide.

Darolutamide and its main metabolite (ORM-15341) showed higher affinity to AR compared to enzalutamide, and hence they could well be effective also in the enzalutamide-resistant CRPC population. In the preclinical setting, with enzalutamide resistant VCaP cells, Kregel and colleagues showed that increased-AR-overexpression was still the major driver in the recurrence of enzalutamide resistant CRPC (Kregel S et al., 2016). In line with results *in vitro*, we have shown significant increase in the full length-AR and also in the AR-V1 and AR-V7 variant expression in the castration-resistant VCaP xenografts in mice after treatment of second-generation antiandrogen enzalutamide (Knuutila M et al., 2018). An increasing amount of data indicates divergent resistance mechanisms and heterogeneity already in assays carried out with enzalutamide-resistant cell lines (Kregel S et al., 2016). More importantly, various heterogenic mechanisms have been observed in individual patients through RNA profiling. In these studies both AR-dependent alterations e.g. AR mutations and increased mRNA of several AR splice variants, and AR-independent alterations such as activation of GR, ncWnt, and the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) signaling pathways, loss of the phosphatase and tensin homolog (PTEN) tumor suppressor gene were detected (Taylor BS et al., 2010, Miyamoto DT et al., 2015). In addition, loss or mutation in the p53 tumor suppressor protein and retinoblastoma tumor suppressor protein (Rb1) has been noticed as important factors, driving resistance to antiandrogens causing neuroendocrine prostate cancer differentiation (Robinson D et al., 2015). Recently, the results from *in vivo* mouse study showed that Rb1 loss facilitates lineage plasticity and metastasis in prostate cancer and p53 causes secondary resistance to therapies targeting the AR signaling axis (Ku SY et al., 2017).

Another reference compound in our studies was galeterone, a steroidal CYP17A1 inhibitor, which has been reported to inhibit wtAR in the nonclinical model with K_i value 460 nM (Soifer HS et al., 2012), roughly equal to our results. However, wtAR-binding affinity of galeterone is more than 50 times weaker compared to darolutamide and more than 10 times weaker as observed with ODM-204 or enzalutamide, our results thus being in line with previously published results (Tran C et al., 2009). These findings challenge the naming of galeterone as an AR antagonist, although we observed inhibition in the proliferation of AR-dependent prostate cancer cell lines at high concentrations of galeterone, again corresponding to previously reported findings (Yu Z et al., 2014a). However, the IC_{50} values were more than 10 times higher than that with darolutamide, ODM-204 or enzalutamide, proposing that reported AR antagonistic properties are not direct properties of galeterone, but instead those of active metabolites (Alyamani M et al., 2017). In conclusion, the presented results have been attained in relatively high concentrations (from 1 to 10 μ M), and it cannot be totally excluded that some of the effects could have appeared via cytotoxicity, insolubility or even be off-target effects of galeterone.

6.3 Darolutamide and ODM-204 do not activate key AR-point mutations present in CRPC

In addition to AR upregulation and amplification, AR-point mutations appear to be a significant drug resistance mechanism in CRPC. In untreated tumors AR mutations have been reported to be relatively rare (Culig Z et al., 2001). However, AR mutations cause treatment failure of the conventional first-generation AR-antagonists, and can promote progression to the CRPC state. After ADT therapy, in the recurrent disease, roughly 15–30% of CRPC cases have AR mutations (Taplin M-E et al., 2003, Taylor BS et al., 2010, Eisermann K et al., 2013, Robinson D et al., 2015). To date, all clinically relevant AR-mutations in the CRPC-state are localized in the LBD of AR, and the structure of the ligand binding pocket and the specificity of the ligand binding of AR appear to be altered by these mutations, consequently allowing other steroid hormones and antiandrogens to activate AR (Veldscholte J et al., 1990a, Taplin M-E et al., 2003).

