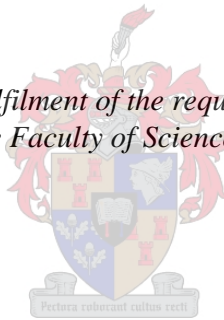# AN INVESTIGATION INTO THE INFLUENCE OF ROOIBOS (*ASPALATHUS LINEARIS*) ON ANDROGEN METABOLISM IN NORMAL AND PROSTATE CANCER CELLS

by
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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Science at Stellenbosch University

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March 2015

Declaration

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Abstract

In this study, the influence of rooibos on the catalytic activity of enzymes 17β-hydroxysteroid dehydrogenase type 3 (17βHSD3), 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3), 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2), 5α-reductase type 1 (SRD5A1) and 5α-reductase type 2 (SRD5A2), which catalyse prostate androgen metabolism, was investigated. The activities of both 17βHSD3 and AKR1C3 heterologously expressed in CHO-K1 and HEK293 cells were inhibited significantly by rooibos, with rooibos reducing the conversion of androstenedione (A4) and 11keto-androstenedione (11KA4) to testosterone (T) and 11keto-testosterone (11KT), respectively. The catalytic activity of 17βHSD2 towards T, 11hydroxy-testosterone (11OHT) and 11KT was also significantly inhibited by rooibos in transiently transfected HEK293 cells. In transiently transfected HEK293 cells rooibos did not inhibit SRD5A1 while the rate of T conversion to dihydrotestosterone (DHT) by SRD5A2 was decreased. Analysis of steroid metabolism in PNT2 cells also suggests that rooibos does not modulate the catalytic activity of endogenously expressed SRD5A towards A4, however, the conversion of T to DHT was reduced. In addition, reductive 17βHSD activity towards A4 was inhibited in the presence of rooibos in both PNT2 and BPH-1 cells. In contrast, the conversion of 11KA4 to 11KT was inhibited in BPH-1, PC-3 and LNCaP cells, with negligible conversion of 11KA4 in PNT2 cells. Interestingly, data suggests inhibition of 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2) activity in the production of androsterone (AST) from 5α–androstenedione (5α-dione), as well as the dehydrogenase reaction of T to A4 in PNT2 cells by rooibos. Androgen metabolism pathways were subsequently investigated in LNCaP cells to determine androgen metabolism by endogenous enzymes. Rooibos resulted in the reduced conversion of A4 in LNCaP cells to the same extent as indomethacin, a known AKR1C3 inhibitor. Rooibos also modulated T, DHT and AST metabolism in LNCaP cells. Furthermore, uridine diphosphate glucuronosyltransferase (UGT) activity in LNCaP cells was inhibited by rooibos, decreasing T-, DHT– and AST-glucuronide formation. These data prompted subsequent investigations into the influence of rooibos at cellular level, and prostate-specific antigen (PSA) levels were assayed in the presence of rooibos. PSA was significantly inhibited by rooibos in the absence and presence of DHT, suggesting possible interaction of rooibos with the mutated androgen receptor (AR) or estrogen receptor-β (ERβ) expressed in LNCaP cells.

Taken together, rooibos inhibited the catalytic activity of key enzymes that catalyse the activation of androgens in the prostate, as well as inhibiting enzymes involved in the conjugation of androgens. At cellular level, PSA levels were also decreased by rooibos, possibly through AR or ERβ interactions – clearly indicating a modulatory role for rooibos in active androgen production.

Opsomming

In hierdie studie was die invloed van rooibos ten opsigte van die katalitiese aktiwiteite van die ensieme 17β-hidroksi-steroïed-dehidrogenase tipe 2, tipe 3 en tipe 5 (17βHSD2, 17βHSD3, AKR1C3), asook 5α-reduktase tipe 1 en tipe 2 (SRD5A1, SRD5A2) ondersoek. Hierdie ensieme is betrokke in die produksie van androgene in die prostaat. Rooibos het die katalitiese aktiwiteit van 17βHSD3 en AKR1C3 in CHO-K1 en HEK293 selle beïnvloed en het vermindere omskakeling van androstenedioon (A4) en 11keto-androstenedioon (11KA4) na testosteroon (T) en 11-keto-testosteroon (11KT), afsonderlik, veroorsaak. Die katalitiese aktiwiteit van 17βHSD2 teenoor T, 11-hidroksie-testosteroon (11OHT) en 11KT was ook beïnvloed in die teenwoordigheid van rooibos in HEK293 selle. Die katalitiese aktiwiteit van SRD5A1 teenoor A4 en T is nie beïnvloed deur rooibos nie, alhoewel dit voorkom asof rooibos die omsettingstempo van T na dihidrotestosteroon (DHT) deur SRD5A2, getransfekteer in HEK293 selle, verminder het. Verdere ondersoeke is in normale prostaat epiteel selle, in die teenwoordigheid van rooibos uitgevoer. Rooibos het geen invloed op die katalitiese aktiwiteit van SRD5A teenoor A4 gehad nie, alhoewel vermindere omskakeling van T na DHT aangetoon kon word. Rooibos het ook die omskakeling van A4 na T in beide PNT2 en BPH-1 selle tot 'n mate geïnhibeer. Die omskakeling van 11KA4 na 11KT was ook verminder in BPH-1, PC-3 en LNCaP selle. Die omskakeling van 11KA4 na 11KT was beduidend laer in PNT2 selle en kon die invloed van rooibos nie aangetoon word nie. Bykomende data toon dat rooibos ook die omskakeling van 5α-androstenedioon (5α-dione) na androsteroon (AST), gekataliseer deur 3α-hidroksi-dehidrogenase tipe 3 (AKR1C2), verminder, gesamentlik met die vermindere omskakeling van T na A4, deur 17βHSD2, in PNT2 selle. Hierdie studie het ook ondersoek ingestel, na die metabolisme van androgene in LNCaP selle. Vermindere A4 metabolisme is in die teenwoordigheid van rooibos asook in die teenwoordigheid van indometasien, 'n bekende AKR1C3 inhibitor, gevind. Rooibos verminder dus die aktiwiteit van reduktiewe 17βHSD in LNCaP selle. Verandering in die metabolisme van T, DHT en AST in LNCaP selle, in die teenwoordigheid van rooibos, is ook gevind. Verdere ondersoek in LNCaP selle het gewys dat rooibos 'n vermindering in die produksie van gekonjugeerde T, DHT en AST veroorsaak. Die studie het die invloed van rooibos op prostaat-spesifieke antigeen (PSA) ook ondersoek. Daar is vasgestel dat rooibos die vlakke van PSA verminder in die afwesigheid en teenwoordigheid van DHT in LNCaP selle. Hierdie resultaat dui op moontlike interaksie van rooibos met die androgeen (AR) of estrogeen-reseptor-β (ERβ), teenwoordig in LNCaP selle.

Rooibos het die katalitiese aktiwiteit van ensieme, wat bydra tot androgeen produksie, geïnhibeer, asook die konjugasie van androgene. Op 'n sellulêre vlak, het rooibos die vlakke van PSA-sekresie verminder, wat moontlike interaksie met die AR en ERβ aandui. Hierdie bevindings dui daarop dat rooibos wel n rol het om te speel in die modulasie van aktiewe androgene in die prostaat.

Dedicated to the most important person, whom without none of this would ever have been possible, the most unbelievably strong woman I know, my mother

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Table of Contents

Abbreviations and symbols

General

ACT	α1-antichymotrypsin
ACTH	Adrenocorticotropin
ADT	Androgen deprivation therapy
AES	Amino-terminal enhancer of split
AOM	Azoxymethanol
AR	Androgen receptor
ARE	Androgen response elements
BCA	Bicinchoninic acid
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
C-terminal	Carboxy terminal
CBP	CREB (cAMP-response element binding) protein
CRH	Corticotropin-releasing hormone
CRPC	Castration-resistant prostate cancer
CYP450	Cytochrome P450
Cyt b_5	Cytochrome b_5
DBD	DNA binding domain
ER(β)	Estrogen receptor (beta)
FSH	Follicle-stimulating hormone
GR	Glucocorticoid receptor
HAT	Histone acetylases
HDAC3	Histone deacetylase 3
HDAC6	Histone deacetylase 6

hCG	Human chorionic gonadotropin
HEY1	Hairy/enhancer-of-split related with YRPW motif 1
HSP	Heat shock protein
LBD	Ligand binding domain
LH	Luteinizing hormone
MR	Mineralocorticoid receptor
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide
N -terminal	Amino terminal
NADPH	Nicotinamide adenine dinucleotide phosphate
NCOR	Nuclear corepressor
PCa	Prostate cancer
PCAF	p300/CBP-associated factor
POR	P450 oxidoreductase
PSA	Prostate-specific antigen
RNA pol II	RNA polymerase II
SMRT	Silencing mediator of retinoid and thyroid
SRC	Nuclear receptor co-activator
StAR	Steroidogenic acute regulatory protein
TF7L2	Transcription factor 7-like 2
TLE	Transducin-like enhancer of split
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
ZF	Zona fasiculata
ZG	Zona glomerulosa
ZR	Zona reticularis

Enzymes

3αHSD	3α-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
11βHSD	11β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
AKR1C2	3α-hydroxysteroid dehydrogenase type 3
AKR1C3	17β-hydroxysteroid dehydrogenase type 5
CYP11A1	Cytochrome P450 cholesterol side chain cleavage
CYP11B1	Cytochrome P450 11β-hydroxylase
CYP11B2	Cytochrome P450 aldosterone synthase
CYP17A1	Cytochrome P450 17α-hydroxylase/17,-20-lyase
CYP21A2	Cytochrome P450 steroid 21-hydroxylase
H6PDH	Hexose-6-phosphate dehydrogenase
RL-HSD	RoDH like 3αHSD
RoDH	Retinol dehydrogenase
SRD5A	5α-reductase
SULT2A1	Sulfotransferase
UGT	Uridine diphosphate glucuronosyltransferase

Steroid hormones

3α-adiol	5α-androstane-3α, 17β-diol
3α-adiol-G	5α-androstane-3α, 17β-diol glucuronide
3β-adiol	5α-androstane-3β-17β-diol
5α-dione	5α-androstanedione
11K-5α-dione	11-keto-5α-androstanedione
11KA4	11keto-androstenedione
11KDHT	5α-dihydro-11-keto-testosterone
11KT	11keto-testosterone
11OH-5α-dione	11β-hydroxy-5α-androstanedione
11OHA4	11β-hydroxyandrostenedione
11OHDHT	5α-dihydro-11β-hydroxytestosterone
11OHT	11β-hydroxytestosterone
17OH allo-pregnanolone	5α-pregnane-3α, 17α-diol-20-one
17OH dihydroprogesterone	5α-pregnane 17α-ol-3, 20-dione
17OH-PREG	17α-hydroxypregnenolone
17OH-PROG	17α-hydroxyprogesterone
A4	Androstenedione
ALDO	Aldosterone
Androstenediol	Androsta-5-ene-3β, 17β-diol
AST	Androsterone
AST-G	Androsterone glucuronide
CORT	Corticosterone
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulphate

DHT	Dihydrotestosterone
DHT-G	Dihydrotestosterone glucuronide
DOC	11-deoxycorticosterone
GnRH	Gonadotropin-releasing hormone
PREG	Pregnenolone
PROG	Progesterone
T	Testosterone
T-G	Testosterone glucuronide

Mathematical symbols

K_m	Michaelis constant
V_{max}	Maximum reaction rate

Chapter 1

Introduction

Prostate cancer (PCa) is the most common malignancy found in men, with a median age for diagnosis, set at 66, and a median age of death set at 88. According to The American Cancer Society, 1 in every 7 men will be diagnosed with PCa during their lifetime, with 1 in every 36 of these men dying from PCa (American Cancer Society, 2014). A patient's cancer stage at diagnosis determines subsequent treatment options, with the cancer termed as localized (stage 1) if it has not metastasised and is located only in the tissue of origin. However, if the cancer has spread, it is termed regional or distant. Localized cancer accounts for ±81% of PCa cases, regional for ±12% and distant for ±4%, with ±3% of cancers being difficult to diagnose within the aforementioned groups. As such, localized and regional cancers have a 100% 5-year relative survival rate, compared to distant cancers which only have a 28% 5-year relative survival rate (National Cancer Institute, 2014).

Androgens play important roles in the pathogenesis of PCa, with diseases of the prostate - both benign prostatic hyperplasia (BPH) and PCa - considered as hormone-dependent diseases (Penning, 2010). Once diagnosed the option of castration is suggested, as a means of depleting the prostate of active androgens which promote cell proliferation. Many treatment strategies, such as castration and androgen-deprivation therapy (ADT), are currently employed against PCa, but irrespective of the treatment regimen, in about 30–40% of cases the cancer can re-emerge (2–5 years later) and is termed: castration resistant prostate cancer (CRPC) (Sharifi & Auchus, 2012). CRPC is in almost all cases fatal, with PSA, which serves as a clinical tool to analyse whether a patient is progressing to PCa, increasing once again with CRPC (Mohler et al., 2004). Cell growth and proliferation as it occurs in the prostate, is either due to the conversion of T into the potent active androgen, DHT, by the enzyme SRD5A or the conversion of 5α-dione, the SRD5A product of A4, to DHT by the enzyme 17βHSD. DHT is able to bind the AR and translocate into the nucleus and subsequently activate AR-dependent gene transcription (Hsing, Chu, & Stanczyk, 2008). Under normal circumstances, the testes provides T that circulates to the prostate, however, with CRPC, it is the adrenal that provides androgen precursors dehydroepiandrosterone (DHEA), A4 and to a lesser extent, T (Luu-The, Bélanger, & Labrie, 2008; Montgomery et al., 2008; Stanbrough et al., 2006; Stein, Goodin, & Dipaola, 2012). In addition, *de novo* steroid biosynthesis has also been suggested in prostatic tissue, representing yet another metabolic route supplying AR ligands to the androgen pool (Cai et al., 2011). Together these routes provide sufficient androgens to support cell growth under normal condition as well as driving PCa, and have also been suggested to drive the progression of CRPC. As mentioned, ADT is currently the treatment strategy against PCa, which include enzyme inhibitors or the use of AR antagonists, in order to reduce androgen

production and inactivate AR transcription. New approaches, however, need to be explored and implemented in an effort to increase survival rates.

There exists epidemiologic and case-controlled evidence that suggests that diet may be a modifier of PCa risk (Ornish et al., 2005; Saxe et al., 2006; Shimizu et al., 1991; Strom et al., 1999). In 2008, Ornish et al., reported a pilot study examining changes in prostate gene expression in men with low-risk PCa who participated in an intensive nutrition and lifestyle intervention, in which 48 up-regulated and 453 down-regulated transcripts, including oncogenes, were identified after the intervention. These gene changes comprised genes involved in tumourigenesis, which included genes involved in protein metabolism and modification, intracellular trafficking and protein phosphorylation. The lifestyle changes included a diet low in fat, comprised of whole-foods and plant-based nutrition, stress maintenance, together with exercise routines as well as attending a psychosocial support group. This study suggested that intensive nutrition and lifestyle changes may modulate gene expression in the prostate (Ornish et al., 2008). The participant's diets were supplemented with soy, fish oil, selenium and vitamin C and E and included soy products rich in polyphenols, as in the case of rooibos. It is interesting to note that the incidence of clinically significant PCa is less prevalent in world populations where people consume a predominant plant-based diet (Hebert et al., 1998; Parkin, Bray, Ferlay, & Pisani, 2005; Parkin, Pisani, & Ferlay, 1999).

Indeed the anti-carcinogenic properties of rooibos have been widely investigated and reported (Larsen et al., 2011; Marnewick et al., 2005). The anti-carcinogenic properties of rooibos in terms of PCa have, however, not been extensively investigated. As PCa is driven by androgens, the only plausible evidence to suggest a role for rooibos in PCa, are reports of rooibos and its role in the modulation of adrenal steroidogenesis together with the inhibition of steroid-metabolizing enzymes (Perold, 2009; Schloms & Swart, 2014; Schloms et al., 2013; Schloms et al., 2012). As mentioned, in the battle against PCa, new treatment approaches against PCa progression are sought and the use of herbal medicinal products preferred to mainstream pharmaceuticals, has led to increased interest in recent years in natural plant products.

Chapter 2 presents an overview of the flavonoid composition of rooibos together with the bioavailability and metabolism of the major polyphenolic compounds of rooibos. The roles that have been attributed to polyphenols and rooibos in steroid-dependent cancer development and the modulation of steroidogenic enzymes will be presented and this chapter concludes with the discussion of potential applications of rooibos in PCa.

Chapter 3 provides an overview of testicular, adrenal and prostatic steroidogenesis and highlights prostate androgen metabolism pathways and the enzyme machinery involved therein. Activities of SRD5As, 17βHSDs and UGTs together with the activation of the AR and downstream implications

thereof will be presented. This chapter furthermore highlights the progression of PCa to CRPC and concludes with a discussion of CRPC treatment strategies.

Chapter 4 and Chapter 5 describe the study into the influence of rooibos on androgen metabolism in normal and PCa cells in which the following aims are addressed:

- to determine the influence of rooibos on cell viability in CHO-K1, COS-1, HEK293 and LNCaP cells;
- to determine the influence of rooibos on the activities of reductive 17βHSD3 and AKR1C3, as well as on the oxidative activity of 17βHSD2, heterologously expressed in CHO-K1 and HEK293 cells;
- to determine the influence of rooibos on the conversion of steroids to their respective 5α-reduced androgens, catalysed via SRD5A1 and SRD5A2, heterologously expressed in U2OS and HEK293 cells;
- to investigate the influence of rooibos on androgen metabolism and endogenous enzyme activity in prostate cell models - PNT2, BPH-1, PC-3 and LNCaP cells;
- to determine PSA levels in the presence and absence of DHT, followed by the investigation into the influence of rooibos on basal and DHT-stimulated PSA levels in LNCaP cells.

The influence of rooibos on the catalytic activity of 17βHSD3, AKR1C3, 17βHSD2, SRD5A1 and SRD5A2, heterologously expressed in cell models, is described in chapter 4. The influence of rooibos on androgen metabolism in normal epithelial, PNT2, and in BPH-1 prostate cells, specifically on the catalytic conversion of androgens by endogenously expressed 17βHSDs and SRD5As, as well as the influence of rooibos on cell viability will be discussed in this chapter.

In chapter 5, the influence of rooibos on prostate androgen metabolism in the androgen-dependent PCa cell line, LNCaP, is discussed. This chapter reports on the metabolism of A4, T, DHT, AST and 3α-adiol in terms of endogenous enzyme activity and the preferential conversion of steroids in metabolic pathways in this cell model. The inhibitory effect of rooibos on endogenously expressed reductive 17βHSDs and the modulation of AKR1C2, as well as UGTs, are subsequently described, together with the influence of rooibos on AR-regulated gene expression of PSA.

Chapter 6 concludes the thesis with an overview of the findings of this study and presents conclusions based on the data presented, together with the potential role of rooibos in therapeutic approaches to PCa.

Chapter 7 presents detailed experimental procedures undertaken throughout this study.

Chapter 2

The bioactivity of rooibos and its potential application in PCa

2.1 Introduction

The discovery that rooibos, a polyphenol-rich herbal tea, soothed Annetjie Theron baby's colic, in 1968, launched rooibos' reputation as a 'health gimmick' in modern times, 300 years after the Khoisan tribe had already discovered that an aromatic tea can be brewed from the wild rooibos plant (Joubert, 2011). Rooibos tea is currently enjoyed in 37 countries, including Germany, the Netherlands, the United Kingdom, Japan and the United States of America, with the aforementioned countries representing 86% of the rooibos export market. Rooibos is consumed as an herbal tea competing with coffee and varieties of *Camellia sinensis*. Rooibos is caffeine free and compared to the before mentioned beverages, very low in tannins. Rooibos grows naturally in the Cederberg area in the Western Province of South Africa and is produced from the stems and leaves of *Aspalathus linearis*, belonging to the genus *Fabaceae*, *Tribe Crotalarieae*, endemic to the Cape Floristic Region (Joubert & de Beer, 2011).

Rooibos was first marketed in 1904 in its fermented 'oxidized' form, with the unfermented 'green' rooibos form currently emerging as a growing market. Green rooibos was first produced during the 1990s to achieve higher antioxidant levels, and is consumed as a functional beverage. Green rooibos extracts are also marketed in the food and cosmetic industries (Joubert, 2011). Green rooibos is popular as it contains high levels of flavonoids and is caffeine-free, and together with fermented rooibos aids in the alleviation of depression, anxiety and insomnia. Its popularity has grown to such a degree that rooibos extracts, usually combined with other ingredients, are available today in tablet form, functioning as dietary supplements.