AR(T878A) is the most broadly studied mutation, originally described in LNCaP cells and first reported in human, during the hydroxyl-flutamide treatment (Veldscholte J et al., 1992). In our studies Darolutamide, ORM-15341 and ODM-204 were found to be full antagonists for this mutated AR, ODM-204 being the most potent one. Furthermore, the AR(W742L) mutation, activated by bicalutamide (Sun C et al., 2006), was also studied. All the novel compounds acted as full antagonists for the mutated receptor. Antagonism against these two mutations is important, because in the CRPC state many of the patients will be treated either with bicalutamide or hydroxyflutamide or both, and according to the current standard of care guidelines (NIH, 2018), the next treatment line for many of those patients will be a second-generation antiandrogen or a CYP17A1 inhibitor. However, progression after abiraterone, the enrichment of AR mutations (T878A) or (L702H) were observed in 13% of tumors, without changes in the AR copy number (Romanel A et al., 2015). Notably, this brings up the possibility that a number of steroids may activate these AR-mutations and promote the progression of the disease. With abiraterone, increased systemic levels of the P might serve as an alternative agonist for this mutation (Watson PA et al., 2015). Based on these relatively small studies but relatively high numbers of observed mutations in CRPC patients, there is an urgent need for new treatments, like darolutamide or ODM-204 which have shown antagonism in key AR mutations.

The third critical mutation, AR(F877L), has been detected in plasma DNA from men with CRPC treated with enzalutamide and apalutamide, strongly suggesting that the AR(F877L) mutation acts as a driver of acquired resistance to second-generation antiandrogens (Korpal M et al., 2013, Nelson WG et al., 2013). Markedly, darolutamide and its main metabolite ORM-15341 were full antagonists in this mutation. In line with our results, Borgmann and colleagues showed that in the LNCaP-derived MR49F cells darolutamide acts as an antagonist to the AR(F877L) mutation (Borgmann H et al., 2018),

which has been earlier described to be resistant to enzalutamide (Joseph JD et al., 2013). Also ODM-204 showed potent antagonism in this AR(F877L) mutation, although at high concentrations ($> 2 \mu\text{M}$), while ODM-204 may tend to switch from antagonism to agonism.

6.4 Darolutamide does not penetrate to brain

The tendency to induce seizures has been recognized as an unexpected problem in discovery and development of the second-generation antiandrogens (Rathkopf D et al., 2011). An off-target activity of gamma-aminobutyric A (GABA-A) inhibition has been presented to explain these side effects (Foster WR et al., 2011). In a phase I/II study with enzalutamide, seizures were reported in three (2%) of 140 CRPC patients receiving higher doses of the drug (Scher HI et al., 2010), and in phase III trials with a lower dose of enzalutamide, seizures have been observed with 0.3-0.6 % prevalence (Scher HI et al., 2012, Slovin S et al., 2017). With apalutamide, seizures have been reported in 0.2 % of the patients receiving the drug (Smith MR et al., 2018). In the PK studies in mice, we established the characteristic of darolutamide and ORM-15341 compared to enzalutamide and apalutamide concerning their potency to cross the blood-brain barrier. In these studies both darolutamide and ORM-15341 showed negligible brain penetration. The small concentrations of darolutamide and ORM-15341 found in the brain were likely to derive from the blood sustained in the vessels when brains were homogenized (brain/plasma ratios of 1.9–3.9%), because no perfusion was done during the sampling. Very recently another pharmacokinetic study was carried out to investigate the brain penetration of radiolabeled darolutamide and enzalutamide in rat. In this study, enzalutamide was present constantly in brain, the highest observed brain/blood-ratio was ~ 0.765 , while with darolutamide it was only ~ 0.074 (Zurth C, 2018).