2.2 Bioavailability and metabolism of the major polyphenolic compounds in rooibos

Plants possess the ability to acquire polyphenolic precursor compounds and to produce flavonoids, which include flavonols, anthocyanins and tannins, through the phenylpropanoid pathway. This pathway forms part of secondary metabolite biosynthesis maintained by plants and is therefore not required to sustain plant life, but contributes to aiding in the plant's defences and reproduction (Kubasek et al., 1992). Flavonoids are polyphenols that occur naturally in fruits, vegetables, teas and herbs, with flavonoid biosynthesis starting from phenylalanine to produce 4-coumaroyl-CoA, via cinnamic acid and *p*-coumaric acid. Condensation of 4-coumaryol-CoA and malonyl-CoA subsequently yields the intermediate chalcones, which are precursors of different flavonoid subgroups, such as flavones, flavanones, isoflavones and flavans (fig. 2.1) (Brožič et al., 2009).

Flavones   Flavanones   Isoflavones   Flavans
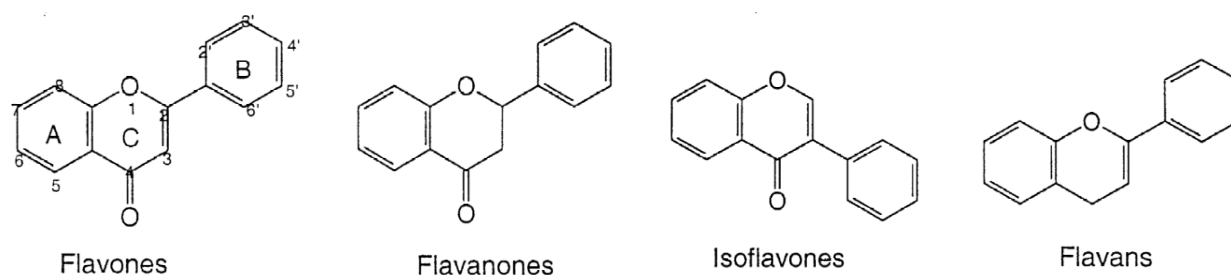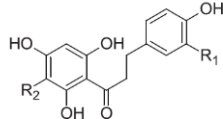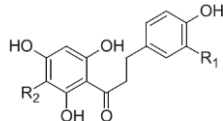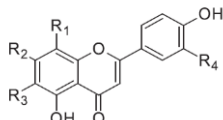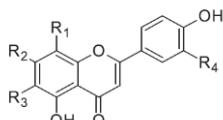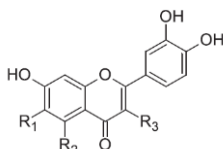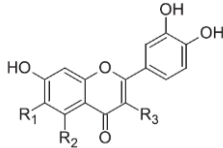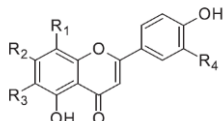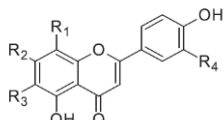
Figure 2.1: General structures of flavonoids.

Over 4000 flavonoids have been identified in foods such as soy, apples, red wine, tea and onions (Zand et al., 2002), with 46 flavonoids identified in rooibos to date (Beelders et al., 2012; Joubert, Gelderblom, Louw, & de Beer, 2008). Flavonoids in rooibos are derivatives of 2-phenyl-4-benzopyrone (flavone), with phenolic hydroxyl or methoxyl substituents at positions 3 – 6 (ring A and C) and 3' – 5' (ring B) of the two aromatic ring systems (fig. 2.1). These moieties are often glycosylated (Kachlicki et al., 2008; Xiao et al., 2009). Rooibos contains two unique polyphenolic compounds, aspalathin, a dihydrochalcone C-glucoside, and aspalalinin, a cyclic dihydrochalcone. Nothofagin is a rare dihydrochalcone C-glucoside also present in rooibos, and one other specie, *Nothofagus fusca* (Joubert & de Beer, 2011). Major flavonoid compounds present in rooibos include flavones, flavanones and flavonols, together with the aforementioned dihydrochalcones, as shown in table 2.1.

Table 2.1: Chemical structures of the major rooibos flavonoids. Reproduced with permission from (Joubert & de Beer, 2011; Schloms & Swart, 2014).

Compound	Polyphenol subgroup and substitution	Structure
Aspalathin	Dihydrochalcone (R_1 = OH; R_2 = C-β-D-glucopyranosyl)	
Nothofagin	Dihydrochalcone (R_1 = H; R_2 = C-β-D-glucopyranosyl)	
Orientin	Flavone (R_1 = C-β-D-glucopyranosyl; R_2, R_4 = OH; R_3 = H)	
Isoorientin	Flavone (R_1 = H; R_2, R_4 = OH; R_3 = C-β-D-glucopyranosyl)	
Quercetin	Flavonol (R_1 = H; R_2, R_3 = OH)	
Rutin	Flavonol (R_1 = H; R_2 = OH; R_3 = O-rutinosyl)	
Isovitexin	Flavone (R1, R_4 = H; R_2 = OH, R_3 = C-β-D-glucopyranosyl)	
Vitexin	Flavone (R_1 = C-β-D-glucopyranosyl; R_2= OH, R_3, R_4 =H)	

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As previously mentioned, rooibos is processed as either unfermented or fermented (oxidised), with a higher polyphenol content found in unfermented 'green' rooibos. This is due to reduced exposure to the sun when the leaves are dried, thus reducing possible oxidative changes. The main polyphenols in unfermented rooibos are: aspalathin, isoorientin, orientin and rutin, with isovitexin, vitexin, isoquercitrin, hyperoside, quercetin, luteolin and chrysoeryol being present at lower levels. Aspalathin is ±50 times higher in the unfermented form of rooibos, compared to the fermented form. The fermentation process results in substantial degradation of aspalathin. One of the changes that occur is the oxidation of aspalathin to its flavanone analogues isoorientin and orientin, with dihydro-isoorientin ((R) and (S)-eriodictyol-6-C-glucoside as intermediates (fig. 2.2). Orientin forms irreversibly from isoorientin, with the latter undergoing opening of its vinyl ester structure to form a chalcone intermediate. Given the degradation and studies reporting poor bioavailability of aspalathin (Breiter et al., 2011; Courts & Williamson, 2009; Kreuz et al., 2008; Stalmach et al., 2009), the importance of this rooibos flavonoid is highlighted by *in vivo* evidence of its bioactivity, including anti-mutagenic effects, its hypoglycaemic activity and moderate phytoestrogenic effects (Joubert & de Beer, 2011).
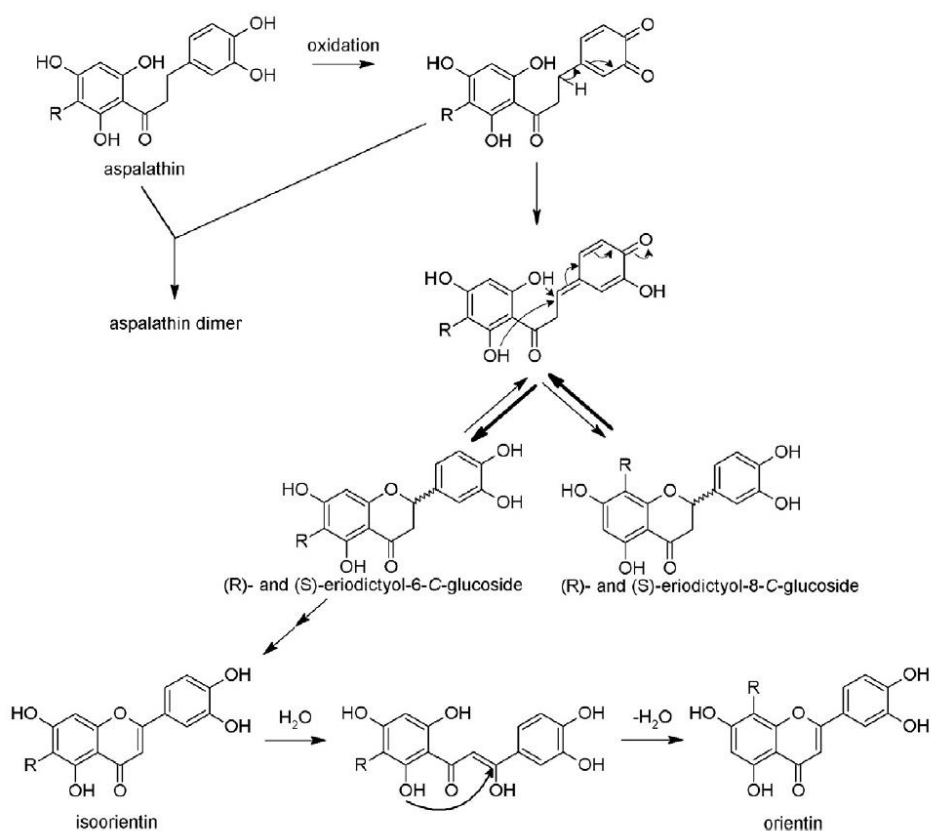
Figure 2.2: Mechanism of aspalathin oxidation. Reproduced with permission from (Joubert & de Beer, 2011).

The C-glycosyl-flavones isoorientin, orientin, vitexin and isovitexin are degraded less when compared with the main flavonol-glycoside rutin, which is partly degraded to form quercetin

(Bramati, Aquilano, & Pietta, 2003). Fermentation therefore causes a definitive shift from a flavonoid rich tea to a flavonoid poor tea extract, resulting in quantitative changes in phenolic composition.

Bioavailability of polyphenols is a concept that incorporates bioaccessibility, absorption, metabolism, tissue distribution and bioactivity. Bioaccessibility refers to the amount of an ingested compound that becomes available for absorption in the gut compared to bioavailability which is the concentration of a compound or its metabolite at the peripheral target tissue (Manach et al., 2004; Scholz & Williamson, 2007; Stahl et al., 2002). The definition of bioavailability often encompasses the liberation, absorption, distribution, metabolism and excretion of a compound. Liberation suggests the release of a compound from its matrix, absorption is the transport of a compound from the site of administration into the systemic circulation and distribution is the transportation of a compound by the systemic circulation to body tissue. Metabolism is the biotransformation of a compound and excretion is the elimination of a compound from the body via renal, biliary- or pulmonary processes (Holst & Williamson, 2008). There exist exogenous and endogenous factors that can influence the bioavailability of compounds. Exogenous factors include the complexity of the food matrix, the dosage and chemical form of the compound and endogenous factors include intestinal transit time, rate of gastric emptying, metabolism and extent of conjugation (Holst & Williamson, 2008).

Many studies have considered the absorption and metabolism of rooibos flavonoids – as their potency and eventual effect is determined by their metabolism. Considering a single oral dose administered to human subjects, researchers reported that an unfermented aqueous drink produced a single methylated and a single methylated-glucuronidated metabolite excreted in urine (Courts & Williamson, 2009), while unfermented and fermented aqueous extracts in ready-to-drink beverages led to the identification of eight metabolites in urine. The eight metabolites identified included: *O*-linked-methyl, sulfate, and glucuronide metabolites of aspalathin and an eriodictyol-*O*-sulfate (Stalmach et al., 2009). No metabolite could, however, be identified in plasma, due to the great affinity of flavonoids to proteins. Notably, in circulation polyphenols are mostly conjugated derivatives extensively bound to albumin (van der Merwe, 2012) with protein-polyphenol complexes forming readily as flavonoids exhibit a high affinity towards proteins, as shown in *in vitro* studies (Manach et al., 1995).

It was subsequently shown that rooibos tea and an isolated active fraction from unfermented rooibos yielded the following metabolites in urine: methylated-, glucuronidated-, methylated-glucuronidated- and sulphated aspalathin derivatives; three aglycone forms of aspalathin glucuronidated at three different positions; the glucuronidated form of nothofagin as well as unmetabolised aspalathin and nothofagin. The main excreted metabolite was methylated aspalathin, which suggests methylation as a significant conjugation pathway. Aspalathin, orientin,

isoorientin, (S)-eriodictyol-8-C-glucoside, vitexin and an isomer of rutin were also detected in plasma. These metabolites were detected 24 h after consumption of the different rooibos drinks and on average 0.26% of total flavonoids consumed were detected in plasma samples after intake of rooibos tea. However, recovery rates were low and marked inter-individual variation in absorption patterns of the human subjects in the study were evident. The data nevertheless indicated flavonoid bioavailability (Breiter et al., 2011).

It is assumed that the intact flavonoids reach the large intestine, whereupon exposure to the action of intestinal bacteria, leads to the metabolites which are detected in urine. This is for instance the case with rutin that is metabolized by bacterial enzymes to form quercetin, which is further metabolised to yield phenolic acids, together with methylated and glucuronidated metabolites (Breiter et al., 2011; Stalmach et al., 2009). Following absorption, flavonoids can be metabolized by both phase I and phase II metabolising enzymes (Williamson et al., 2000). Cytochrome P450 (CYP450) enzymes are phase I monooxygenases fundamental in metabolism of drugs and foreign compounds and mediate flavonoid metabolism (Moon et al., 2006; van der Merwe, 2012). The CYP450-mediated oxidation, however, has not been shown to be as important as glucuronidation and sulphation *in vivo* or in intact cells (Walle, 2004). Metabolism by phase II enzymes includes UGTs, sulfotransferases (SULT) and catechol-O-methyltransferase, leading to mono- or multiple glucuronidated, sulphated and methylated conjugates (Kroon et al., 2004; Zhang et al., 2007). Glucuronidation is, however, considered as one of the most important metabolic pathways in the liver and intestine (Zhang et al., 2007), with sulphation having a higher affinity, but a lower-capacity pathway compared to glucuronidation, resulting in a shift from sulphation towards glucuronidation when the ingested dose increases (Koster et al., 1981).

The question remains whether these phenolic compounds reach peripheral target tissue in their intact form, at physiological relevant concentrations, or whether effects are due to their metabolites. In addition increasing the dosage does not necessarily mean that more compounds will reach specific peripheral target tissues, as polyphenols taken in high dosages have been linked to hepatotoxic effects, potential drug interactions as well as estrogenic effects (Mennen et al., 2005). These side effects are, however, only seen when high doses of supplements are taken and do not occur with the daily consumption of teas. Therefore, even though the bioavailability and toxicity level of flavonoid ingestion is uncertain, the bioactivity of polyphenols and rooibos extracts has been reported extensively and will be discussed further.

2.3 Physiological activity of polyphenols

Flavonoids are phenolic compounds characterized by their diaryl nucleus – mimicking the chemical structures of natural human steroid hormones, hence the scientific interest in these compounds, and their potential application in the prevention of hormone-dependent cancers. Potential targets of

phytoestrogens include steroid hormone receptors, steroidogenic enzymes, elements involved in signal transduction and apoptotic pathways as well as DNA processing mechanisms.

2.3.1 The influence of polyphenols and rooibos extract on hormone-dependent cancers

The first association between flavonoids and steroid-hormone dependent cancers was made when studies highlighted the low incidence of cancers, such as breast and colon cancer, in Asian countries, with their diet consisting predominantly of high polyphenol soy-based foods. Flavonoids present in significant amounts in the human diet, include: soy isoflavones (genistein, daidzein and biochanin A), flavonols (quercetin, myricetin, kaempferol) and flavones (luteolin and apigenin). An ecological study found a negative correlation between soy consumption and the incidence of cancer. The specific cancers included in the study were; breast, colon, cervix and ovary, however, data did not show a negative correlation with PCa (Messina et al., 1994). Another study which included 34 000 vegetarians and omnivores, showed a significantly reduced risk of colon cancer and reduced PCa rates (Zand et al., 2002). Another study undertaken with 83 PCa patients found that a higher intake of phytoestrogens, including isoflavones and flavonoids, had a slightly protective effect on PCa risk (Strom et al., 1999).

The number of hydroxyl substitutions found on the chemical structure of a flavonoid has been linked to their anti-oxidant properties, with hydroxyl substitutions at C3' and C4' (fig. 2.1, ring B) being shown to be important to peroxyl radical absorbing activity. However, pro-oxidant activities have also been linked to flavonoids (Cao et al., 1997). Structure-function relationships are important as diphenolic structures and hydroxyl groups at C7 (fig. 2.1, ring A) or C4' (fig.2.1, ring B) of the flavonoid molecule are necessary for their estrogenic activity. Quercetin, one of the flavonoids found in rooibos, possesses a hydroxy group at C3 (fig. 2.1, ring C), hindering binding to the ER, accounting for its low binding affinity (Zand et al., 2002). These data leads one to question whether quercetin might have a potential role to play in AR binding and if this flavonoid would possess androgenic activity.

The androgenic and anti-androgenic activity of flavonoids has only recently been investigated. An investigation into the anti-androgenic properties of rooibos was conducted with TM3 mouse testicular Leydig cells treated with fermented and unfermented extracts under both basal and stimulated conditions. Stimulated conditions were achieved in cell cultures by adding human chorionic gonadotropin (hCG). Both unfermented and fermented rooibos extract resulted in a decrease in T production, under both hCG stimulated (3.9 – 31.8%) and basal (16.3 – 37.9%) conditions. Using a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay it was shown that rooibos maintained cell viability at 0.25 – 1 mg/mL, but reached cytotoxic levels at 5 mg/mL. The mechanism by which T production was inhibited was suggested to be by the inhibition of CYP11A1 (cytochrome P450 short-chain cleavage enzyme), the reduction of cyclic adenine monophosphate levels and the inhibition of 3β-hydroxysteroid dehydrogenase (3βHSD)

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and/or 17βHSD (Opuwari & Monsees, 2014). This study showing the anti-androgenic activity of rooibos, suggest a role for rooibos in T-dependent cancers, and that rooibos influences steroid-metabolizing enzymes and pathways.

Flavonoids have also been shown to influence cell growth. In T47-D breast cancer cells, 20 μM genistein and quercetin markedly inhibited growth, with quercetin being able to inhibit cell growth over the entire concentration range of 100 nM to 20 μM. The suggestive mechanism seems to be through apoptosis, due to the detected chromatin fragmentation (Wang et al., 1994). Another study showed that the core structure of the flavones, 2-phenyl-4H-1-benzopyran-4-one, were highly selective towards transformed cells only, showing a role for flavonoids in chemotherapeutic uses (Wenzel et al., 2000). To date no structure-function relationships have been observed for effects of flavonoids on proliferation. Cell cycle arrest has been suggested to form part of this inhibition, specifically for quercetin. This compound was shown to arrest the cell cycle at the G1 and S-phase boundary, which is involved in gene expression and protein synthesis as well as in cell DNA synthesis (Zand et al., 2002).

The anti-cancer properties of flavonoids was already reported in 1980 when a significant reduction of mammary cancer by X-irradiation was demonstrated in rats fed a raw soybean diet (Troll et al., 1980). In this study 74% of the control rats, on a casein diet, developed tumours while 44% of the experimental rats developed tumours. Another study evaluated the anti-carcinogenic effects in the colon of quercetin and rutin. Female mice were fed a diet supplemented with quercetin (0.1, 0.5 or 2.0%) or rutin (1.0 or 4.0%) for 50 weeks to assess inhibition of azoxymethanol (AOM)-induced colonic neoplasia. No changes were observed in mice fed flavonoid-supplemented diets without AOM treatment. However, both quercetin (2.0%) and rutin (4.0%) significantly inhibited hyper-proliferation and the shift of S-phase cells to middle and upper portions of the crypts in AOM-treated mice (Deschner et al., 1991).

Although a number of *in vivo* studies have investigated the effects of flavonoids on cancer, very few studies have the potential of utilizing a flavonoid rich diet to reduce PCa risk (Zand et al., 2002). However, various studies have been conducted in PCa cell models. In a study conducted in two androgen-dependent PCa human prostate tumour cell lines, the AR-positive LNCaP and the AR-negative PC-3 cell lines, the effect of a flavonoid rich diet on PCa was investigated. Four phytoestrogens, genistein, daidzein, coumestrol and equol, inhibited cell growth in both cell lines, exposed to 100 μM for three or six days. Growth inhibition was achieved at lower phytoestrogen concentrations in LNCaP cells than in PC-3 cells, suggesting a potential role for the interaction of phytoestrogens and the AR. The authors suggested the mutated AR present in LNCaP cells to be relaxed in specificity, allowing other ligands such as phytoestrogens to bind. However, when phytoestrogen concentrations reached 100 μM, the inhibition of cell growth was similar in both cell lines, suggesting a non-receptor-related action (Mitchell et al., 2009). Zand et al., (2000; 2001),

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evaluated the inhibition of PSA, a biomarker of cancer progression in breast cancer and PCa cells, the latter transfected with human AR, showing the inhibitory effect of 72 flavonoids at 10 µM (Zand et al., 2001, 2000). Another group showed inhibition of PSA secretion by genistein in LNCaP cells. This result was seen at all concentrations, however, in androgen independent VeCaP prostate cells, genistein was only able to suppress PSA production at high concentrations. The inhibition of cell proliferation, however, was independent of PSA signalling pathways in VeCaP cells (Davis et al., 2000). Similar androgen action was also obtained with green tea polyphenols (Gupta et al., 1999).

A plausible link between phytoestrogen intake and PCa risk was suggested in a case-control study, which included 83 cases and 107 frequency-matched controls. The flavonoids in this study were provided for the most part by cranberry juice/cranberries, black tea, onions and apples. This study suggested an inverse association between PCa risk, coumestrol and two isoflavonoids, genistein and daidzein. The authors concluded with the suggestion that at-risk populations should modify their diet behaviour (Strom et al., 1999).

The synergistic effect of flavonoids against hormone-dependent cancers, such as PCa, has not been extensively investigated. However, a recent study into the synergistic effect of flavonoids demonstrated improved efficacy when PCa cells were exposed to a combination of flavonoid compounds. A combination of genistein, quercetin and biochanin A was used in this study, either as single, double combination or triple combination. The combination of 8.33 µM of genistein, quercetin and biochanin A was shown to be more potent than single compound exposure in inhibiting the growth of LNCaP cells, as well as DU-145 and PC-3 PCa cells. Although mutations of the AR occur in PCa progression, increased ER expression occurs concurrently. The authors therefore suggested that the action of phytoestrogens was mediated through ER and AR dependent pathways, since the phytoestrogen combination inhibited cell proliferation even in the presence of fulvestrant, a potent estrogen antagonist. They concluded that a combination of phytoestrogens exhibiting anti-cancer properties could significantly increase the efficacy of individual compounds resulting in improved efficacy at achievable physiological concentrations (Kumar et al., 2011).

Taken together, polyphenols, flavonoids and rooibos definitively have a role to play in the modulation of the development and progression of hormone-dependent cancers, especially PCa. The mechanism by which they act, whether through estrogenic or androgenic activity, modulation of ER and/or AR activation, cell cycle arrest or apoptotic pathways, remain unclear.

2.3.2 The modulation of steroidogenic enzymes by polyphenolic compounds

As the estrogenic activity of flavonoids and polyphenols are dependent on their phytoestrogenicity given their chemical structures are similar to those of natural occurring hormones, so too their

androgenic activity relies on this characteristic. There exists the strong possibility that the modulation of the catalytic activity of steroidogenic enzymes by flavonoids is a contributing factor to the mechanism, underlying their anti-cancer properties.

A recent study by Schloms et al., (2012), focused on the influence of rooibos and dihydrochalcones on adrenal steroidogenesis, investigating the influence of these compounds on steroid hormone biosynthesis. Results showed that under both basal and forskolin stimulated conditions, rooibos, aspalathin and nothofagin decreased total steroid production in H295R cells, a human adrenal carcinoma cell line. In addition rooibos inhibited the levels of 11β-hydroxyandrostenedione (11OHA4), A4 and T, suggesting modulation of endogenous enzyme activity. This study also showed the inhibition of the catalytic activity of heterologously expressed CYP17A1 (17α-hydroxylase/17,-20-lyase) towards pregnenolone (PREG) and CYP21A2 (cytochrome P450 steroid 21-hydroxylase) towards progesterone (PROG) (table 2.2), by rooibos extracts, aspalathin and nothofagin in COS-1 cells (Schloms et al., 2012). These results are comparable to results reported by Ohno et al. (2002) and Perold (2009). Ohno et al., (2002), showed that flavonoid compounds, such as diadzein, genistein and 6-hydroxyflavone, selectively inhibit key steroidogenic enzymes including: 3βHSD type 2 (3βHSD2) and CYP21A2 in H295R cells (Ohno et al., 2002). Perold (2009), also showed inhibition of CYP21A2 activity by flavonoids, rutin, vitexin and orientin in COS-1 cells transiently transfected with baboon CYP21A2 (Perold, 2009).

Another study conducted by Schloms & Swart, (2014), determined the inhibitory effect of rooibos and its phenolic compounds on the enzymes 3βHSD2, CYP17A1, CYP21A2 and CYP11B1 (cytochrome P450 11β-hydroxylase). All the flavonoids significantly inhibited 3βHSD2 and CYP17A1, while the inhibition of CYP21A2 and CYP11B1 was both substrate and flavonoid specific. The dihydrochalcones, aspalathin and nothofagin, inhibited the activity of CYP21A2 but not that of CYP11B1. Rutin, orientin and vitexin inhibited CYP11B1, albeit at low levels (20%), however, no inhibition was detected with CYP21A2. In contrast rooibos inhibited both CYP21A2 and CYP17A1 significantly. This study also reported that rutin had the highest inhibitory effect on steroid production in forskolin stimulated H295R cells. In addition nothofagin and vitexin inhibited overall steroid production more so compared to aspalathin and orientin. This study also suggests 17βHSD inhibition due to reduced basal T levels that were observed in H295R cells, although the adrenal is not the primary site for 17βHSD expression. All the flavonoids decreased T production, with rutin exerting the greatest inhibitory effect (Schloms & Swart, 2014).

Table 2.2: Steroidogenic enzymes: general conversions and localization in human tissue (Luu-The, 2001; Mostaghel, 2014; Payne & Hales, 2004; Walle & Walle, 2002)

Steroidogenic enzyme	General conversion	Tissue expression
Cytochrome P450 enzymes		
CYP11B1	11-deoxycortisol → cortisol	Adrenal gland (zona fasiculata, zona reticularis), skin
CYP17A1	PREG → 17OH-PREG PROG → 17OH-PROG 17OH-PREG → DHEA	Adrenal gland (zona fasiculata, zona reticularis), testis, skin
CYP21A2	PROG → deoxycorticosterone 17OH-PROG → deoxycortisol	Adrenal gland (zona glomerulosa, zona fasiculata), skin
Hydroxysteroid dehydrogenases		
3αHSD2	DHT → 3α-adiol	Prostatic tissue, testis
3βHSD2	PREG → PROG 17OH-PREG → 17OH-PROG DHEA → A4	Adrenal gland (zona glomerulosa, zona fasiculata, zona reticularis), testis, skin
11βHSD1	Cortisone → cortisol	Adrenal gland (zona glomerulosa, zona fasiculata, zona reticularis), testis
11βHSD2	Cortisol → cortisone	Adrenal gland (zona glomerulosa, zona fasiculata, zona reticularis)
17βHSD1	Estrone → estradiol	Ovary, mammary gland
17βHSD3	A4 → T	Adrenal gland (zona reticularis), testis, prostatic tissue
AKR1C3	A4 → T	Adrenal gland (zona reticularis), testis, prostatic tissue
Uridine diphosphate glucuronosyltransferase		
UGT1A1	Glucuronidation of various endogenous and exogenous compounds	Hepatic cells, Intestinal cells

As the inhibitory mechanism of flavonoid compounds are not fully understood, structural-relationships are central in understanding the inhibitory effect. As many flavonoids are phytoestrogens and their chemical structures are similar to that of natural estrogens and androgens, it is possible for the phenolic compounds in rooibos to either bind the active site or co-factor redox partner binding sites of the specific enzyme, thereby influencing the enzyme's catalytic activity. A study showed the inhibition of human CYP450 by green tea catechins, specifically epigallocatechin gallate, which was achieved by the inhibition of the enzyme activity of CYP450 and also partially by inhibition of human nicotinamide adenine dinucleotide phosphate (NADPH)-CYP450 reductase (Muto, Fujita, Yamazaki, & Kamataki, 2001), a complex that forms when P450 enzymes receive electrons from NADPH via a single 2-flavin protein termed P450 oxidoreductase (POR) (Miller, 2005). According to Middleton et al. (2000), enzymes of importance as targets of polyphenols are enzymes with NADPH as a cofactor, as is the case for CYP450 enzymes as well as reductive 17βHSD and SRD5A enzymes (Middleton Jr., Kandaswami, & Theoharides, 2000).

The study by Schloms et al., (2013), suggested that structural differences regarding the number and position of hydroxyl and glucose moieties as well as structural flexibility could impact the manner in which these flavonoids influence the activity of adrenal steroidogenic enzymes. This could possibly account for the inhibition of T production by rutin in H295R cells, presumably by inhibiting the catalytic activity of 17βHSD (Schloms et al., 2013). In a recent study, inhibitors of 17βHSD3 and AKR1C3, which are key enzymes involved in adrenal and prostate androgen metabolism were screened virtually. It was shown that the substrate binding domain of 17βHSD3 and AKR1C3 were able to accommodate structurally diverse substrates which bind to different regions in the active site. The crystal structure of 17βHSD3 and AKR1C3 was depicted with rutin, bound at the base of the substrate binding domain, with water molecules forming a network of hydrogen bonds with the flavonoid (Schuster et al., 2011). Ohno et al., (2002), also report on structural relationships, suggesting the hydroxy groups at C6 on ring A and at C4' on ring B (fig. 2.1) play an important role in the inhibition of steroidogenic enzymes (Ohno et al., 2002). Another study showed that a range of flavones with hydroxy groups at positions C6 or C7 of ring A and C4' on ring B (fig. 2.1) inhibited 3βHSD2 (Hasegawa, Nakagawa, Sato, Tachikawa, & Yamato, 2013). Schloms & Swart, (2014), suggested that a hydroxy group on position C4' on ring B (fig. 2.1) of the flavonoid may be involved in the binding of these compounds in the active pocket of steroidogenic CYP450 enzymes, with hydrogen bonds stabilizing the orientation of flavonoid compounds in the active site. They also pointed out that conclusions could not be drawn regarding the orientation of flavonoids within the active site, and as the type of inhibition was uncertain, the binding of flavonoids to the same site as the substrate could not be assumed (Schloms & Swart, 2014).

Focusing on the steroidogenic 17βHSD enzymes, another study showed flavonoids as inhibitors of 17βHSD type 1 (17βHSD1). They focused on 17βHSD1 as this enzyme is involved in the conversion of estrone to estradiol (table 2.2), a potent ligand for ERs, playing a pivotal role in

estrogen-dependent diseases. They reported on 10 flavonoids, which included a flavonoid with a hydroxy group on C7 of ring A and on C4' of ring B (fig. 2.1), with this flavonoid showing more than 70% inhibition of 17βHSD1 at 6 µM. This study reported that inhibitors possess one or two substituents on B ring, with one methoxy group on the flavone ring (fig. 2.1) (Brožič et al., 2009).

Krazeisen et al., (2001), specifically considered the enzyme AKR1C3, and showed the best phytoestrogen inhibitors of AKR1C3 were; zearalone, coumestrol, biochanin A and quercetin at concentrations ranging from 100 nM to 20 µM, influencing the conversion of A4 to T (table 2.2), involved in androgen metabolism. They suggested that within the flavones, the inhibitor potency increased with increasing hydroxy groups on ring A, the most effective being a hydroxy group at C7 (fig. 2.1). They noted that under reductive conditions, a double bond in ring C (fig. 2.1), as is the case with flavones, increased the inhibitory effect. In contrast, the flavanones, with no double bond in ring C, show a stronger inhibitory effect on the oxidative activity of AKR1C3. For both reductive and oxidative 17βHSD activities, inhibitor potency decreases when ring B is found at C3 (fig. 2.1), as it is in isoflavones. In addition the isoflavones, methylation of hydroxy group at C4 on ring B (fig. 2.1), results in greater inhibitor activity. They also suggested that the phytoestrogens bind to the hydrophilic redox partner binding site of the enzyme and not to the substrate binding site. However, the hydroxy group at C7 (fig. 2.1) of the flavonoids may bind to the active site (Krazeisen, Breitling, Moller, & Adamski, 2001).

PCa is a hormone-dependent cancer, and PSA is the most common biomarker to date, to assess PCa progression. PSA is an AR regulated gene and is therefore associated with cell proliferation and growth, which is also dependent on the AR activation. Prostate steroid metabolism results in active androgens which activate the AR. The prostate has enzymatic machinery in place regulating the levels of active androgens. These enzymes comprise of 3α-hydroxysteroid dehydrogenases (3αHSDs) and UGTs (phase II inactivation). A study was undertaken to investigate the influence of biochanin A on UGTs and also PSA production in PCa cells. Biochanin A, the precursor of genistein, increased the production of the glucuronidated form of T at twice the normal rate in LNCaP cells, and subsequently decreased the T-stimulated release of PSA by 40%. They also showed a 10-fold increase in glucuronidated T formation, as well as an increase in UGT transcript after 5 days treatment with 5 µM biochanin A. Interestingly, the same result was not observed with the addition of genistein or quercetin in LNCaP cells. Quercetin only resulted in a 2-fold increase in glucuronidated T formation (Sun, Plouzek, Henry, Wang, & Phang, 1998). Zhang et al., (2007), also reported that polyphenols induced phase II enzymes, UGTs, together with Walle & Walle (2002), that reported that UGT1A1 (table 2.2) was induced by flavonoids (Walle & Walle, 2002; Zhang et al., 2007).

A mechanism controlling the ratio of the concentration of biologically active cortisol, which is able to bind the glucocorticoid and mineralocorticoid receptor (GR, MR) and its inactive 11-keto form,

cortisone, is orchestrated by 11β-hydroxysteroid dehydrogenase (11βHSD) type 1 (table 2.2). 11βHSD1 is therefore essential for GR and MR activation. 11βHSD type 2 (11βHSD2) catalyses the oxidation of cortisol (table 2.2), thereby preventing activation of the GR and MR by cortisol (Schweizer, Atanasov, Frey, & Odermatt, 2003). In human studies, a significant reduction in the cortisol:cortisone ratio of both male and female subjects following rooibos consumption, suggesting that rooibos favours the inactivation of cortisol. This could be attributed to the modulation of 11βHSD activity. This prompted the authors to examine the effect of rooibos on both 11βHSD isozymes at cellular level. Co-transfection of 11βHSD1 and hexose-6-phosphate dehydrogenase (H6PDH) in CHO-K1 cells simulated *in vivo* conditions in which the oxoreductase activity of 11βHSD1 predominates, while nearly eliminating the dehydrogenase activity. Rooibos significantly reduced the cortisol:cortisone ratio in cells expressing 11βHSD1, suggesting that rooibos modulates 11βHSD1 activity. Furthermore, the authors found that 11βHSD2 activity was unaffected (Schloms & Swart, 2014). This result was also found by Schweizer et al., (2003), where a rapid screening of inhibitors of 11βHSD found flavanones to selectively inhibit 11βHSD1 and not 11βHSD2. They concluded that the C2 and C3 double bond (ring C) (fig. 2.1) reduced the inhibitory effect of flavones for 11βHSD1 and 11βHSD2 activities (Schweizer et al., 2003). However, as previously mentioned, this chemical characteristic increases the inhibitory effect of flavones, found in rooibos, on 17βHSD activity.

Taking into account all the above mentioned flavonoid and enzyme interactions, a strong case for the role flavonoids in diseases driven by steroid hormones is provided, as the modulation of steroid-metabolizing enzymes favouring the production of an inactive steroid over an active steroid, could lead to decreased risk in the development of hormone-dependent cancers, such as PCa.

2.4 Potential health applications of Rooibos in PCa

Rooibos's potential in the pharmaceutical domain is determined by its bioactivity that encompasses its antioxidant capacity, chemopreventive potential, immune boosting effects, anti-allergic actions, together with recent findings of cardiovascular protection and aiding in the treatment of metabolic diseases (Breiter et al., 2011; HesselIng & Joubert, 1982; Khan & Gilani, 2006; Komatsu et al., 1994; Kunishiro et al., 2001; Schulz et al., 2003). In recent years, the shift towards natural and herbal medicinal products, away from mainstream pharmaceuticals, by the general public has led to increased interest in indigenous natural plant products which included rooibos. Sales of herb and botanical dietary supplements in the United States increased ± 5.5% in 2012, with this growth being greater than the 5% increase in sales noted for the year 2011. The 2012 increase marks the 9th year in a row since 2004 in which herb sales increased over the previous year (Lindstrom et al., 2013). Furthermore, total retail sales of teas in the United States increased by 5.9% in 2013, with rooibos showing a double-digit annual sales increase in its loose form (Keating et al., 2013).

The nutraceutical value of rooibos lies in its application as an anti-diabetic agent (Kawano et al., 2009; Larsen et al., 2011), in topical skin products (Marnewick et al., 2005) and in the treatment of neurological and psychiatric disorders of the central nervous system (Frank & Dimpfel, 2010). Rooibos is commonly used to treat asthma, eczema, headache, nausea and mild depression. Although the anti-cancer and anti-mutagenic properties of rooibos has been widely reported and linked to the anti-oxidant capacity of rooibos (Marnewick et al., 2000; Sasaki et al., 1993; Shimoi et al., 1996), few studies have linked the bioactivity of rooibos to the human endocrine system. Our group has shown that rooibos influences adrenal steroidogenic enzymes (Schloms & Swart, 2014; Schloms et al., 2013, 2012), modulating steroid hormone biosynthesis and may as such have applications as a natural herbal product aiding metabolic diseases driven by hormonal imbalances. As rooibos affects steroidogenic enzymes, rooibos may impact hormone driven cancers such as PCa, since PCa development is dependent on steroid hormone biosynthesis and subsequent steroid receptor activation.

As previously mentioned multiple studies, including our group, have shown specific inhibition of CYP17A1, CYP21A2 and 3βHSD2 steroidogenic enzymes, both in cell systems and transfected cells. Notably, these aforementioned enzymes, together with CYP11A1 and SRD5A1 and SRD5A2 have been shown to be expressed in the skin (Dumont et al., 1992; Slominski et al., 1996; Thiboutot et al., 2003; Thigpen et al., 1993). CYP11A1 have been shown on the gene and protein level (Slominski et al., 2004), evidence that the skin itself is able to allow cutaneous steroidogenesis. SRD5A1 is expressed in newborn skin and then again in nongenital skin after puberty, compared to SRD5A2 that is expressed in fetal genital skin (Thigpen et al., 1993). Studies have shown conversion of cholesterol to PREG, but only if a 3βHSD inhibitor is added, therefore PREG metabolism is evident in the skin (Slominski et al., 2004). The skin is, thus, capable of *in situ* steroid biosynthesis and is an extra-adrenal site for steroid production (Slominski et al., 2007). Rooibos has been reported to slow down tumour growth and to decrease skin cancer tumour size (Marnewick et al., 2005), an anti-cancer effect of rooibos which could be linked to the inhibition of steroidogenic enzymes, possibly contributing to the mechanism of action.

The level of consumption and manner in which rooibos is administered, be it infusions or as a supplementary tablet, that would result in anti-carcinogenic effects is still unclear. Techniques for quantifying flavonoid uptake and absorption are, however, improving together with more comprehensive databases being available for analyses. In the case of animal studies, the high levels of flavonoid compounds required to elicit protective effects are at concentrations humans are unable to attain through whole food consumption. Consumption of nutraceuticals or functional foods has thus been suggested to supplement the daily intake of flavonoid compounds. Although rooibos has the potential to contribute towards healthy living when used as a dietary supplement, there may be risks involved in excessive polyphenol consumption. For example, commonly found on the internet, are recommendations of a daily intake of 1-6 tablets containing 300 mg quercetin,

1 g citrus flavonoids or 20 mg resveratrol. This, however, leads to a flavonoid consumption ±100 times greater than would otherwise be present in a western diet (Mennen et al., 2005), levels which could possibly be toxic.

A consequence of excessive flavonoid consumption, for example, is that of reduced iron absorption in populations with marginal iron stores, as polyphenols inhibit non-heme iron absorption and may lead to iron depletion (Temme & Van Hoydonck, 2002). In addition the safety of the flavonoid supplement may also be influenced by the method of extraction of polyphenols from source material as well as subsequent processing. It was, for example, reported that a hydroalcoholic extract of tea buds, sold as a slimming supplement, led to severe liver toxicity (Van der Woude et al., 2003).