The results strongly suggest that darolutamide, with only negligible brain penetration, has a low risk of inducing seizures in CRPC patients. This is also supported by clinical data from phase I study, where patients with a history of seizures or any predisposing conditions were allowed to participate (Massard C et al., 2016), in contrast to enzalutamide and apalutamide studies (Scher HI et al., 2012, Rathkopf D, 2013, Beer TM et al., 2014a), and no seizures were observed (Massard C et al., 2016). Low brain penetration increases safety and tolerability profile of the drug and consequently is a clear benefit for the patients (Shore ND, 2017). This is important as the second-generation antiandrogens are currently also used in earlier disease states, such as hormone-sensitive and nonmetastatic diseases. This trend can be observed from the design of ongoing clinical trials e.g. PROSPER (NCT02003924), SPARTAN (NCT01946204) and ARAMIS (NCT02200614). In practice this means that these new treatments will be used

for an increasing number of patients in the future, and thus, the safety aspects of the new drugs are becoming more and more significant.

6.5 The *in vivo* efficacy of darolutamide and ODM-204

In our studies, the *in vivo* efficacy of novel drug candidates was studied in mice, which were inoculated subcutaneously with the VCaP human prostate cancer cells, originally derived from a vertebral bone metastasis of a patient with CRPC (Korenchuk S et al., 2001). VCaP xenograft was selected to the disease model for the present project, as there is clear evidence that these cells represent many important properties of CRPC: being androgen sensitive, presenting with high endogenous levels of AR due to AR amplification, secreting PSA, showing upregulation of full length AR and its splice variants (AR-V1 and AR-V7) after castration. Furthermore, they also carry clinically relevant AR-driven TMPRSS2-ERG translocation, and they have been reported to express multiple enzymes of steroid biosynthesis enabling the synthesis of androgens (Loberg RD et al., 2006, Cai C et al., 2009, Cai C et al., 2011a, Knuutila M et al., 2014). Finally, tumors grown in intact animals respond to castration but show recurrence within a sufficient time frame, which allows the modeling of different states of prostate cancer in both hormone sensitive and CRPC states.

6.5.1 *Darolutamide has superior antitumor activity in nonclinical CRPC model*

Darolutamide showed strong antitumor activity in castration-resistant VCaP xenografts. In this model, ORX of the mice stabilized tumor growth at first, but after a few weeks, the tumors started to grow again, demonstrating the castration-resistant state. The oral dosing of darolutamide and enzalutamide were initiated when tumors reached the same tumor volumes as they had before castration. In line with the *in vitro* binding and proliferation inhibition results, darolutamide given orally (50 mg/kg qd or bid) showed a more potent antitumor activity than similarly dosed enzalutamide (20 mg/kg, qd), even though the plasma exposure for enzalutamide was higher than that for darolutamide. In line with data reported in this study, enzalutamide has been shown to inhibit orthotopically inoculated CRPC VCaP tumors in nude mice, detected by sequential serum PSA analysis (Knuutila M et al., 2018). Although significant efficacy was still observed at the end of the study, a clear trend for growing PSA values were seen after four weeks of dosing (Knuutila M et al., 2018), similarly as in the subcutaneous tumors in this study. This significantly better efficacy observed for darolutamide compared to enzalutamide in the castration-resistant VCaP model might also indicate better clinical effectiveness of darolutamide. Enzalutamide increased the serum T levels in mice. In line with this, in

enzalutamide-treated CRPC patients, elevated levels of T and estradiol in circulation and increased T in the bone marrow were reported (Efstathiou E et al., 2011, Smith MR et al., 2013). Importantly, darolutamide does not increase serum T levels in mice, in concert with the findings in darolutamide-treated patients, showing castrate levels of the serum T (Fizazi K et al., 2014).

In addition to darolutamide and enzalutamide, also ODM-204 and a reference compound (abiraterone) were tested in the castration-resistant VCaP model. ODM-204 showed significant inhibition of tumor growth. However, the inhibition was only modest, compared with the antitumor activity of darolutamide. Albeit the fact that abiraterone has a significant clinical impact, in our study abiraterone did not show any antitumor activity in the castration-resistant model. There are only few examples from the nonclinical prostate cancer efficacy models, where tumor growth has been successfully inhibited with abiraterone, and in those reports responses have been short-termed (Mostaghel EA et al., 2011, Lam H-M et al., 2017). The recurrence mechanisms in nonclinical models have been proposed to be caused by upregulation of CYP17A1 and by an increase of expression of AR and AR splice variants. In addition, the complex metabolism of abiraterone via steroidogenic enzymes has been reported to produce both AR agonistic (Li Z et al., 2016) and AR antagonistic metabolites (Li Z et al., 2015). These agonistic metabolites, such as 5- α -abiraterone is formed by SRD5A enzymes from $\Delta 4$ -abiraterone, which has been shown to be AR antagonist (Li Z et al., 2016).

6.5.2 ODM-204 potently inhibits VCaP tumor growth in intact VCaP mice

Interestingly, the growth of VCaP tumors grafted to the intact animals was powerfully inhibited by ODM-204. To our surprise, abiraterone did not work in this efficacy model, albeit with a similar dose-range abiraterone has been shown to efficiently decrease androgen-dependent tissue weights in intact male mice (Duc I et al., 2003). This may be a consequence of the high levels of AR in the VCaP cells. The antitumor activity with ODM-204 shown in the intact mice suggests that the compound may have a good response rate in clinical populations in an early state of the disease, perhaps among the pre-chemo and hormone sensitive prostate cancer patients. The results from our intact rat study supported this hypothesis, since ODM-204 significantly potentiated the effects of GnRH-agonist by further decreasing the weights of androgen sensitive tissues and reduced serum T levels in a dose-dependent manner, without any increase in LH. However, there is a slight concern about the observed activation of AR(F877L) mutation at high concentrations, which may cause a progression of the disease in patients harboring this mutation. Importantly the mutation has been detected in a small fraction of CRPC patients progressing after enzalutamide and apalutamide treatments (Korpala M et al., 2013, Nelson WG et al., 2013).

6.5.3 ODM-204 is a potent nonsteroidal CYP17A1 inhibitor

To date due to the wide clinical use of abiraterone, CYP17A1 inhibition is a well validated mechanism and strategy in the treatment of CRPC (Attard G et al., 2009, de Bono JS et al., 2011). Abiraterone inhibits efficiently and equally well both of the reactions catalyzed by CYP17A1 (17-hydroxylase and 17,20-lyase) in the adrenals and in the tumor tissue (Potter GA et al., 1995). In addition to abiraterone there have been also several other CYP17A1 inhibitors in clinical development, e.g. galeterone, orteronel (TAK-700) and VT-464 being the most advanced. Unfortunately the development of both galeterone and orteronel has been discontinued recently due to the lack of efficacy in OS, which was the primary endpoint in phase III trials (Fizazi K et al., 2015b, NIH, 2017).

The studies modeling CYP17A1 inhibition *in vivo* were conducted in intact mature male rats and monkeys after single doses of ODM-204. The models were developed and modified based on the data from previous studies carried out with CYP17A1 inhibitors (Hodgson YM et al., 1982, Duc I et al., 2003, Haidar S et al., 2003, Yamaoka M et al., 2012). In our studies ODM-204 showed inhibition equal to galeterone towards 17-alpha-hydroxylation and 17,20-lyation. However, abiraterone was significantly more potent than either ODM-204 or galeterone, having 100-fold better IC₅₀ values both in microsomal and cellular assays. Although abiraterone is an extremely potent CYP17A1 inhibitor *in vitro*, the efficacy *in vivo* in mice has been reported to be modest and the acetate form of abiraterone has been recommended for oral dosing due to its improved bioavailability and more favorable pharmacokinetic profile (Barrie SE et al., 1994, Ang JE et al., 2009). However, previous studies with abiraterone have shown a dose-dependent reduction in the androgen-dependent organ weights and serum T and LH levels with dose range 40-200 mg/kg qd being in line with our data (Duc I et al., 2003, Haidar S et al., 2003).