Flavonoids have also been reported to inhibit thyroid peroxidase and impede thyroid hormone biosynthesis as was shown by the administration of vitexin to rats, resulting in increased thyroid weight and decreased plasma thyroid hormone levels (Doerge & Sheehan, 2002; Ferreira et al., 2002). However, two clinical studies failed to show significant effects on thyroid hormones after a 3-6 month period of isoflavone-containing soy proteins consumption, among adults (Duncan et al., 1999; Persky et al., 2002). Another point of concern is that polyphenol consumption could affect drug bioavailability and pharmacokinetics, with drugs such as benzodiazepines showing an increase in plasma concentrations with a single dose of grapefruit juice (Lilja et al., 2000). It was also shown that green tea catechins, comprising 1.0% or 0.1% of the diet, enhanced tumour development in the colon of F344 male rats (Hirose et al., 2001). Epidemiologic studies have, however, not shown any carcinogenic effects of polyphenols to date (Hertog et al., 1993). It should be noted that the dose which would result in a positive effect *in vitro* may have a different effect *in vivo*. Furthermore, a dose applied in an experimental study may be unachievable *in vivo*, since consumption would perhaps not reach comparable levels together with factors such as, bioavailability and transport the target sites being uncertain (Mennen et al., 2005).

Studies regarding the efficacy and safety are therefore central to future research strategies regarding the use of herbal products, specifically herbal products aimed at being used in functional foods, nutraceuticals or other therapeutic applications (Van der Merwe, 2012).

2.5 Summary

It is apparent that the manner in which flavonoids may prevent steroid-hormone dependent cancers, such as PCa, include modulation of steroid hormones, inhibition of proliferation and anti-carcinogenic and antioxidant activities, as was illustrated by the data reported. It is furthermore clear that the specific modulation of steroidogenic enzymes can be attributed to polyphenols either, binding in the active site or to redox partner binding sites of the enzymes. The main structural characteristics to which these inhibitory effects are proposed to be attributed to are the number and position of hydroxyl moieties, together with the structural flexibility of the flavonoid carbon backbone.

Reports to date suggest that there is a potential role for rooibos in steroid hormone dependent cancers, such as PCa, which may be beneficial. Investigating the effect of rooibos on steroid hormone levels and on steroidogenic enzymes will contribute not only to the basic understanding of steroid metabolism in the prostate but also to the understanding of the cellular mechanisms underlying rooibos's anti-cancer properties.

Chapter 3

Androgen hormones in PCa: biosynthesis of C19 steroids and metabolism in the prostate and in PCa

3.1 Introduction

In 1966, Huggins and Hodges won the Nobel Prize for medicine with their observation that orchiectomy (surgical castration) led to regression of metastatic PCa. Their finding demonstrated that nearly all PCas require circulating androgens for survival (Huggins & Bergenstal, 1952; Huggins, 1946). Androgens are C19 steroid hormones that induce the differentiation of the male reproductive organs as well as male secondary sex characteristics, and are formed primarily in the testes. In addition, in men, androgens are also formed in peripheral tissues, including the prostate and the skin (Hsing et al., 2002).

All steroids are derived from 27-carbon cholesterol, which when oxidised forms 21-carbon steroids, represented by glucocorticoids and mineralocorticoids. These 21-carbon steroids can subsequently be further oxidised to 19-carbon and 18-carbon steroids, represented by androgens and estrogens, respectively. These steps occur due to intricate enzyme machinery present in adrenal and gonadal tissue. These two tissues are able to convert cholesterol to PREG, which represents the first step for the subsequent production of glucocorticoids, mineralocorticoids, androgens and estrogens. Two of the most important hormones in adult males are T and DHT. T is primarily produced by Leydig cells in the testes and is released into circulation. In target tissue T is reduced to DHT which is the most potent natural androgen (Hsing et al., 2002).

The androgen pathway – from the generation of T and DHT up until the transcription of AR-regulated genes – is a prerequisite for the development and differentiation of the normal prostate, as well as for the development and progression of PCa (Sharifi & Auchus, 2012). The androgen signalling pathway is intimately involved in tumour initiation and invasion, and the subsequent development of metastatic disease. The mechanisms that regulate the androgen axis, which include the generation of ligands, AR expression and AR-regulated gene responses, therefore all represent potential sites of intervention for the development of new pharmacologic therapies to be employed against PCa (Sharifi, 2012).

This chapter will summarise steroid metabolism, as it occurs in the prostate, and will focus on pathways and enzymes that drive prostate steroid metabolism and the implication thereof in PCa.

3.2 Physiology of prostatic tissue

The principal function of the prostate is to provide proteins and ions that form the bulk of the seminal fluid. Fertility is impaired in the absence of the prostate. The primary functions of the

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prostatic secretions relate to semen gelation, coagulation, and liquefaction with the prostate and seminal vesicles producing most of the ejaculate. The adult human prostate is a tubuloalveolar gland composed of ducts lined with a pseudostratified columnar epithelium. Lining the ducts are tall columnar and secretory cells with basal nuclei, with a nearly continuous layer of basal epithelial cells lying on top of the epithelial basement membrane (Hayward & Cunha, 2000) (fig. 3.1).
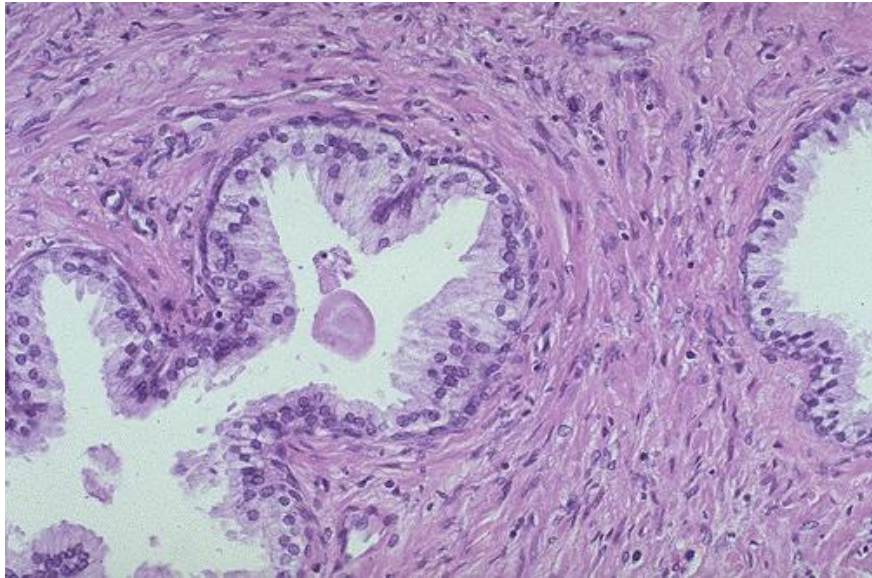
Figure 3.1: Physiology of normal prostatic tissue, representing corpora amylacea in the gland lumen (pink laminated concretions) and infoldings of the columnar epithelium (Prostate, 2014.)

The development of the prostate begins with the growth of prostatic buds at about 10 weeks of fetal development in humans. ARs in the urogenital sinus mesenchyme are stimulated by testicular androgens to induce epithelial budding, proliferation and differentiation to form ductal structures. Androgenic stimulation is therefore an absolute requirement for prostatic development. At puberty there is a growth spurt characterised by an increase in prostatic wet weight but only a small increase in the number of ductal tips. At this stage the prostate grows from weighing 2 g to ±20 g, representing a phase of exponential growth, corresponding to the rise in serum T reaching adult levels. Mean prostatic weight subsequently stabilizes and remains fairly constant until the end of the third decade of life, when mean prostatic weight begins to rise slowly. The prostate is, however, a slow growing organ, with this slow rate of growth balanced by a slow rate of apoptosis, resulting in a growth-dominant gland (Hayward & Cunha, 2000). However, the disruption of this balance is reflected by the onset of pathogenesis of BPH.

Prostate diseases are chronic diseases developing over time. As males age, there is a slow decline in circulating T from the gonads and diseases of the prostate, such as BPH and PCa become prevalent in the aging male. Both conditions are androgen dependent with BPH affecting 90% of all men above the age of 80 while PCa, occurs in men older than 50 years of age. BPH and PCa are two separate diseases. BPH originates in the transitional zone, where obstruction of the

urinary bladder occurs due to outgrowth of the stromal and epithelial cells. In contrast, PCa originates in the peripheral zone (outer prostate), which allows PCa to metastasize into the adjacent lymph glands and bone. Of interest, both diseases are dependent upon intra-tumoural conversion of T to the higher-affinity ligand DHT (Penning, 2010).

ADT is currently the primary treatment followed in PCa, and involves medical and surgical castration in order to reduce circulating T levels. This approach is initially effective, however, most cases progress to CRPC. CRPC is androgen dependent and proliferates in the absence of testicular T (Sharifi & Auchus, 2012). There is growing evidence that it is not only testicular T contributing towards PCa but also C19 steroids of adrenal origin that play an important role in the proliferation of CRPC, as these metabolites may serve as substrates for the intratumoral biosynthesis of potent AR agonists (Luu-The et al., 2008; Montgomery et al., 2008; Stanbrough et al., 2006; Stein, Goodin, & Dipaola, 2012).

3.3 Androgen biosynthesis

The generation of active androgens in the prostate is dependent on C19 steroids from the testes and adrenal. Following castration, and the elimination of all testicular androgens, 30-50% of DHT remains in the prostate, denoting the importance of adrenal androgens (Labrie, 1993; Larsen et al., 1990). Furthermore, the *de novo* production of androgens in the prostate has been suggested to play a role in the pathogenesis and development of PCa (Lopez-Otin & Diamandis, 1998).

3.3.1 Androgen biosynthesis in the testes

Testicular Leydig cells account for ±95% of androgen biosynthesis and secretion, and are the main site of T production in men under normal physiological conditions. T regulates spermatogenesis, sperm maturation and sexual function in the adult male. The hypothalamic-pituitary-gonadal axis is controlled by pulses from gonadotropin-releasing hormone (GnRH). Each pulse of GnRH results in a pulse of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gonadotropes. LH stimulates T production from Leydig cells, and consequently T provides negative feedback at the hypothalamus and pituitary to suppress LH and T production (Sharifi & Auchus, 2012).

Testicular biosynthesis of T begins with the transfer of a 27-carbon cholesterol molecule from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). Cholesterol is converted to the C21 steroid, PREG, via CYP11A1 utilizing ferredoxin reductase which receives electrons from a phosphorylated cofactor (NADPH) and transfers electrons to a soluble iron-sulfur protein ferredoxin in the mitochondrial matrix (fig. 3.2). Once PREG is produced from cholesterol, it may undergo 17α-hydroxylation by CYP17A1 to yield 17α-hydroxypregnenolone (17OH-PREG). CYP17A1 is the microsomal P450 enzyme that catalyses both 17α-hydroxylase and 17,-20-lyase activities, the latter resulting in the conversion of 17OH-

PREG to DHEA. The 17,-20-lyase activity of CYP17A1 is augmented by the presence of the small hemoprotein, cytochrome b_5 (cyt b_5). Testicular cells express two hydroxysteroid dehydrogenases which are essential to T production. Leydig cells express 3βHSD2 which converts 3β-hydroxy-Δ^5-steroids to their 3-keto-Δ^4-congeners, converting DHEA to A4. Secondly, 17βHSD3, directs steroidogenesis towards T, with the main substrates for 17βHSD3 being A4 and DHEA. T is produced directly from A4, while androsta-5-ene-3β, 17β-diol (androstenediol) is produced from DHEA and is subsequently converted to T via 3βHSD2 (Miller & Auchus, 2011; Sharifi & Auchus, 2012). T is subsequently secreted into circulation and upon reaching peripheral tissues, such as the prostate, is able to exert physiological effects.
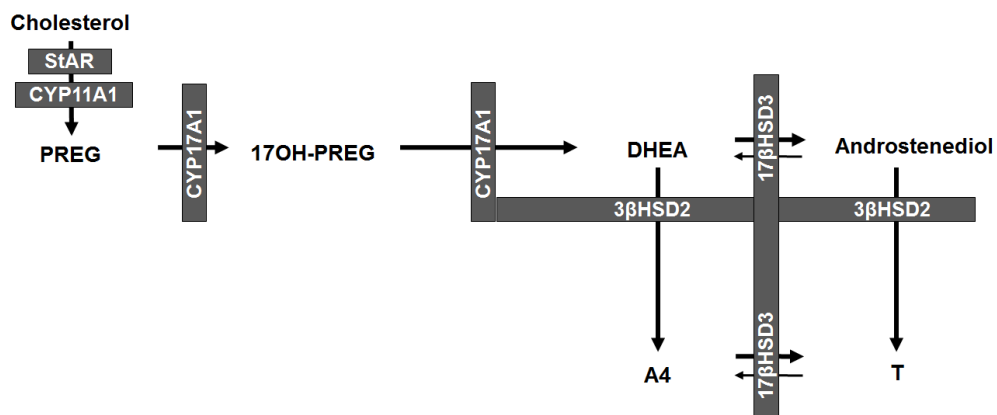
Figure 3.2: T biosynthesis in testicular Leydig cells.

3.3.2 Androgen precursor biosynthesis in the adrenal

Similar to testicular Leydig cells, steroid biosynthesis in the adrenal begins with the transfer of a 27-carbon cholesterol molecule from the outer to the inner mitochondrial membrane by StAR. However, due to the expression of steroidogenic enzymes in the adrenal cortex *viz.*: steroidogenic CYP450 enzymes (type 1, in mitochondria and type 2, in endoplasmic reticulum) and hydroxysteroid dehydrogenases (belonging to either the aldo-keto reductases or short-chain dehydrogenase reductase families) the adrenal also produces mineralocorticoids and glucucorticoids besides the androgen precursors, in a zone-specific manner, driven by the expression of specific enzymes and catalytic co-factors.

The steroid hormone output of the adrenal is important in the maintenance of hormonal homeostasis. Corticotropin-releasing hormone (CRH) from the hypothalamus stimulates adrenocorticotropin (ACTH) release from the pituitary corticotropes, derived from proteolytic processing of the precursor proopiomelanocortin. ACTH subsequently stimulates the zona fasiculata (ZF) and the zona reticularis (ZR) cells to produce cortisol and dehydroepiandrosterone sulphate (DHEA-S), respectively. It is possible for cortisol to exert negative feedback on CRH and ACTH. In addition cortisol can bind and activate the MR and GR and various metabolic pathways

lead to cortisol inactivation. In contrast, DHEA-S itself is not an AR agonist (Sharifi & Auchus, 2012), and does not contribute significantly to the androgen precursor pool as the regeneration of DHEA from DHEA-S seems to be a path rarely taken in adults, with low levels of steroid sulfatase expression and activity having been reported in human tissue (Miki et al., 2002).

The first step in adrenal steroidogenesis is catalysed by CYP11A1 which is expressed in the mitochondria and converts cholesterol to PREG (fig. 3.3). The production of PREG occurs in the three zones of the adrenal cortex *viz*., zona glomerulosa (ZG), ZF and ZR, with androgen precursor production occurring only in the ZR. Once PREG is produced it may undergo 17α-hydroxylation by CYP17A1, a microsomal P450 enzyme, to yield 17OH-PREG, or it may be converted to PROG, the first biologically important steroid in the pathway. 3βHSD2 converts PREG to PROG, 17OH-PREG to 17α-hydroxyprogesterone (17OH-PROG), DHEA to A4, and androstenediol to T. Although the SULT2A1 sulfonates most of the DHEA formed, A4 and low levels of T and other 19-carbon steroids are produced by the adrenal. Furthermore, CYP17A1 catalyses the subsequent conversion of 17OH-PREG to DHEA, also augmented by cyt b_5 as in Leydig cells. The 17,-20-lyase activity is enhanced by cyt b_5 by promoting the interaction between CYP17A1 and POR. Especially during adrenarche, cyt b_5 is up-regulated, together with the down-regulation of 3βHSD2, thereby ensuring the 17,-20-lyase reaction converts 17OH-PREG to DHEA, since 17OH-PROG is not a substrate for the 17,-20-lyase reaction (Auchus, Lee, & Miller, 1998; Pandey & Miller, 2005). This 17,-20-lyase reaction is therefore vital for the production of all androgens and androgen precursors. The characteristic 17α-hydroxylase and 17,-20-lyase activities of CYP17A1 place this enzyme at a key branch point in steroid hormone biosynthesis. Neither activity of CYP17A1 is present in the adrenal ZG, hence PREG is converted to the mineralocorticoid, aldosterone. In the ZF, the 17α-hydroxylase activity is present, but 17,-20-lyase activity is not, hence PREG is converted to the glucocorticoid, cortisol. In the ZR, both activities are present, enabling the conversion of PREG to androgens. The principal factor regulating the 17,-20-lyase reaction is electron transport from NADPH via POR. All microsomal (type 2) CYP450 enzymes, which include steroidogenic CYP17A1 and CYP21A2 receive electrons from POR, a membrane-bound flavoprotein which differs from the mitochondrial flavoprotein, ferredoxin reductase, supplying electrons to the mitochondrial (type 1) P450 enzyme. POR receives two electrons from NADPH and transfers one electron at a time to P450.

Microsomal CYP21A2 catalyse the 21-hydroxylation of the Δ^4 steroids, PROG to 11-deoxycorticosterone (DOC), and 17OH-PROG to 11-deoxycortisol, in the biosynthesis of mineralocorticoids and glucocorticoids, respectively (Sharifi & Auchus, 2012).

Zona glomerulosa	Zona fasciculata	Zona reticularis
Mineralocorticoids	Glucocorticoids	Androgen precursors
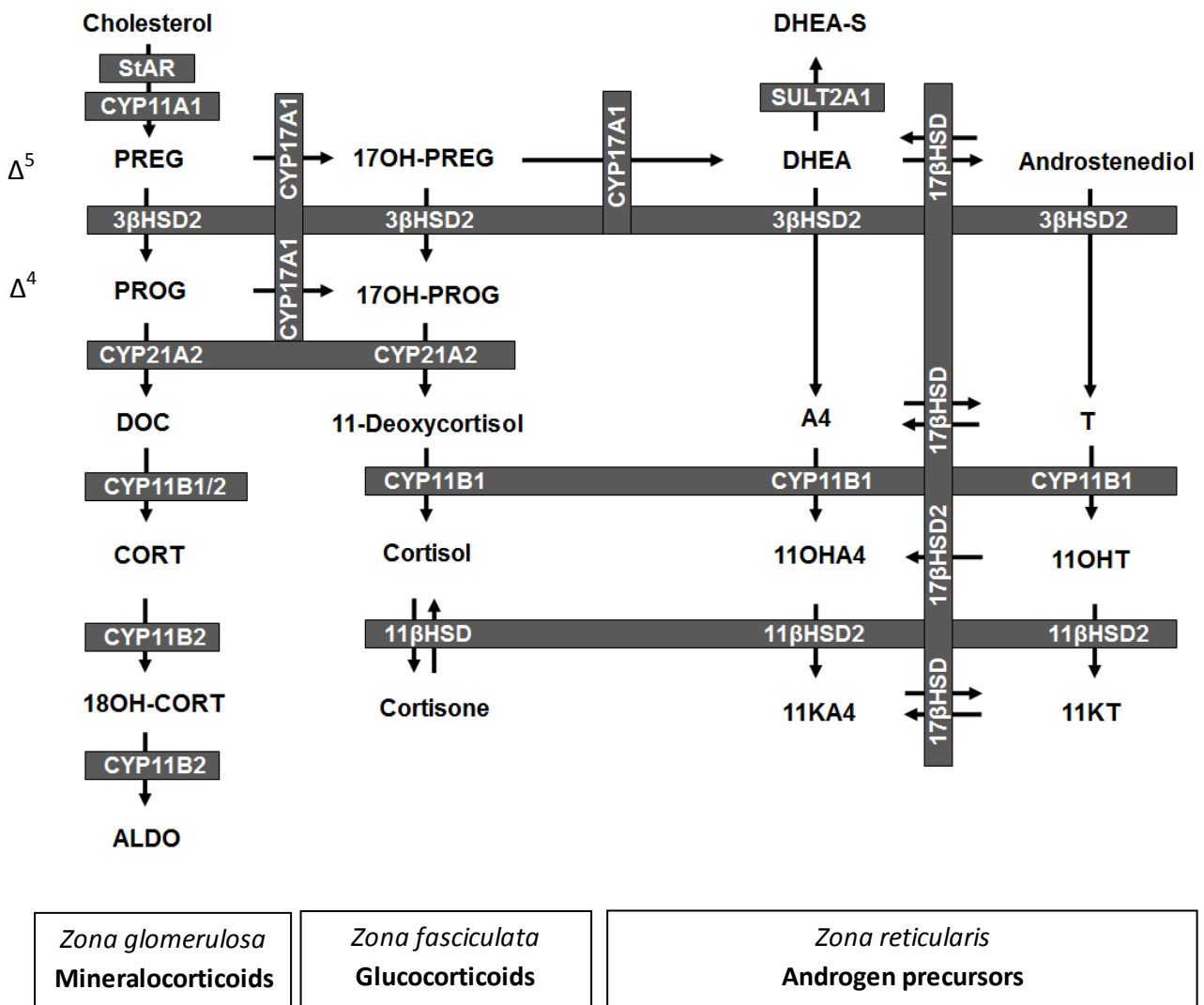
Figure 3.3: Steroid hormone biosynthesis pathways represented in the zones of the adrenal cortex.