Monkeys have similar endocrine regulation and secretion of adrenal steroids to that in human (Abbott DH et al., 2009). Furthermore, unlike rodents in our studies, CYP17A1 enzyme in the monkeys and human are structurally very similar (Swart AC et al., 2002, Uno Y et al., 2014). In line with *in vitro* results, already a single oral dose in monkeys resulted in dose-dependent inhibition of the main androgens T and DHEA secreted from testicles and adrenals, respectively. However, due to the decreased androgen levels an increase in systemic LH levels was observed. Equal findings have also been reported in nonclinical studies with orteronel (TAK-700), and in the toxicological studies of abiraterone (Aziz RM, 2011, Yamaoka M et al., 2012). Interestingly, as an indication of potent inhibition of CYP17A1, serum P concentrations in the intact male monkeys increased rapidly with oral doses of ODM-204. The elevation in serum P was 2-3.5-fold with ODM-204, while with abiraterone as high as 14-35-fold increase has been reported after three weeks of dosing (250-1000 mg/kg qd) (Aziz RM, 2011). This substantial

increase in P levels may have a role in the CRPC progression, while P may act as a substrate for the intratumoral steroid biosynthesis, but also to work as a weak AR agonist by itself (Kumagai J et al., 2013b, Ando T et al., 2018). Already in the phase I dose-escalation study conducted with abiraterone without glucocorticoid supplement, significant increases in hormones in the mineralocorticoid pathway e.g. in the deoxycortisone, 10-fold and corticosterone, 40-fold were observed, most likely due to increased P concentration in the adrenal glands. This has been inhibited by concomitant dosing of a low dose of dexamethasone (0.5 mg/day), which more completely than standard of care, prednisone (5 mg/kg bid), suppresses the secretion of ACTH (Attard G et al., 2008). The recent reports underpin this hypothesis; by changing prednisone to dexamethasone in patients recurring with abiraterone (Lorente D et al., 2014, Venkitaraman R et al., 2015), a significant part (up to 40%) of the patients have shown PSA response and showed more complete suppression of ACTH, which leads to decreased production of steroid hormones capable to activate the progression of CRPC. However, this may also promote the development of AR mutations, especially AR(L702H); which has been recognized to be activated by glucocorticoids (Krishnan AV et al., 2002, van de Wijngaart DJ et al., 2010).

Only scarce clinical data is available showing the possible effect of the CYP17A1 inhibitors on the serum P levels in CRPC patients. Recently, a small phase II combination study with abiraterone and dutasteride (McKay RR et al., 2017) showed the connection between abiraterone and P levels; patients with higher exposures to abiraterone were reported to have higher serum P levels compared with individuals with lower abiraterone exposures. Moreover, increased serum P concentrations were reported already during ADT, although a clear accumulation of circulating P and pregnenolone was observed with abiraterone treated patients (Snaterse G et al., 2017). Importantly, recent data has also clearly shown that in spite of potent inhibition of CYP17A1 with abiraterone, DHEA, DHEA-S and A4 can be detected in the bloodstream (Taplin ME et al., 2014, Snaterse G et al., 2017). In the presence of high levels of AR, as progression of the disease, including resistance to abiraterone, these steroids may directly activate AR or they can also be converted to more active androgens, such as T and/or DHT.

6.6 Mouse adrenal hormones contribute to the growth of castration-resistant VCaP tumors

The synthesis of T and DHT in the tumors is one significant mechanism in the progression of CRPC. The old data that mouse adrenal glands lack the expression CYP17A1 enzyme and do not produce androgens (van Weerden WM et al., 1992), has markedly affected the preclinical research of CRPC, and studies have focused on measuring only the intratumoral synthesis of androgens (Montgomery RB et al., 2008, Chang K-H et al.,

2011, Pfeiffer MJ et al., 2011, Fiandalo MV et al., 2014). In our xenograft study the effect of adrenal hormones on CRPC growth was studied by surgical interventions, by performing castration (ORX) alone or castration and adrenalectomy (ORX+ADX) to mice. The effects of the surgical interventions were then compared to an intact control group. To our knowledge, this was the first study showing that mouse adrenal glands produce biologically significant amounts of the highly potent androgens, T and DHT, and further validating the role of adrenal hormones in promoting the growth of VCaP tumors. The growth of tumors in the ORX+ADX mice was significantly slower compared to the ORX mice and the decline in the serum PSA value in the ORX+ADX group was remarkably better than in the ORX mice. Because of the importance of glucocorticoids in the endocrine homeostasis, we also confirmed that the reduced growth of VCaP tumors is not caused by decreased welfare of mice. For this, we next treated the ORX+ADX mice with a physiological dose of corticosterone, and this treatment had no effect on the growth of the VCaP tumors. Finally, the expression of certain enzymes of androgen biosynthesis (*Akr1c3*, *Srd5a1* and *Srd5a2*), including enzymes with *Hsd17b*-activity (Stuchbery R et al., 2017) were verified by RT-qPCR and the observed increased expression after ORX was in line with the steroid analysis indicating activation of the synthesis of steroid hormones.

Interestingly, ORX significantly increased the weights of adrenal glands, indicating a consequent activation of adrenal cortex. Identical increase of adrenal gland weights have been observed in the ORX mice with abiraterone (Duc I et al., 2003), and an identical finding was reported in rats almost four decades ago (Malendowicz LK, 1979). In the adrenals the expression of *Lhr* displayed also a close correlation with the expression of *Cyp17a1*, and this finding was in line with previously presented results, where ORX resulted in a marked induction of *Lhr* expression in the mouse adrenal gland (Kero J et al., 2000), suggesting that in ORX mice, LH could promote early steps of adrenal steroidogenesis.

In a few nonclinical studies, systemic P levels have been measured in mice after orchidectomy (Locke JA et al., 2008, Nilsson ME et al., 2015), although its origin has not been entirely clarified, and in the xenograft studies with ORX mice observed circulating P was suggested to be produced by the tumors (Dillard PR et al., 2008, Locke JA et al., 2009). The present data shows that majority of P is produced in adrenals both in intact and ORX mice carrying VCaP xenografts. Moreover, the data indicates that the adrenal glands significantly contribute to the levels of circulating T and DHT. The clear decline after ORX+ADX in both circulating and intra-tumoral levels of androgens suggests that hormones secreted by the adrenal glands may act as AR agonists and promote the growth of VCaP tumors. Data obtained in ORX mice also suggests that P secreted by the adrenal glands may have a dual role: it can act as a precursor for the intra-tumoral androgen production in castration-resistant VCaP tumors, but it can also act as an AR agonist in

circumstances with high AR expression, such as in CRPC (Culig Z et al., 1993, Heinlein CA et al., 2004). In addition, the human AR with the AR(T878A) mutation is strongly stimulated by P (Fenton MA et al., 1997), and this mutation is also reported to be one possible reason for abiraterone resistance (Chen EJ et al., 2015). Notably, P has been shown to be a substrate for SRD5A enzymes in the 5 α -dione pathway for DHT synthesis, bypassing the need of T production as an intermediate (Chang K-H et al., 2011). Based on the present results, the intratumoral concentration of DHT is unaltered after the ORX and even after a significant decrease in DHT after ORX+ADX, the detected levels are high enough to activate AR and stimulate tumor growth. The role of tumor steroid biosynthesis in the androgen-dependent growth of the VCaP tumors in ORX mice is also supported by the observed increase in AR expression and with changes in the levels of androgen-dependent gene expression including *NOV*, *FKBP5* and *ST6GALNAC1* in the tumors already after the ORX. Furthermore these changes were more significant in ORX+ADX mice. Similar changes on the gene expression for these genes have been observed with castration-resistant VCaP tumors with enzalutamide (Knuutila M et al., 2018). Both *NOV* and *ST6GalNAc1* are directly regulated by AR and have been observed in clinical specimens as well strengthening the hypothesis that mouse adrenal glands contribute to the androgen dependent growth of VCaP tumors in ORX mice (Wu L et al., 2014, Munkley J et al., 2015).