The final steps in the biosynthesis of glucocorticoids and mineralocorticoids are catalysed by two closely related mitochondrial enzymes, CYP11B1 and CYP11B2 (P450c11AS, aldosterone synthase). Both forms of CYP11B use ferredoxin and ferredoxin reductase to accept electrons from NADPH to allow catalysis. The more abundant of the two isoenzymes is CYP11B1, which is the classical 11β-hydroxylase that converts 11-deoxycortisol to cortisol and DOC to corticosterone (CORT), and is expressed predominantly in the ZF, and to a lesser extent in the ZR and in the ZG. Our research group has recently shown that the conversion of adrenal A4 to 11OHA4 and T to 11OHT (Schloms et al., 2012), is catalysed by CYP11B1. The less abundant CYP11B2, is expressed in the ZG only, where it exhibits 11β-hydroxylase, 18-hydroxylase and 18-methyl oxidase activities, and thus catalyses the conversion of DOC to aldosterone (Sharifi & Auchus, 2012). CYP11B2 also catalyses the C11-hydroxylation of A4 and T, and although the level of the 11β-hydroxylation of T may be negligible in the adrenal due to the zone specific enzyme expression, it is quite possible that circulating T may be hydroxylated by CYP11B2 in the ZG and thus contribute to 11OHT production (Swart et al., 2013a).

Although 17βHSDs, termed 17-oxidoreductases or 17-ketosteroid reductases, interconvert DHEA and androstenediol, as well as A4 and T, expression levels are, low in the adrenal. 17βHSD2 is a microsomal oxidase that uses NAD^+ in the catalysis of the conversion and inactivation of androgens (T to A4 and 11OHT to 11OHA4), while AKR1C3, catalyses the conversion of A4 to T and DHEA to androstenediol (Miller & Auchus, 2011, and the references therein). In the adrenal, the interconversion of 11KA4 and 11KT is also possible due to the presence of 17βHSDs in the ZR (Rege & Rainey, 2012; Rege et al., 2013).

The adrenal also expresses low levels of the two isoenzymes of 11βHSD which catalyse the interconversion of cortisol and cortisone. These isoenzymes exhibit oxidase and reductase activity, depending on whether $NADP^+$ or NADPH is available as co-factor. 11βHSD1 catalyses both the oxidation of cortisol to cortisone using $NADP^+$ as co-factor and the reduction of cortisone to cortisol using NADPH co-factor and H6PDH as co-enzyme. In the endoplasmic reticulum, the ratio of NADPH to $NADP^+$ is maintained by H6PDH. 11βHSD2 catalyses the oxidation of cortisol to cortisone only, using NAD^+, and functions best with low concentrations of steroid (Miller & Auchus, 2011). Furthermore, it is also possible for 11βHSD to mediate the interconversion between 11OHA4 and 11KA4 as well as 11OHT and 11KT (Swart et al., 2013a).

It was recently shown that 17OH-PROG produced in the adrenal may also be converted to DHT, in clinical cases of 21-hydroxylase deficiency. This alternative 'backdoor' route produces DHT without going through the conventional intermediates DHEA, A4 or T (fig. 3.4). PROG and 17OH-PROG accumulate in the absence of CYP21A2, with this pathway being initiated when either PROG or 17OH-PROG are reduced by SRD5A1 or SRD5A2. Once 5α-pregnane-3α, 17α-diol-20-one (17OH allo-pregnanolone) is formed it can be converted by the 17,-20-lyase activity of CYP17A1 to AST. 17βHSD3 and AKR1C3 subsequently converts AST to 5α-androstane-3α, 17β-diol (3α-adiol), and finally 3αHSD oxidises 3α-adiol to DHT (Auchus, 2004; Kamrath et al., 2012).
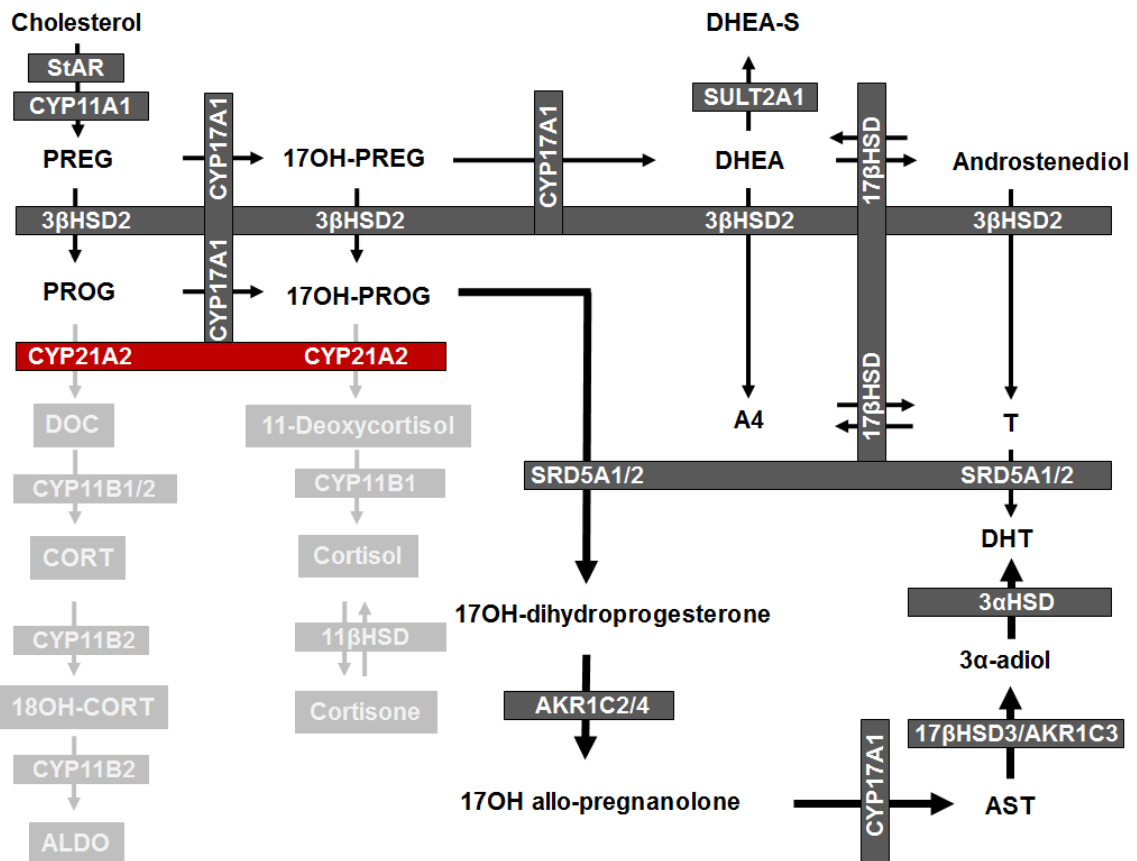
Figure 3.4: The biosynthesis of DHT via the 'backdoor' pathway.

The 'backdoor' pathway is possible wherein C21 steroids are acted on by the reductive activity of SRD5A1 and/or SRD5A2 and AKR1C2 prior to the lyase activity of CYP17A1, with reports of 17OH-PROG being a better substrate for SRD5A1 than A4 and T (Auchus, 2009). It has, to date, not been identified which isoenzymes are responsible for the 5α-reduction of 17OH-PROG in patients with 21-hydroxylase deficiency. Interestingly, 5α-pregnane 17α-ol-3, 20-dione (17OH-dihydroprogesterone) is a poor substrate for the lyase activity of CYP17A1, therefore biosynthesis proceeds via AKR1C2, which yields 17OH allo-pregnanolone, an excellent substrate for the lyase activity of CYP17A1. It is likely that the 'backdoor' pathway contributes to DHT biosynthesis in androgen excess disorders, particularly in those in which 17OH-PROG accumulates, such as polycystic ovary syndrome, 3βHSD deficiency and Antley-Bixler syndrome characterised by disordered steroidogenesis due to POR deficiency (Ghayee & Auchus, 2007). The adrenal and testes express all the required enzymes for the conversion of 17OH-dihydroprogesterone to DHT (Dufort, Rheault, Huang, Soucy, & Luu-The, 1999; Fluck et al., 2011; Penning et al., 2000). Of interest, the adrenals express negligible SRD5A with SRD5A2 being expressed at low levels in fetal testes and, at high levels in adult testes, while SRD5A1 is expressed at higher levels in the fetal testes than in the adult testes (Fluck et al., 2011). In addition *in situ* steroid biosynthesis, in the skin is widely reported, as the skin cells express CYP11B1, CYP17A1, 3βHSD, SRD5A1 and SRD5A2 (Dumont et al., 1992; Slominski et al., 1996; Slominski et al., 2004; Thiboutot et al., 2003;

Thigpen et al., 1993). The 'backdoor' pathway could therefore function within skin cells, albeit only to produce 17OH-dihydroprogesterone, which could subsequently be released into circulation and be metabolised within the 'backdoor' pathway in peripheral tissue, yielding DHT.

The adrenals produce androgen precursors which are secreted into circulation and exert physiological effects in peripheral target tissue, such as the skin, liver and prostate. These steroid precursors include DHEA(S), A4, T and 11OHA4 and have been implicated in the recurrence of androgen dependent PCa, subsequent to castration having dramatically reduced circulating testicular T. Adrenal steroidogenesis therefore provides androgen precursors that may drive the recurrence of PCa, following ADT.

3.3.3 *De novo* androgen biosynthesis in the prostate

It was shown that AR activity in castration-resistant VeCaP (wild type AR positive androgen dependent PCa cells) xenografts is driven by CYP17A1 and AKR1C3-dependent intratumoural *de novo* androgen biosynthesis. *De novo* androgen biosynthesis within CRPC cells may thus yield adequate C19 steroids, capable of restoring AR transcriptional activity (Cai et al., 2011). Studies carried out in LNCaP cells have also shown that cholesterol biosynthesis and metabolism increased as tumours progressed subsequent to androgen deprivation (Bauman et al., 2006; Dillard et al., 2008; Ettinger et al., 2004; Leon et al., 2010; Locke et al., 2008). Although mRNA transcripts encoding CYP11A1 and CYP17A1, required for *de novo* biosynthesis of steroids from cholesterol, have been detected in PCa cell lines, *de novo* T production using radioactive tracers, was shown to be very low with androgen levels unable to restore AR activity in LNCaP cells (Dillard et al., 2008; Locke et al., 2008). It has furthermore also recently been shown that *de novo* steroidogenesis is not possible and not able to support AR transactivation, together with cells growth in PCa cells (Kumagai et al., 2013).

It is therefore possible that *de novo* androgen biosynthesis from cholesterol in CRPC tissue serves as an alternative source of androgens, other than the testicular and adrenal C19 steroids. However, the abundance of adrenal precursors in serum, and the requirement for only two to three enzymes for the conversion of these C19 precursors to T and DHT, suggests that the adrenals are the main source of intratumoural androgens in CRPC (Sharifi et al., 2010).

3.4 Androgen metabolism in the prostate

Steroids in circulation are bound to plasma proteins. The unbound hormones are available for uptake by target tissues, such as the prostate. The uptake of circulating androgens and subsequent metabolism to active steroids by peripheral target tissues such as breast, prostate and skin, (termed intracrinology), involves the paracrine diffusion and conversion of steroid substrates among neighbouring cell types with different enzyme capacities (Mostaghel, 2013).

Steroid metabolism in prostatic tissue and steroid-dependent PCa cells does not necessarily follow a single dominant pathway, for instance the conventional C19 pathway. Alternate metabolic pathways may differ somewhat from the conventional pathway converging at DHT. Competing pathways and enzymes may also exist, and the flux within various routes may vary depending on the available precursors and other conditions, such as redox-partners required for catalysis. In addition, multiple androgen precursors are available to PCa cells, reducing the number of enzymes needed to generate AR ligands (Sharifi & Auchus, 2012).

3.4.1 The conventional C19 pathway

DHT, regarded as the most potent natural androgen, is generally accepted to be produced by the reduction of T by SRD5A1 and SRD5A2 enzymes (fig. 3.5). Cell growth and proliferation as it occurs in the prostate, is due to DHT which is approximately 10-fold more potent than T (Hsing et al., 2008). Therefore, the conventional C19 pathway primarily refers to the production of T by the testes, and the subsequent biosynthesis of DHT from T in the prostate.
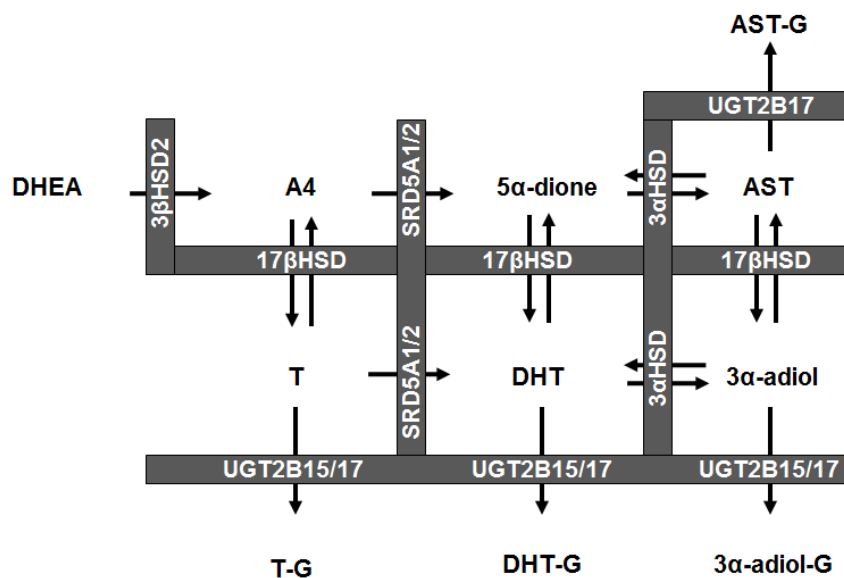
Figure 3.5: C19 steroid metabolism in the conventional C19 pathway.

In normal physiology the testes provides T that is taken up by the prostate, with adrenal androgens making a minimal contribution. The first treatment strategy employed to treat PCa is ADT, as mentioned above, which depletes the body of circulating testicular T. However, it is the adrenal androgens, DHEA, A4 and to a lesser extent T (Rege et al., 2013; Xing et al., 2011), which contribute towards the androgen pool when PCa recurs when patients relapse. Uptake of these three androgens into the prostate cells results in A4 and T being interconverted by 17βHSDs and A4 being produced from DHEA via 3βHSD2. 17βHSDs play a dominant role in converting A4 to T, and in so doing activate T. Both 17βHSD3 and AKR1C3 has been identified in prostate tissue.

Together with these reductases, 17βHSD2 is also present in the prostate, and converts T and DHT to A4 and 5α-dione, respectively (Wu et al., 1993). In the conventional pathway it is thought that A4 is converted to T and that T is subsequently reduced via SRD5A1 and SRD5A2 to produce DHT (3.4.2).

3.4.2 The 5α-dione pathway

The conversion of T to DHT, via SRD5A was thought to be the predominant conversion in the biosynthesis of DHT during prostate androgen metabolism. The alternative to DHT biosynthesis through T is the 5α-reduction of A4 to 5α-dione, which is subsequently reduced at C17 to form DHT (fig. 3.6). Chang et al., (2011), recently showed that A4 is preferentially reduced to 5α-dione by SRD5A1, rather than being reduced to T, in multiple models of CRPC, as well as freshly biopsied tissue from patients with metastatic CRPC (Chang et al., 2011).
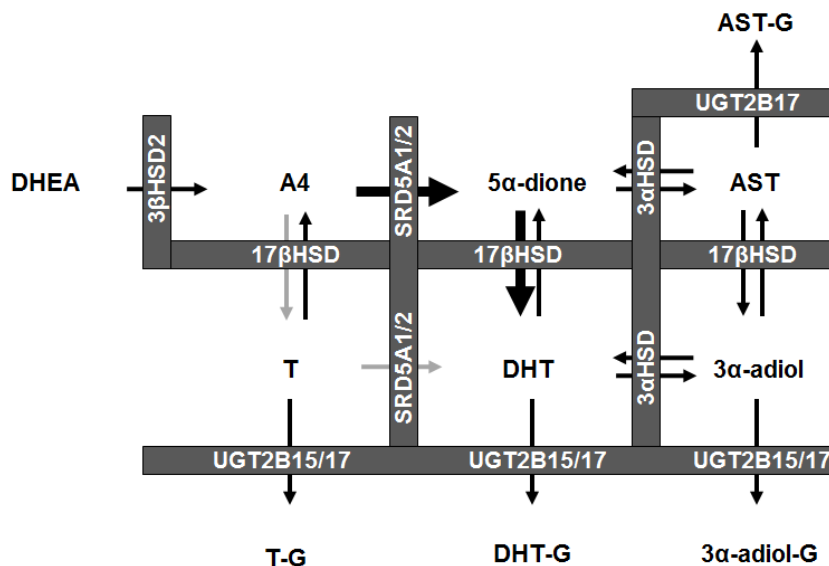
Figure 3.6: C19 steroid metabolism in the 'alternative' 5α-dione pathway.

Therefore the preferred route of adrenal precursors being metabolised to DHT is, A4 to 5α-dione (via SRD5A1), followed by 5α-dione to DHT (via 17βHSD), rather than from A4 to T (via 17βHSD) followed by T to DHT (via SRD5A) as in the conventional C19 pathway. It was shown that silencing the expression of *SRD5A1* blocked the conversion of A4 to 5α-dione and the eventual biosynthesis of DHT in CRPC. These data suggested the up-regulation of SRD5A1 described in multiple clinical studies of CRPC tissue, served to increase flux from A4 to 5α-dione, rather than T to DHT (Chang et al., 2011; Sharifi, 2012).

Inactivation of active androgens can follow in two steps, the first step is catalysed by AKR1C2, followed by inactivation catalysed by UGT2B15 and UGT2B17. AKR1C2 catalyse the reversible reduction of DHT and 5α-dione, to 3α-adiol and AST, respectively, which exhibit only weak

androgenic activity. The presence of AKR1C1 also results in the β-conformations of these steroids, 3β-adiol and epi-AST, being produced (Muthusamy et al., 2011). The enzyme mediating the reverse conversion of 3α-adiol and AST is thought to be the retinol dehydrogenase (RoDH) like 3αHSDs (RL-HSDs, 17βHSD6) (Bauman et al., 2006). In the second step, the conjugating UGT enzymes terminate the androgen signal by irreversibly catalysing the glucuronidation of 3α-adiol and AST, together with T and DHT (Chouinard et al., 2007).

3.4.3 The 11OHA4 pathway

In addition to DHEA(S), A4 and T, the adrenal produces substantial amounts of the C19 steroid, 11OHA4, which is the product of CYP11B1 catalysed hydroxylation of A4 (Schloms et al., 2012). The possibility that 11OHA4 may serve as a potential precursor in the biosynthesis of potent androgens in mammals has been overlooked, until recently when it was reported that 11OHA4 is converted to active androgens by the 11OHA4 pathway (fig. 3.7) (Storbeck et al., 2013). It was recently shown that CYP11B1, as well as CYP11B2 catalyse the hydroxylation of A4 and T to 11OHA4 and 11OHT, respectively. In addition, 11βHSD2, also expressed in the adrenal, catalyses the conversion of these hydroxy-steroids to their keto-forms, producing 11KA4 and 11KT, respectively (Swart et al., 2013a). Furthermore AKR1C3 converts A4 to T, and is also able to produce 11KT from 11KA4, once 11KA4 is produced in the adrenals. The levels of 11KA4, 11OHT and 11KT produced by the adrenal are, however, significantly lower than that of 11OHA4 (Rege et al., 2013). Therefore, once in circulation, 11OHA4 will reach peripheral tissues, such as the prostate and be metabolised, thus contributing additional steroid metabolites to the androgen pool. Interestingly, it has been shown that CYP11B1 and CYP11B2 are expressed in human primary prostate carcinomas and metastatic primary prostate carcinomas (Biancolella et al., 2007; Mitsiades et al., 2012), which suggests PCa cells may be capable of producing 11OHA4 and 11OHT from A4 and T, respectively.
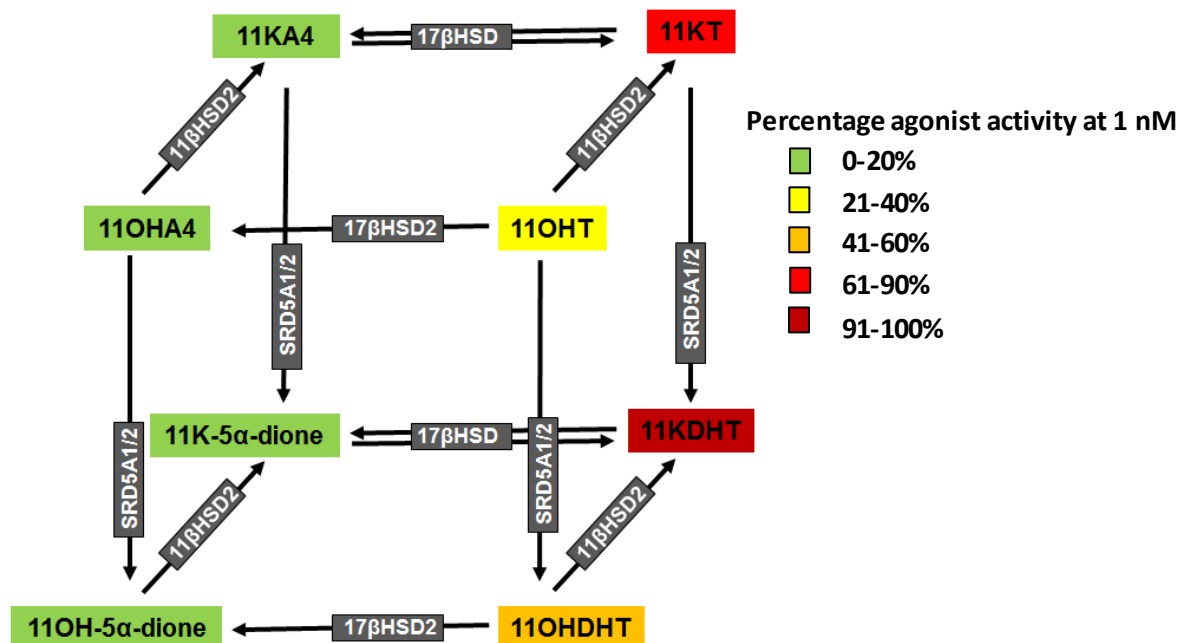
Figure 3.7: C19 steroid metabolism in the 11OHA4 pathway. Shading indicates AR transactivation relative to that of 1 nM DHT. Reproduced with permission from (Bloem et al., 2013).

Adrenal 11OHA4 is metabolized by three enzymes, *viz.* 17βHSDs, 11βHSDs and SRD5As in the prostate, as shown in LNCaP cells. In the pathway, the conversion of 11OHA4 to produce 11KA4 is catalysed by 11βHSD2, which in turn is utilized by AKR1C3 and/or 17βHSD3 to produce 11KT. 11KT and its dihydro-form, the novel steroid 5α-dihydro-11-keto-testosterone (11KDHT), have been shown to have androgenic activity, with the 5α-reduced form of 11KT possessing agonist activity towards the AR comparable to that of DHT (fig. 3.7). It is also possible for 11OHA4 to be reduced by SRD5A to its dihydro-form, 11β-hydroxy-5α-androstanedione (11OH-5α-dione), which is subsequently catalysed by 11βHSD2 to produce 11-keto-5α-androstanedione (11K-5α-dione), followed by the reduction by 17βHSDs leading again to the production of 11KDHT. 11KA4 and 11OHT are also metabolized to their novel 5α-reduced forms by SRD5A1 and SRD5A2, *viz.*: 11K-5α-dione and 5α-dihydro-11β-hydroxytestosterone (11OHDHT), respectively. 11OHA4, 11KA4, 11OH-5α-dione and 11K-5α-dione exhibit negligible androgenic activity at 1 nM, however, when 17βHSDs metabolise these steroids, their androgenic activity increased. While 11OHT at 1 nM exhibited minimal androgenic activity, the reduction of this metabolite to 11OHDHT increased the androgenic activity. 11KT showed partial agonist activity towards the AR, comparable to that of T, and as mentioned previously, 11KDHT is a full agonist, thereby implicating it as a role player in CRPC. These data showed that the C11-keto forms of A4, T and DHT, were more androgenic than their respective 11-hydroxy forms, indicating that a keto group at C11 may be preferential for optimal interaction with the AR ligand binding domain (Storbeck et al., 2013).

In LNCaP cells, which express only low levels of SRD5A, 11OHA4 was shown to be preferentially converted to 11KA4 by 11βHSD2. As mentioned previously two isoenzymes of 11βHSD exist,

11βHSD1 expression in the prostate remains questionable, but 11βHSD2 has been detected in LNCaP cells (Dovio et al., 2009; Page, Warriar, & Govindan, 1994), and although 11βHSD2 does not have an active role in the metabolism of C19 steroids without a C11 hydroxy- or C11 keto-group, this enzyme has a definitive role in the 11OHA4 pathway. Studies with COS-1 cells transfected with SRD5A showed that although the 11-hydroxy and 11-keto A4 and T steroids may all serve as substrates for SRD5A, 11OHA4 and 11KA4 are the preferred substrates, yielding 11OH-5α-dione and 11K-5α-dione, respectively. This data is comparable to the 'alternative' 5α-dione pathway in which the 5α-reduction of A4 is preferred over that of T (3.4.2). Studies with 17βHSD showed that the hydroxy or keto group at C11 position influences the ability of the 17βHSD isoforms in accepting these metabolites as substrates. The reductive activity of the 17βHSDs was only detected for the 11-keto steroids, while the reverse reaction exhibited no preference for either a hydroxy or a keto group at C11 and catalysed the conversion of both C11-hydroxy and C11-keto substrates. It was also observed that the metabolism of the 5α-reduced by 17βHSD mimics that of their Δ^4 parent compounds. Due to the substrate preferences of the enzymes involved, the principal metabolites produced by the 11OHA4 pathway are 11KT and the novel steroid 11KDHT as confirmed in LNCaP cells. This finding has significant implications in CRPC. 11OHA4 thus serves as an additional source of adrenal derived androgens, other than DHEA(S), A4 and T, which may drive CRPC through the activation of the AR (Bloem et al., 2013; Storbeck et al., 2013; Swart et al., 2013a).

Whether the 5α-reduced steroids, in the 11OHA4 pathway, are reduced by AKR1C2 at C3 has not to date been established, together with the reverse reaction catalysed by 17βHSD6. The catalytic activity of UGT2B15 and UGT2B17 towards 11OHT, 11KT, the 5α-reduced steroids 11OHDHT and 11KDHT as well as the possible 3α-reduced steroids, have also not been established.

3.5 Prostatic steroidogenic and inactivating enzymes

The localization of AKR1C3, 3βHSD2 and the AR in the prostate have been firmly established (Akinola et al., 1996; Bonkhoff & Remberger, 1993; Dumont et al., 1992; Loda et al., 1994; Martel et al., 1992; Pelletier & Tong, 1992) (fig. 3.8). It has been shown by using *in situ* hybridization and immunocytochemistry, that AKR1C3 is expressed in basal cells, the fibroblasts dispersed throughout the stroma or in association with the wall of blood vessels and the endothelial cells lining blood vessels. Furthermore, the localization and expression of 3βHSD2 was reported to be similar to that of AKR1C3 and in all cells expressing 3βHSD2, staining was restricted to the cytoplasm, with no significant nuclear staining being detected. The AR appeared to be localized exclusively in the nuclei of prostate cells in all the specimens which included: BPH and normal prostate tissue as well as cultured epithelial cells. In cultured epithelial cells AKR1C3 showed similar pattern of expression as in the epithelial cells of BPH and normal prostate tissue (El-alfy et al., 1999). Previous studies reporting on the localization of SRD5A1 and SRD5A2, showed that the enzymes are produced by both epithelial and stromal cells in the prostate (Aumüller et al., 1996;

Bruchovsky et al., 1996; Habib et al., 1998; Pelletier, Luu-The et al., 1998). The presence of AKR1C3, 3βHSD and SRD5A isoenzymes in basal cells suggests that the cell type is actively involved in androgen production and is not simply a secretory cell, producing precursors for the luminal cells.
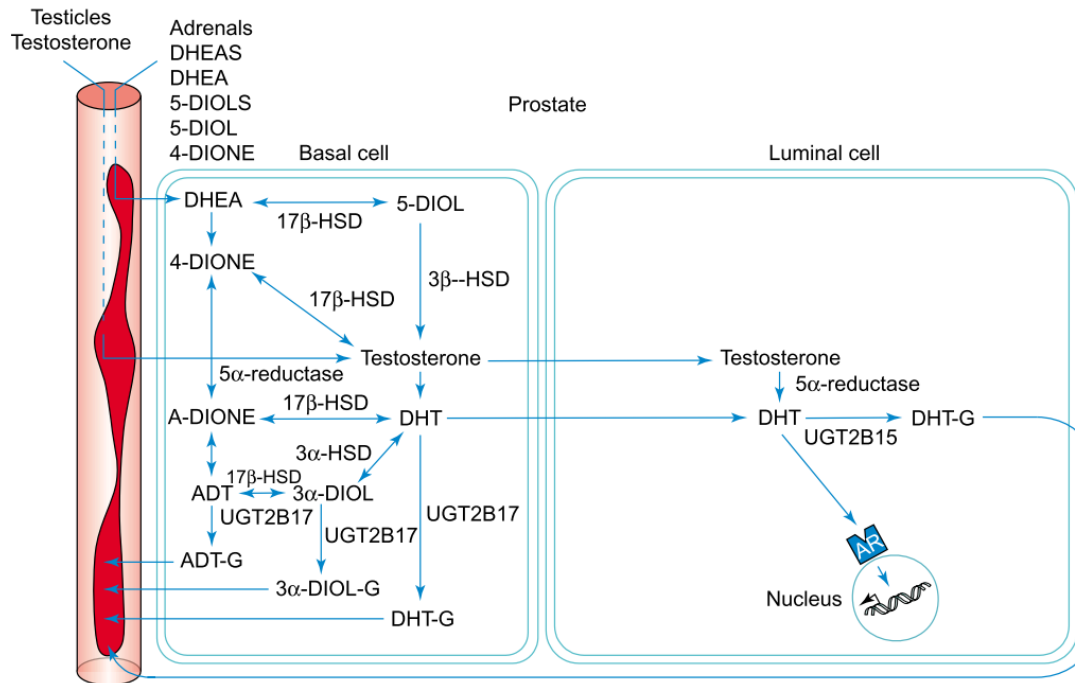
Figure 3.8: Androgen metabolism and enzyme location in the human prostate. Abbreviations as follows: DHEAS, dehydroepiandrosterone sulphate; DHEA, dehydroepiandrosterone; 5-DIOLS, androst-5-ene-3β, 17β-diol sulphate; 5-DIOL, androst-5-ene-3β, 17β-diol; 4-DIONE, androstenedione; A-DIONE, androstanedione; DHT, dihydrotestosterone; 3α-DIOL, androstane-3α, 17β-diol; ADT, androsterone; ADT-G, androsterone-glucuronide; 3α-DIOL-G, androstane-3α, 17β-diol -glucuronide; DHT-G, dihydrotestosterone-glucuronide. Reproduced with permission from (Bélanger et al., 2003).

It has been suggested that T synthesized in the basal cells, due to the presence of both AKR1C3 and 3βHSD2, reaches the luminal cells, in a paracrine manner, and is ultimately transformed into DHT in the luminal cells, where androgenic effects are exerted and AR is highly expressed. DHT, produced in the luminal cells by SRD5As, would subsequently exert its effects in the luminal cells, which can thus be viewed as an intracrine activity and suggests a two-cell mechanism of androgen formation in the human prostate (El-alfy et al., 1999). In this context it is, however, also important to consider the inactivation and conjugation of active androgens. AKR1C2 is expressed in the basal cells, where the enzyme catalyses the reduction of DHT to 3α-adiol while UGT2B15 is expressed in the luminal cells and UGT2B17 is expressed in the basal cells of the human prostate. The levels of DHT are therefore modulated independently by UGT2B15 in the luminal cells and by UGT2B17 in the basal cells (Barbier & Bélanger, 2008).

3.5.1 The SRD5A enzymes

In 1968 the intraprostatic conversion of T to DHT was characterized which established the importance of the enzyme SRD5A and of DHT (Bruchovsky & Wilson, 1968). SRD5A enzymes, are NADPH-dependent enzymes that act upon C19 and C21 steroids with C4-C5 double bonds in their carbon-backbones (Soronen et al., 2004), irreversibly reducing the double bonds to produce 5α-reduced steroids.

Three isoforms of SRD5A exist, with SRD5A2 representing the predominant isoform in normal prostatic tissue and SRD5A1 showing increased expression in cancerous prostatic tissue (Chang et al., 2011; Stanbrough et al., 2006). Although *SRD5A1* expression levels have also been reported to remain unchanged, while *SRD5A2* levels are reduced, a definite shift from the predominant expression of *SRD5A2* to that of *SRD5A1* exists in recurrent PCa (Titus et al., 2005). The third isoform, SRD5A type 3 (SRD5A3), has been suggested to be associated with DHT production and AR activation in hormone refractory PCa (Uemura et al., 2008). Its role, however, has not been elucidated, but a shift away from SRD5A2 to increased expression of *SRD5A1* and *SRD5A3* in CRPC has also been described (Azzouni et al., 2012; Godoy et al., 2011). Increased expression of *SRD5A3* was shown in metastatic PCa (Mitsiades et al., 2012) and mean mRNA levels in CRPC suggest relative gene expression gradients of *SRD5A3>SRD5A1>SRD5A2* (Titus & Mohler, 2009). Dutasteride, a bispecific SRD5A1 and SRD5A2 inhibitor, to date the most effective SRD5A inhibitor, does not block SRD5A3, suggesting that SRD5A3 may be a possible approach in targeting prostate androgen metabolism in CRPC.

SRD5As are expressed in androgen-responsive tissues and in androgen-stimulated benign prostate tissue with expression levels being higher in clinically higher graded PCa compared to lower graded PCa (Thomas et al., 2008). SRD5A1 and SRD5A2 are expressed mainly in the cytoplasm of PCa cells, while SRD5A1 is located in the nucleus in benign prostate cells. SRD5A1 and SRD5A2 share approximately 50% amino acid sequence identity and possess similar substrate specificity (Titus & Mohler, 2009). However, SRD5A1 has a lower affinity (K_m = 1-5 μM) for substrates such as T and A4 (Andersson & Russell, 1990), compared to SRD5A2 having a higher affinity (K_m = 4-50 nM) (Faller et al., 1993; Thigpen et al., 1993) for these substrates (Russell & Wilson, 1994).

Although SRD5As are widely accepted as catalysing the conversion of T to DHT, the conversion of A4 to 5α-dione has been shown to be the preferred pathway to DHT production in CRPC (Chang et al., 2011; Sharifi, 2012). Within the 11OHA4 pathway both SRD5A1 and SRD5A2, preferably convert A4 metabolites, 11OHA4 and 11KA4, to their respective dihydro steroids, while also catalysing the 5α-reduction of 11OHT and 11KT. These isoenzymes thus play a significant role in the production of active androgens, the C19 5α-reduced steroids and the novel full AR agonist steroid hormone 11KDHT (Storbeck et al., 2013).

3.5.2 The 17βHSD enzymes

The 17βHSDs catalyse the reduction of the keto group at the C17 position of the steroid nucleus, thereby modulating the potency of these androgen steroids, with the hydroxy-forms being the active form of the steroid (Mindnich et al., 2004). To date 14 types of 17βHSDs have been isolated, with types 1, 3, 5 and 7 catalysing the reductive reactions and types 2, 4 and 8 catalysing the oxidative reactions. 17βHSD3 and AKR1C3 are the most important isoforms in prostate steroid metabolism. Of interest, the reductive 17βHSD activity requires NADPH while the oxidative 17βHSD activity requires NAD$^+$ (Luu-The, 2001). Interestingly, although 17βHSD3 and AKR1C3 share a high degree of homology (Labrie et al., 2000), 17βHSD3 belongs to the short-chain alcohol dehydrogenase reductase superfamily, and AKR1C3 belongs to the aldo-keto reductase family 1. Both isoforms function in a unidirectional manner, predominantly determined by their respective cofactors (Luu-The, 2001).

17βHSD3 prefers NADPH as a cofactor and its primary activity is reductive, catalysing the production of T in the testes, in the adrenals (minimal) and in peripheral tissues, such as the prostate (Mindnich et al., 2004; Moeller & Adamski, 2009; Montgomery et al., 2008). However, in the production of T, the role of AKR1C3 is more prominent in the adrenals and prostate. Indeed, the adrenal cortex expresses AKR1C3, with definitive levels detected in the ZR (Nakamura et al., 2009). This is not surprising as the cells of the ZR are the site of androgen precursor biosynthesis. In the prostate, AKR1C3 is also the predominant isoform contributing to T production (Luu-The, 2001). Of interest, both 17βHSDs catalyse the production of androgens other than T as discussed in the sections above.

A major role in PCa for both 17βHSD2 and 17βHSD3 in PCa tissue was proposed by a study which showed higher expression levels of the *HSD17B3* gene in malignant prostatic tissue compared to normal prostate tissue, while the opposite was shown in the case of 17βHSD2. No significant differences in AKR1C3 gene expression in cancerous and noncancerous tissue were detected (Koh et al., 2002). Similar results were reported in another study in which the authors reported results showing a significant increase in the expression of 17βHSD3 in castration resistant metastases (Montgomery et al., 2008).

Conversely, numerous studies have shown the up-regulation of AKR1C3 in PCas, with the expression increasing as the disease progresses (Fung et al., 2006; Lin et al., 2004; Luu-The et al., 2008; Mitsiades et al., 2012; Montgomery et al., 2008; Penning et al., 2006; Stanbrough et al., 2006). The upregulation of AKR1C3 would contribute to intracellular biosynthesis of AR ligands and subsequently cell growth and proliferation through AR activation (Byrns et al., 2012). Furthermore, AKR1C3 was recently identified as a co-activator of the AR, further emphasizing the role of this enzyme in PCa progression, as it may have a dual role of in the promotion of ligand biosynthesis and AR activation. In this study AKR1C3 stimulated the growth of both androgen-

dependent PCa and CRPC xenografts, with reactivation of androgen signalling occurring concurrently. AKR1C3 was shown to interact directly with the AR in PCa cells and CRPC xenografts and was recruited to the promoter of PSA, an androgen-responsive gene. It was suggested that AKR1C3 may be a nuclear hormone receptor co-activator which could be targeted pharmacologically (Yepuru et al., 2013).

AKR1C2, also a hydroxysteroid dehydrogenase expressed in the prostate, reduces the C19 steroid C3-keto-group to a C3-hydroxy-group (Penning & Byrns, 2009), with the latter subsequently serving as substrates for UGT2B17. The reduction of the keto group at C3 permits further conjugation of not only T ligands but allows the glucuronidation of A4 metabolites at C3. Reduction of A4 metabolites, which would otherwise not be conjugated, thus facilitates the secretion of not only AR ligands but also potential ligands. In this manner AKR1C2 can therefore regulate the occupancy of the AR.

3.5.3 The UGT enzymes

UGTs are enzymes found in many organs of the human body, and in the prostate, these enzymes catalyse the transfer reaction of glucuronic acid from uridine diphosphoglucuronic acid to steroid hormones. In the prostate, this irreversible conversion, following the first inactivation step catalysed by the enzyme AKR1C2, represents the second inactivation step of the active C19 steroids, abolishing their affinity for the AR. UGTs also catalyse reactions with substrates other than steroid hormones, such as lipid-soluble drugs and environmental chemicals. Two superfamilies of UGTs exist, namely: UGT1 and UGT2. The UGT1 family is generally known to conjugate planar and bulky phenol substrates and estrogens and is expressed in the liver and gastrointestinal tract (Strassburg et al., 1998; Strassburg et al., 1998). In contrast UGT2 is divided into two subfamilies, where the UGT2A subfamily is expressed in the olfactory epithelium and have activity towards at least five odorant compounds (Lazard et al., 1991), while the UGT2B subfamily is expressed in the liver, skin, kidney and prostate and catalyse the conjugation of C19 steroids, phenols and fatty acids (Beaulieu et al., 1996). UGTs are therefore critical in facilitating the secretion of steroid-conjugates into the circulation, and UGT2B regulating active steroid hormones in the prostate (Tukey & Strassburg, 2000).