Nevertheless, the non-classical androgens, such as 11-ketoT and 11-ketoDHT, have activated AR with similar affinity as T and DHT (Pretorius E et al., 2016). Also, the role of ligands from the glucocorticoid pathway, 11-deoxycorticosterone and its direct metabolite 5 α -dihydrocorticosterone, which have been reported to activate both wtAR and mutated AR (Uemura M et al., 2010) cannot be forgotten. The formation of 5 α -dihydrocorticosterone has been suggested to occur via SRD5A1, the same enzyme which converts T to DHT and has been reported to be present both in the nonclinical and clinical tumor samples in all states of prostate cancer. The expression of *SRD5A1* enzyme was significantly increased in the VCaP tumors in the ORX+ADX mice, similarly as shown in abiraterone-treated animals using the LuCaP PDX model (Mostaghel EA et al., 2011). In our study the expression of *AKR1C3* was also increased in tumors after the ORX or ORX+ADX intervention, indicating improved androgen metabolism in the VCaP tumors. *AKR1C3* is an important enzyme in the androgen biosynthesis and its induction has been suggested to contribute to drug resistance in CRPC patients treated with abiraterone and enzalutamide (Tian Y et al., 2014, Liu C et al., 2015). These findings increase the value of the CRPC VCaP model.

6.7 Future directions

Despite the significant progression and new promising therapies approved for the treatment of castration-resistant prostate cancer, mechanisms of resistance also evolve for these new drugs. Multiple resistance mechanisms, both AR-independent and -dependent have been identified and novel targeted therapies are being developed. These include new interesting approaches applying e.g. immuno-oncology, epigenetics and DNA-repairing. However, even in the late-state disease the transcriptional activity of AR is one of the resistance mechanisms after current treatments. In spite of the central role of AR itself, its mutations and constitutively active variants as well as the main pathways of androgen biosynthesis in the testicles and adrenal cortex have been recognized, and large amounts of novel data on local steroid biosynthesis in CRPC have been gathered. These pathways and steroidal ligands activating AR are not essentially solved and it is possible that these pathways become dominant and persistent, perhaps even essential for tumor growth in the CRPC state.

One possible novel target to control the altered steroid hormone production in CRPC is CYP11A1 enzyme. It is the first and rate-limiting enzyme in the whole steroid biosynthesis, catalyzing the conversion of cholesterol to pregnenolone. Inhibition of this enzyme is expected to completely suppress the production of all steroidal hormones, including gluco- and mineralocorticoids and importantly also all steroids that currently are known to activate AR. From the drug development point of view CYP11A1 enzyme is interesting due to its very slow rate of catalysis (Tuckey RC et al., 1993). Furthermore it is not highly induced in the tumor models after antiandrogen treatments compared to CYP17A1, which has significantly induced in nonclinical CRPC models (Cai C et al., 2011b, Mostaghel EA et al., 2011, Knuutila M et al., 2014) or in the mouse adrenals after ORX in our study. CYP11A1 enzyme, at least at the mRNA level, has been detected in metastatic CRPC bone marrow biopsies (Stanbrough M et al., 2006b). In addition, based on the results presented by Montgomery and colleagues the CYP11A1 levels are slightly increased in CRPC tumors (Montgomery RB et al., 2008). Recently this enzyme has also been suggested to be a plasma biomarker for biochemical recurrence from the samples taken at prostatectomy (Horning AM et al., 2015). As shown in many reports, the steroid biosynthesis in CRPC, both in the adrenal glands and tumors is extremely complex, and it can vary and mutations in the enzymes can occur. In addition, new data are constantly published showing how various different ligands and intermediates activate the hypersensitive AR in CRPC. The potent and selective CYP11A1 inhibitor in combination with gluco- and mineralocorticoid replacement therapy could offer a new treatment option for prostate cancer patients.