Steroid metabolites with a hydroxy-group at C17 or C3 on the C19 steroid nucleus are suitable substrates, in the catalyses by UGTs of hydrogen release and glucuronic acid binding to the oxygen moiety. In the prostate, enzymes 17βHSD and AKR1C2 yield steroids possessing 17β-hydroxy and 3α-hydroxy groups, respectively. UGTs are able to conjugate the 17β-hydroxy and 3α-hydroxy groups or both, depending on which isoform is expressed. In the C19 pathway within prostate steroid metabolism, AKR1C2 catalyses the first inactivation step, and produces steroids AST from 5α-dione, and 3α-adiol from DHT (fig. 3.5), as previously mentioned. Both AST and 3α-adiol are ideal substrates for UGTs endogenously expressed in the prostate. In addition, the

upstream conversion of A4 to T and 5α-dione to DHT by 17βHSD, results in steroid metabolites possessing a hydroxy group at C17 thus also presenting steroids for conjugation (Bélanger et al., 2003; Byrns et al., 2012; Chouinard et al., 2007).

In the prostate with UGT2B enzymes catalysing the conjugation of these afore mentioned C19 steroids, two isoforms are of importance *viz.*: UGT2B15 and UGT2B17. These isoforms share 95% sequence identity at the amino-terminal, which has been proposed to contain the domain determining aglycone, as well as substrate specificity. UGT2B17 expressed in HEK293 cells exhibited a substrate preference of 3α-adiol>DHT>T>AST (table 3.1) when glucuronide formation was detected at 15-, 14-, 9- and 5 pmol/min/mg protein. It was shown that UGT2B17 only was capable of conjugating the 3α-hydroxyl group of AST with UGT2B15 being unable to catalyse the conjugation of the 3α-hydroxy group of the metabolite (Beaulieu et al., 1996).

The importance of UGTs was highlighted by Chouinard et al., (2007), when the endogenous UGT2B15 and UGT2B17 was knocked out in LNCaP cells, resulting in increased active DHT and decreased DHT-G levels, leading to increased cell proliferation and increased expression of eight androgen-sensitive genes (Chouinard et al., 2007). The predominant form circulating in adult males is 3α-adiol-glucuronide (3α-adiol-G) which acts as a predictor of prostate volume (Vandenput et al., 2007). UGT2B15 preferably conjugates 3α-adiol, and also T and DHT. UGT2B17 only conjugates AST, together with T and DHT. UGT2B17 also conjugates 3α-adiol, however, not as readily UGT2B15 (Turgeon et al., 2001), with the conjugation of 3α-adiol by UGT2B15 being 1.8-fold greater than that of UGT2B17 (table 3.1).

Table 3.1: Relative V_{max} of UGT2B15 and UGT2B17 for androgen metabolites in the C19 pathway. Reproduced with permission from (Turgeon et al., 2001).

Substrates	UGT2B relative V_{max} (pmol/min/mg protein)	
	UGT2B15	*UGT2B17*
T	4.4 ± 1.0	36.7 ± 5.6
DHT	10.4 ± 2.7	63.1 ± 4.1
AST	-	21.7 ± 2.7
3α-adiol	125.4 ± 25.3	70.8 ± 18.3

The conjugation of the C19 steroid metabolites in the 11OHA4 pathway has not been clarified to date. It has been reported that 11-hydroxy C19-steroids were poor substrates for glucuronidation by UGT2B17 in LNCaP cells, with or without the 3α-hydroxy or the 17β-hydroxy group being present. Although the conversion rate of 11hydroxy-AST was similar to that of AST by UGT2B17 it was nevertheless proposed that, due to the enzyme exhibiting a high degree of stereospecificity in terms of the C3-hydroxy group it may be that the presence of the 11(α/β)-hydroxy group would

interfere with the inactivation of these C19 steroids (Beaulieu et al., 1996). Impeded conjugation may possibly occur in the 11OHA4 pathway, implying that androgens remain active and capable of binding the AR, allowing these steroids to remain in the androgen pool. This suggestion leads one to question whether only the C11 hydroxy-group may hinder conjugation and whether the C11 keto-group may also influence the specificity of the UGTs.

UGT2B15 and UGT2B17 are differentially regulated during PCa progression, with the latter being significantly higher in cancerous tissues when compared to protein levels in BPH and prostate intraepithelial neoplasia samples (Paquet et al., 2012). Another study reported an increased expression of UGT2B15 in androgen-independent PCa (Stanbrough et al., 2006). Again, when comparing CRPC with primary tumours, CRPC samples showed increased expression of UGT2B15 (3.5 fold). However, increased expression of both UGT2B15 and UGT2B17 in CRPC has also been reported (Mostaghel, 2013). Whether it is therefore UGT2B15 or both UGT2B15 and UGT2B17 which are up-regulated in the progression of PCa, higher UGT levels indicate an adaptation of prostatic tissue in response to increased levels of active androgens, due to the up-regulation of enzymes such as AKR1C3, converting A4 to T. The adaptation suggests a mechanism by which the cell's conjugating ability is increased to compensate for increased active androgens present. It is also possible that UGT expression is increased due to both *UGT2B15* and *UGT2B17*, being primary androgen-regulated genes. Since they require the AR for both basal and androgen-regulated expression (Bao et al., 2008), AR mutations and reduced AR-ligand specificity may result in their induction and transcription, unrelated to the amount of active androgens, but specific to the receptor and steroid ligand present in the cell.

3.6 Activation of AR and downstream implications in PCa

The AR protein is encoded by the *AR* gene on the Xq11-12 chromosome (Hsing et al., 2002). The AR is a member of the steroid hormone receptor family of ligand-activated nuclear transcription factor and contains four functional regions, *viz.*: an amino terminal regulatory domain (amino terminal), a DNA-binding domain (DBD), a hinge region containing a nuclear localization signal, and a carboxy-terminal ligand-binding domain (LBD) (fig 3.9). ARs localized in the cytoplasm are unligated, but bound to heat shock proteins (HSPs), which stabilize the ARs tertiary structure in a conformation that allows androgen binding. Once ligand bound, the AR dissociates from the HSPs, dimerizes and subsequent to tyrosine kinase phosphorylation, is translocated into the nucleus. In the nucleus, the AR forms an active transcription complex by binding the androgen response elements (AREs) located in the promoter and enhancer regions of target genes (Taplin, 2007).
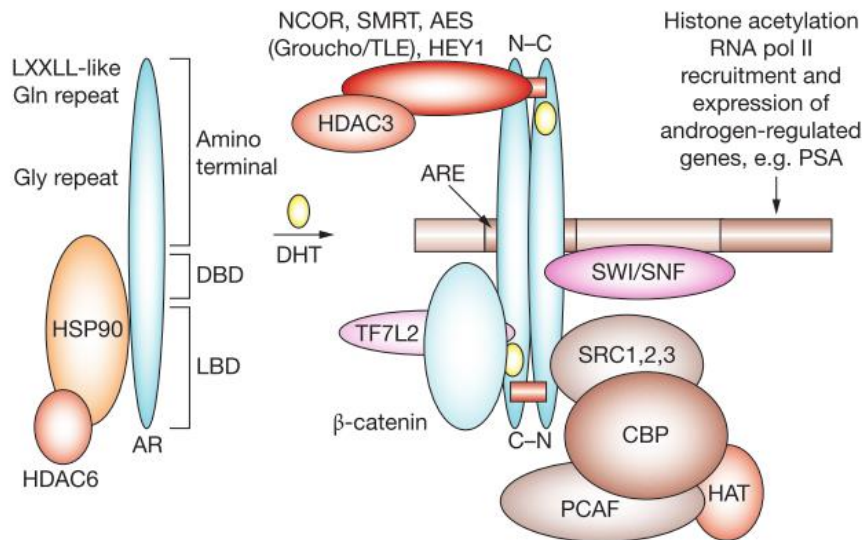
Figure 3.9: AR structure and function, representing the binding of DHT and the formation of a transcription complex of co-regulatory proteins and transcription of androgen-regulated genes. Abbreviations are as follows: AES, amino-terminal enhancer of split; C, carboxy terminal; CBP, CREB-binding protein; HAT, histone acetylases; HDAC3, histone deacetylase 3; HDAC6, histone deacetylase 6; HEY1, hairy/enhancer-of-split related with YRPW motif 1; HSP90, heat shock protein 90; N, amino terminal; NCOR, nuclear corepressor; PCAF, p300/CBP- associated factor; PSA, prostate-specific antigen; RNA pol II, RNA polymerase II; SMRT, silencing mediator of retinoid and thyroid; SRC, nuclear receptor co-activator; TF7L2, transcription factor 7-like 2; TLE, transducin-like enhancer of split. Reproduced with permission from (Taplin, 2007).

The AR plays a central role in normal and PCa cell growth and proliferation. The AR is expressed in all stages of PCa, with somatic mutations of the *AR* gene involved in the progression and aggressiveness of PCa. Therefore, with the onset of PCa, ADT - castration, as well as AR blockage through usage of AR antagonists – is included in the treatment regime. When PCa recurs it is likely to be more aggressive, with AR amplification reported in one third of the cases, demonstrating an adaptation within the PCa cells (Holzbeierlein et al., 2004; Taplin et al., 1995). Furthermore, AR mutations can also occur which allow activation of the AR by weak androgens which may not have activated the AR prior to CRPC. AR mutation frequencies ranging from 0 to 44% and 0 to 50% have been reported for androgen-dependent PCa and CRPC, respectively (Mohler et al., 2004). In addition, it also possible for increased expression of transcriptional co-activator proteins, as another adaptation in PCa cells, to allow the activation of signal transduction pathways which would enhance AR responses to weak androgens (Yuan & Balk, 2009).

Together with increased AR expression, an increase in AR-regulated genes such as PSA, also occur. Transcription of PSA is positively regulated by the AR, and consistently expressed in PCa. PSA, a serum marker for PCa and treatment response, is a serine protease protein which is androgen-regulated. PSA, a member of the kallikrein family proteases, is a major part of semen (0.5 – 2 mg/mL), with the function to cleave semenogelins in the seminal coagulum. PSA is

produced by prostate ductal and acinar epithelium and is secreted into prostatic ducts as an inactive 244-amino acid pro-enzyme (proPSA) that is activated by cleavage of seven N-terminal amino acids. PSA that enters circulation is rapidly inactivated in the lumen by proteolysis and circulates as free PSA, or is bound by protease inhibitors, including α1-antichymotrypsin (ACT) (Balk et al., 2003).

The normal physiology of the human prostate reflects a single layer of secretory epithelial cells, which are surrounded by a continuous layer of basal cells and a basement membrane. There is a concomitant disruption of the basal cell layer and basement membrane with the onset of PCa, and the loss of normal glandular architecture appears to allow direct access of PSA to peripheral circulation, as shown in Fig. 3.10 (Balk et al., 2003; Hayward & Cunha, 2000). Partial basal cell loss has been reported in prostate intraepithelial neoplasia, while complete basal cell loss is characteristic of PCa.
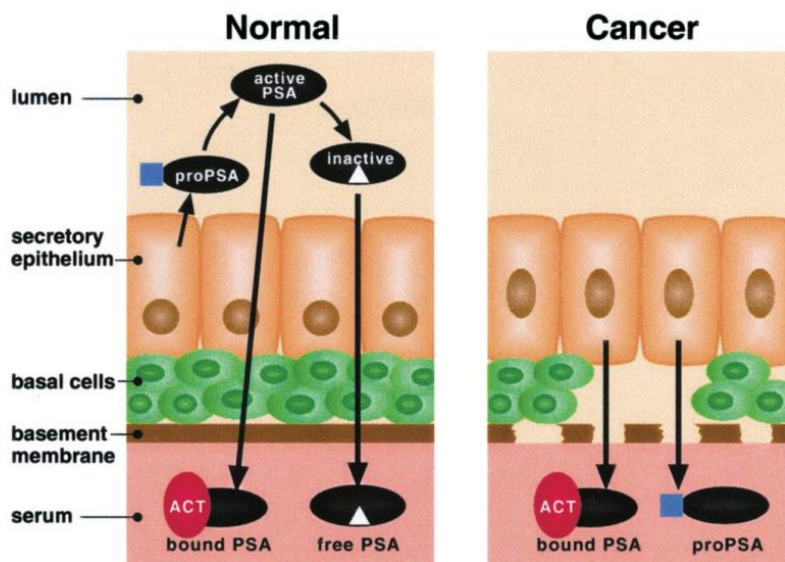
Figure 3.10: Model of PSA activation in normal prostate epithelium versus cancer. Reproduced with permission from (Balk et al., 2003).

Total PSA serum levels are increased in PCa, hence the screening of PSA as an indicator of PCa progression. Analyses of free and total PSA levels can increase the screening accuracy of PSA in PCa, allowing for discrimination between levels detected in the case of the healthy prostate and levels detected in PCa, as the PSA index (free PSA: total PSA) has been reported to be decreased in PCa (Catalona et al., 1998). An initial decline in PSA levels, in response to ADT, is partly due to tumour cell death and partly due to reduced AR-stimulated PSA production by surviving tumour cells. However, PSA expression continues to be maintained even after ADT. The AR is therefore still stabilized and activated by tissue androgens. Indeed, the presence of PSA in CRPC consistently correlates with the presence of an activated AR, as differential expression, subtractive

hybridization and cDNA microarrays showed PSA expression before and after castration in tumour models of androgen-dependent PCa (Mohler et al., 2004).

In castrate tumours DHT levels, between 0.5 and 1.0 ng/g, are sufficient to activate the AR, which subsequently stimulate the expression of AR-driven genes, such as PSA, and thereby mediates tumour growth and progression of PCa to CRPC (Culig et al., 1999; Gregory et al., 1998; Gregory et al., 2001; Mohler et al., 2004). Two cellular events occur in prostate cells: cellular proliferation and PSA production – however, cellular proliferation has been shown at low DHT concentrations, and high PSA levels at high DHT concentrations. It is necessary to note, that although the secretion of PSA in prostate cells is stimulated by androgens (DHT), it does not correlate with cell proliferation. A study by Lee et al., (1995), showed that at low DHT concentrations LNCaP cells proliferate at a high rate, but at high DHT concentrations, cell proliferation decreases with a concomitant rise in PSA levels (Lee et al., 1995).

In addition ERs should also be mentioned, especially ERβ, which is anti-proliferative and proapoptotic (Imamov et al., 2004) and is expressed in both normal prostate as well as in PCa (Lai et al., 2004; Leav et al., 2001). ERβ was reported to be predominantly localized in basal cells and to a lesser extent in stromal cell nuclei (Leav et al., 2001). AR mutations occur in invasive prostate carcinomas (Haapala et al., 2001; Marcelli et al., 2000), as mentioned above, however, increased estrogen sensitiveness as a result of increased expression of ERs occurs concurrently (Lai et al., 2004). Furthermore, it is possible for ERβ to antagonize androgen-dependent prostate and PCa proliferation, as prostatic hyperplasia occurred in ERβ knock-out mice (Krege et al., 1998). Of interest, ERβ expression increases in PCa progression, although it has also been reported that ERβ expression was diminished in high-grade dysplasia and grade 4/5 carcinoma of the peripheral zone of the prostate. The down-regulation of ERβ expression occurring during the late stage of prostatic carcinogenesis, possibly contributes the loss of control over growth processes mediated by ERβ and subsequently allows the proliferative stimuli to be reactivated mediated through the AR (Leav et al., 2001).

3.7 The progression of PCa to CRPC

The pathways that drive normal prostate development ultimately drive PCa development as well as CRPC. CRPC is the recurrence of PCa after ADT and usually occurs 2-5 years after initial ADT treatment. Despite a 94% reduction in serum T with medical castration, intraprostatic T and DHT are only reduced by 70% and 80%, respectively. The apparent availability of precursors for the biosynthesis of residual intraprostatic androgens in the absence of gonadal T provides a clue as to the mechanisms of resistance to gonadal T depletion (Sharifi & Auchus, 2012). A rise in PSA levels, that decline initially with ADT, often precedes radiographic changes and is therefore employed as a signal for the development and progression of CRPC. Increased PSA shows unsuccessful repression of AR signalling despite ADT. In CRPC this can be attributed to: AR

mutations in the LBD, which allows the conversion of antagonists to agonists or reduces the specificity of the AR to allow stimulation by other ligands; AR gene amplification; expression of mutated AR variants that are constitutively active; and finally the production of the most potent agonist, DHT (fig. 3.11) (Attar et al., 2009; Sharifi, 2012).
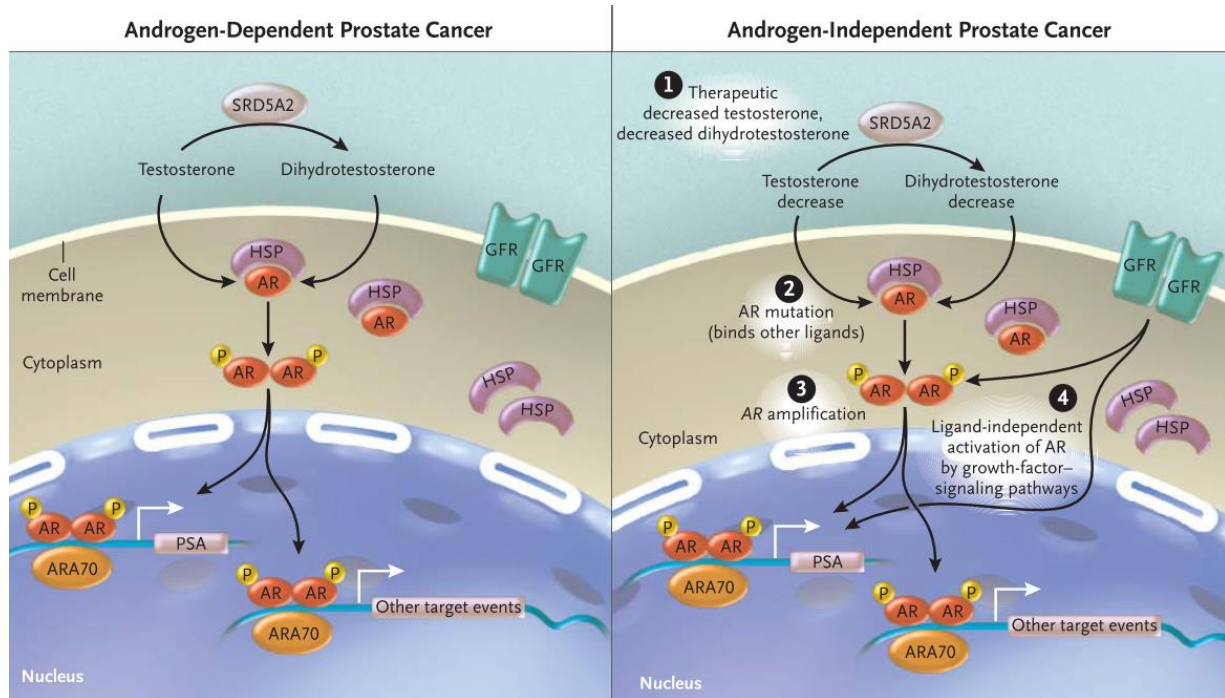

Figure 3.11: Progression of androgen-dependent PCa to CRPC, significant of PCa driven by androgen-independent factors. Reproduced with permission from (Nelson, De Marzo, & Isaacs, 2003), Copyright Massachusetts Medical Society.

Studies suggest that intratumoural DHT concentrations are approximately 1 nM, abundantly sufficient to drive CRPC progression. In addition, studies have shown the concentration of T to be higher than that of DHT (Montgomery et al., 2008; Titus et al., 2005), which suggests that the agonist activity of T could also play a role in CRPC progression. Elevated T:DHT ratios indicate inefficient conversion from T to DHT, which may suggest an alternative dominant pathway. In fact T:DHT ratios in CRPC are similar to those in men treated with a SRD5A inhibitor, finasteride (McConnell et al., 1992). It has therefore been suggested that research into nuclear androgen concentrations in which the AR and agonist ligand are complexed, should be investigated to allow complete evaluation of CRPC parameters (Sharifi & Auchus, 2012).