7 CONCLUSIONS

In summary, darolutamide is a high-activity, next-generation AR antagonist, which antagonizes also AR mutants known to mediate resistance to first- and second-generation antiandrogens. Due to its high affinity it also acts as an antagonist in AR overexpressing cells and impairs nuclear translocation of the receptor. In nonclinical *in vitro* and *in vivo* models of CRPC, darolutamide is more efficacious than other tested antiandrogens and it does not stimulate an androgen feedback loop at the hypothalamic-pituitary-gonadal axis. The unique structure and the presented promising nonclinical properties of darolutamide, observed already in phase I/II studies, make darolutamide a promising new drug that may provide hope for the patients, having properties differing from current antiandrogens and working as antagonist in cases where currently used drugs have lost their effectiveness or even replacing the current antiandrogens since it provides more long-lasting efficacy than the present standard of care.

ODM-204 is the first nonsteroidal, optimized potent inhibitor of CYP17A1 and AR antagonist. It has activity similar to that of galeterone towards CYP17A1, and it also blocks AR action to the same degree as enzalutamide. ODM-204 was shown to efficiently inhibit steroid biosynthesis in rodents and primates, and presented promising antitumoral activity in animal models of human prostate cancer. In addition, ODM-204 has uniquely balanced dual-inhibitory properties, which may provide benefits in CRPC patients. With a single agent, ODM-204 can avoid the drug interaction potential and changes in the drug exposures involved in treatment combinations with enzalutamide and abiraterone, suggesting that ODM-204 has therapeutic potential to be an effective treatment for prostate cancer.

The nonclinical research related to steroid biosynthesis in CRPC has been highly concentrated to capability of xenografted tumors to produce androgens, while the role of hormones secreted by the adrenal gland has been largely forgotten. We, in contrast to the current view, proved that adrenal glands of intact and orchidectomized mice synthesize physiologically relevant levels of androgen synthesis intermediates and active androgens having significant contribution to the growth of VCaP xenografts in the CRPC mouse model.

This thesis provides novel data from the nonclinical development of new drugs for different states of CRPC. In addition, it offers remarkable new data about the role of adrenal gland steroid production in the CRPC xenograft model, providing new tools for further drug development and novel information of CRPC androgen synthesis pathways.

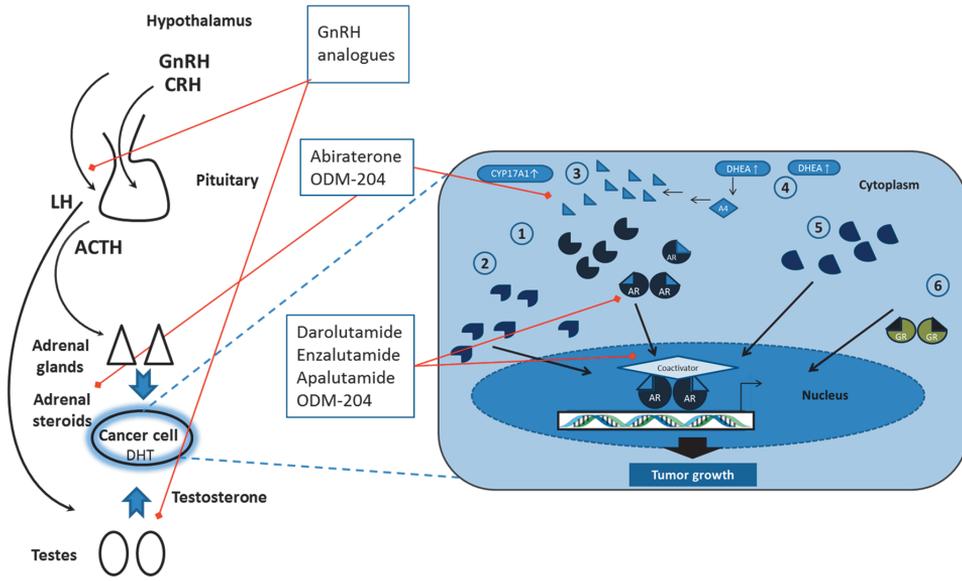


Figure 11: Androgen synthesis and signaling pathways. Mode of actions of selected therapies used for androgen deprivation therapy and blocking androgen actions.

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