Progression of CRPC may also be due to modified steroid-metabolizing enzyme activity, due to subcellular localization, possible post-translational changes, availability of co-factors and ratios of enzyme to redox partner proteins, cellular redox status, competition between enzymes, as well as the availability and preference for alternative steroid substrates (Mizrachi & Auchus, 2009; Sharifi, 2012). As mentioned previously, SRD5A2 which is the dominant isoenzyme in normal prostate, is down-regulated in CRPC, together with the concurrent up-regulation of SRD5A1 (Montgomery et

al., 2008; Stanbrough et al., 2006; Titus et al., 2005). Evidence showing that the preferred pathway in CRPC is the 5α-dione pathway, suggests that together with the up-regulation of SRD5A1 and the preference of this enzyme for A4 as substrate, DHT biosynthesis continues unhindered (Chang et al., 2011). The 3-keto reduction and inactivation of DHT to 3α-adiol is a reversible reaction, which together with the conversion of AST to 5α-dione, might also play a role in CRPC progression, especially if glucuronidation of 5α- and 3α-reduced steroids is impaired in CRPC (Sharifi & Auchus, 2012).

3.8 ADT and CRPC treatment therapies

Strategies employed in the treatment of hormone-dependent cancers include: ADT and administration of inhibitors of either the steroid-metabolizing enzymes or antagonist activity at steroid receptor level. ADT was first accomplished by surgical castration, but can also be achieved using long-acting GnRH agonists (leuprolide acetate and goserelin) and GnRH antagonists such as degarelix. Androgen deprivation leads to regression characterised by a loss of secretory function and a reduction in glandular size of the prostate in adults. Regression is due to widespread apoptosis in the prostate and most extensive in distal regions of prostatic ducts. However, castration simultaneously depletes androgens from both epithelial and stromal AR and the stroma of the adult prostate undergoes changes toward a more fibroblastic, less muscular phenotype. Interestingly, stimulation by androgens of the regressed prostate results in new development, growth and secretory activity, including PSA secretion (Hayward & Cunha, 2000).

Approaches other than or in addition to ADT include: estrogens (diethylstilbestrol) and progestins, together with AR antagonists (flutamide, bicalutamide, nilutamide and a next-generation AR antagonist, Enzalutamide) (fig. 3.12). Included in the aforementioned list are specific enzyme inhibitors that modulate C19 steroid concentrations, including abiraterone, a potent inhibitor of CYP17A1 (Sharifi & Auchus, 2012). Exogenous androgen administration will suppress pulsations from GnRH and LH, and subsequently impair testicular T biosynthesis, and lead to testicular atrophy – but the androgen itself would stimulate PCa growth. Conversely, estrogens and progestin accomplish GnRH and LH suppression, without AR activation. As mentioned previously GnRH release from the hypothalamus leads to the subsequent release of LH from the pituitary. The precise pulsatile rhythm of GnRH release is essential for LH release, and desensitization occurs with continued GnRH stimulation – the rationale behind the use of GnRH agonists in therapeutic treatments employed in PCa, where long-acting GnRH agonists lead to LH and T suppression. The GnRH antagonist, degarelix, together with GnRH agonists has been used preferentially instead of estrogens and progestin, as the former show less risk in the development of gynecomastia and thromboembolism. Furthermore potent synthetic GR agonists, dexamethasone and prednisone, suppress CRH and ACTH release, lowering the production of cortisol, DHEA(S) and C19 androgens (Sharifi & Auchus, 2012), and abiraterone, which forms an irreversible complex between the iron atom of the heme cofactor in the active site of CYP17A1 and

an azole nitrogen atom of the inhibitor (Potter, Banie, Jarman, & Rowlands, 1995), is currently the preferred therapeutic approach for the inhibition of androgen biosynthesis.
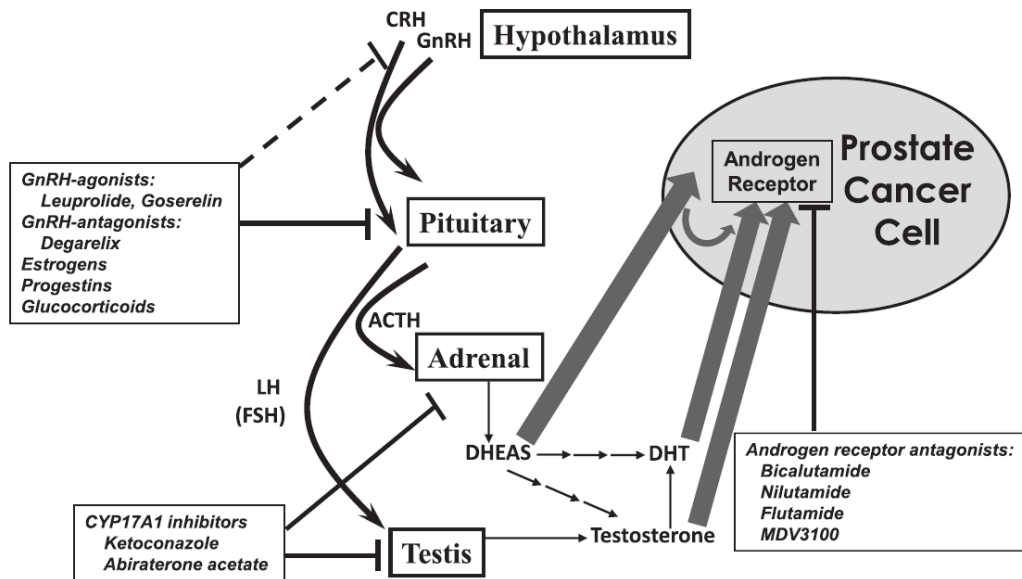

Figure 3.12: Hypothalamic-pituitary-adrenal and –testicular axes and current pharmacologic inhibition mechanisms. Flat line ends indicate inhibitory action. The dotted line indicates that steroids but not GnRH analogues act on hypothalamus. Bold arrows indicate stimulation and thins arrows indicate synthesis. Block arrows indicate receptor activation. Reproduced with permission from (Sharifi & Auchus, 2012).

Inherited genetic deficiencies in three genes have shown to impede or completely block normal prostate development. Firstly, mutations in *HSD17B3*, which encodes 17βHSD3, impair T biosynthesis in the fetal testes, resulting in female external genitalia (Andersson et al., 1996). Secondly, mutations in *SRD5A2*, which encodes the dominantly expressed SRD5A isoenzyme in normal prostate, causes pseudohermaphroditism and interfere with normal prostate development (Andersson et al., 1991; Hayward & Cunha, 2000). Thirdly, AR mutations that block activation and transcription – whether these affect the LBD or DBD – also suppress virilisation and normal prostate development (Mcphaul, 1999). These genetic deficiencies yield irrefutable evidence for the requirement of the two biosynthetic steps for the biosynthesis of DHT, and of a nuclear receptor for the normal development of normal prostatic tissue (Sharifi & Auchus, 2012), while emphasizing DHT biosynthesis as a target for the treatment of PCa. Therefore apart from CYP17A1 inhibition, by abiraterone, the inhibition of other relevant enzymes will hamper CPRC progression.

Lifestyle factors also need to be considered in the treatment of PCa as well as CRPC. These factors include: body size, physical activity, cardiovascular disease, smoking and diet (fig. 3.13). Diet may possibly prevent early onset of PCa development or curb the recurrence of PCa, due to possible gene-environment interactions, such as complex biological interactions among steroid

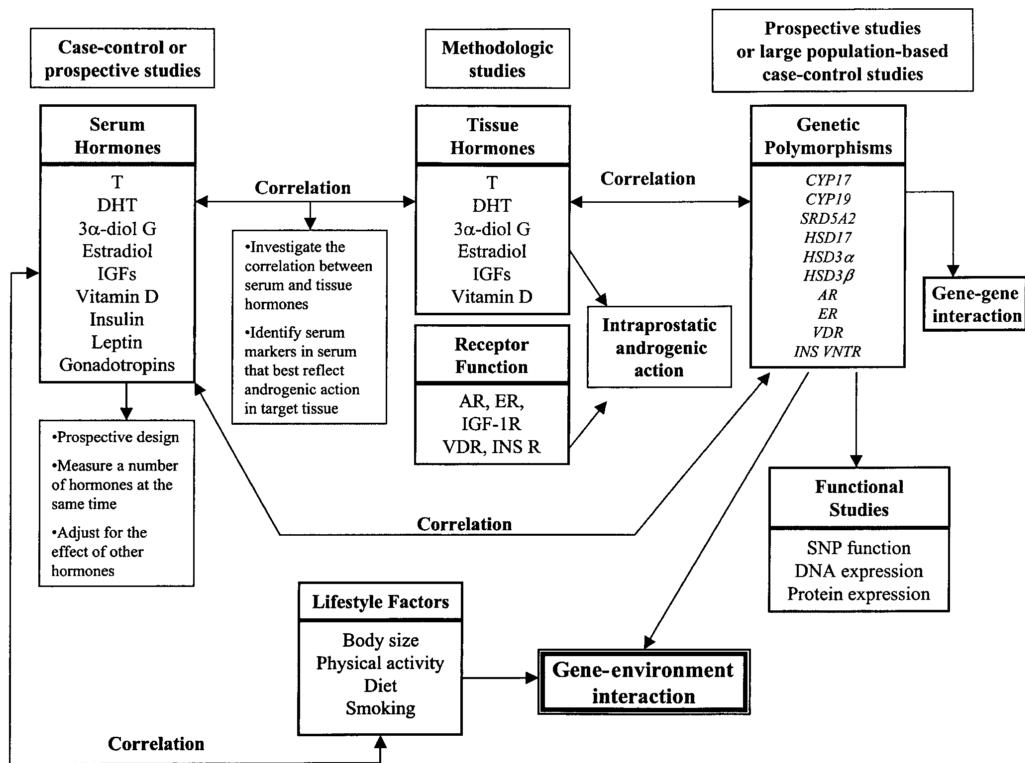hormones, steroid-metabolizing enzymes, receptor proteins, and exogenous factors (Hsing et al., 2002).

Figure 3.13: Possible future research on hormones and PCa. This shows the possible role of *HSD17B*, *SRD5A2* and the *AR* genes have to play in PCa development and the involvement of lifestyle factors, including diet. Reproduced with permission from (Hsing et al., 2002).

Diet and lifestyle interventions in men with early-stage PCa have been shown to decrease PSA levels as well as the rate at which PSA levels increase. These interventions therefore provide some evidence that comprehensive lifestyle changes may have therapeutic potential in early PCas (Ornish et al., 2001; Ornish et al., 2005; Saxe et al., 2006). Following castration, a therapeutic approach employing AR antagonists and enzyme inhibitors combined with lifestyle changes may thus lend itself to greater success in the treatment of CRPC development and progression.

3.9 Summary

It is clear that the development of the prostate is controlled by steroid hormones with intricate pathways mediating steroid biosynthesis utilizing complex enzyme machinery to drive cell proliferation and growth. In the transition from PCa to CRPC, specific enzymes are up-regulated, the implication of which is evident in the survival benefit conferred by obstructing DHT biosynthesis through the inhibition of upstream enzymes. These findings constitutes the strongest clinical evidence implicating an important role of enzymatic changes in CRPC at the current time (Sharifi & Auchus, 2012). Although to date, certain treatment strategies employed are targeting enzymes, thus reducing active androgens, together with AR antagonists, CRPC remains a devastating

disease, and more advanced treatment is required. A therapeutical approach combining clinical treatment strategies targeting PCa and its progression with dietary and lifestyle changes may potentially have enhanced therapeutic applications. In this scenario, it may be possible to include rooibos in a dietary approach. As was discussed in the previous chapter, rooibos and polyphenol compounds have been shown to have anti-carcinogenic and antioxidant activities. In addition, rooibos has been shown to have an effect on steroid hormone dependent cancers, including PCa, in which polyphenols possibly exert androgenic or anti-androgenic effects via receptors.

Owing to the central role prostatic enzymes play not only in the activation of steroids from the precursor androgen pool but also in the progression of PCa and the recurrence of PCa, it is thus important to investigate the effect of rooibos on 17βHSD and SRD5A enzymes which have been shown to play a prominent role in PCa in order to elucidate the anti-carcinogenic properties of rooibos. This investigation will be presented in chapter 4.

Chapter 4

An investigation into the influence of rooibos on 17βHSD and SRD5A enzymes

4.1 Introduction

The production of active androgens in the prostate, is key to cell growth and proliferation, in both normal and PCa tissue (Sharifi & Auchus, 2012). Active androgens activate AR-dependent transcription subsequent to binding the AR and translocating into the nucleus. Androgen metabolism in the prostate is initiated by the uptake of circulating T from the testes, or by the uptake of circulating DHEA, A4 and T from the adrenals. DHEA is metabolised to A4 by 3βHSD2 and the interconversion of A4 and T is subsequently catalysed by 17βHSDs (fig. 4.1 a). SRD5A1 and SRD5A2 present in the prostate, convert both A4 and T to their 5α-reduced derivatives, with the preferred conversion of A4 to 5α-dione occurring in the 'alternative' 5α-dione pathway (Chang et al., 2011; Sharifi, 2012), followed by the conversion of 5α-dione to DHT by 17βHSD3 or AKR1C3. DHT, the most potent androgen known to date, may bind the AR and activate AR-driven genes, or inactivating enzymes may metabolise DHT to 3α/β-adiol and DHT-G, respectively (Penning, 2010).
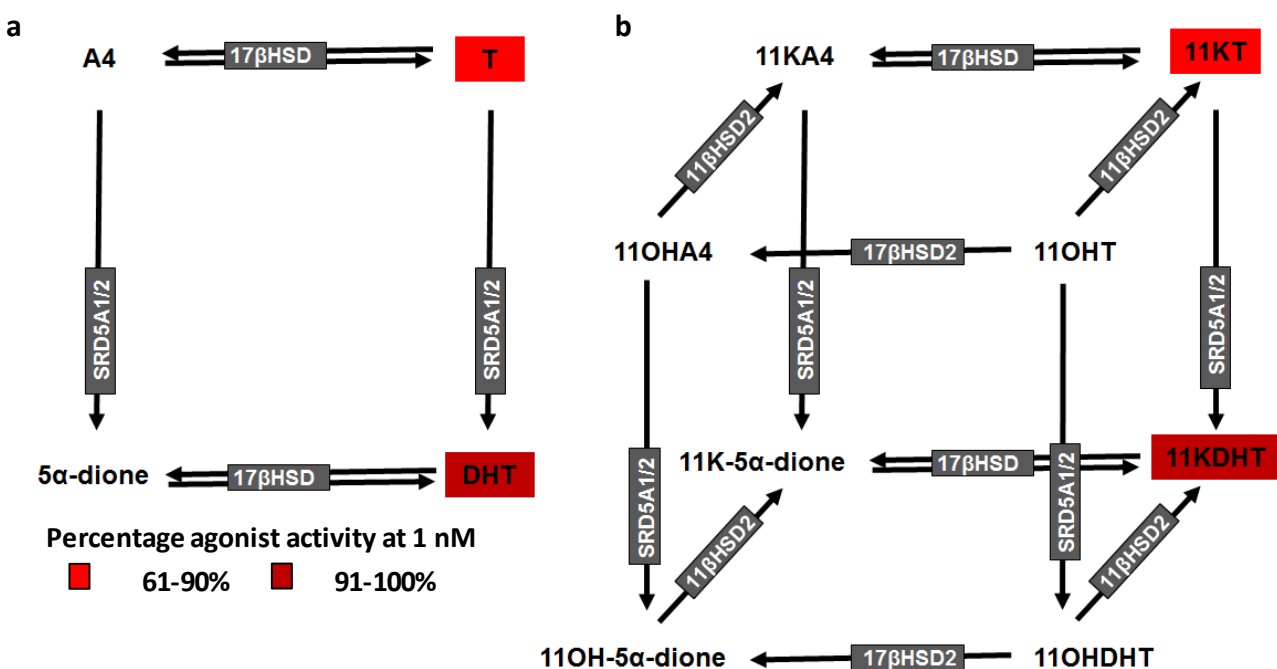

Figure 4.1: Prostate androgen metabolism in (a) the C19 pathway; and (b) the 11OHA4 pathway. Boxed enzymes common to both pathways and shading indicates AR transactivation relative to that of 1 nM DHT.

The 11OHA4 pathway which also contributes to prostate androgen metabolism was recently identified (Storbeck et al., 2013). While 11OHA4 is one of the major C19 steroid hormones

produced by the adrenal (Rege et al., 2013), this pathway is also initiated by the uptake of other circulating adrenal androgens, 11KA4, 11OHT and 11KT. Together with the peripheral interconversion of 11OHA4 and 11KA4 as well as 11OHT and 11KT by 11βHSD, the conversion of these C11-hydroxy metabolites may also be catalysed by 11βHSD2 in the prostate (Swart et al., 2013a). Similar to the C19 pathway, these metabolites may be further metabolised by SRD5A and 17βHSD (fig. 4.1 b). Once the 5α-reduced steroid metabolites are produced, their metabolism by 17βHSD and 11βHSD2 mimic that of their Δ^4 parent compounds, and comparable to the 5α-dione pathway, both 11OHA4 and 11KA4 are preferred substrates for SRD5A. 11KT and its 5α-reduced form, the novel steroid 11KDHT, have been shown to have androgenic activity, with 11KDHT and 11KT possessing agonist activity towards the AR comparable to that of DHT and T, respectively (fig. 4.1 a/b) (Storbeck et al., 2013).

The peripheral metabolism of A4 to DHT, via the conventional C19 and 'alternative' 5α-dione pathways, relies on the activity of 17βHSD and SRD5A (Chang et al., 2011) as does the peripheral metabolism of 11KA4 to 11KDHT via the 11OHA4 pathway. The 17βHSD enzymes act on androgens at their C17 position on the steroid nucleus, thereby modulating their potency, by allowing the interconversion between the active 17β-hydroxysteroids and less active 17-ketosteroids (Gianfrilli et al., 2014). Both 17βHSD3 and AKR1C3 function primarily as reductases and convert the androgen precursor, A4, to T (Mindnich et al., 2004). Both 17βHSDs are expressed in normal and cancerous prostatic tissue, with reported up-regulation of AKR1C3 as PCa progresses (Fung et al., 2006; Lin et al., 2004; Luu-The et al., 2008; Mitsiades et al., 2012; Montgomery et al., 2008; Penning et al., 2006; Stanbrough et al., 2006). 17βHSD2, an oxidative 17βHSD, converts the reverse reaction of 17-ketosteroids to 17β-hydroxysteroids, and is expressed in BPH (Gianfrilli et al., 2014) and reported in PCa cells, with the conversion of oestradiol to oestrone reported in LNCaP and PC-3 cells (Miettinen et al., 1996). SRD5As are enzymes that act upon C19 and C21 steroids that have double bonds between C4 and C5 on the steroid nucleus (Soronen et al., 2004), and thereby irreversibly reduce the double bonds of steroids, producing 5α-reduced steroids. Both isoforms SRD5A1 and SRD5A2 are expressed in peripheral tissue, such as the prostate and skin, with the up-regulation of SRD5A1 reported in PCa (Chang et al., 2011; Stanbrough et al., 2006; Titus et al., 2005).

In recent years, diet and lifestyle interventions in men, with early-stage PCa, have been considered as a possible treatment strategy against PCa progression (Ornish et al., 2008). This has led to the attention being placed on the influence of, amongst others, polyphenolic compounds on steroidogenesis. Flavonoids are phenolic compounds characterized by their diaryl nucleus – mimicking the chemical structures of natural steroid hormones, hence the scientific interest in these compounds, and their potential application in the prevention of hormone-dependent cancers. The anti-cancer properties of flavonoids were already reported in 1980 when a significant reduction of mammary cancer by X-irradiation was demonstrated in rats fed a raw soybean diet. The study

reported 74% of the control rats, on a casein diet, developed tumours while only 44% of the experimental rats developed tumours (Troll et al., 1980). Furthermore, it has been shown in 83 PCa patients that a higher intake of phytoestrogens, including isoflavones and flavonoids, had a moderate protective effect on PCa risk (Strom et al., 1999). Rooibos, a polyphenol-rich herbal tea produced from the South African fynbos plant *Aspalathus linearis*, has been previously shown to inhibit the steroid production in an adrenal cancer cell line and the catalytic activity of key enzymes involved in adrenal steroidogenesis, with specific rooibos flavonoids contributing to the inhibitory effects (Perold, 2009; Schloms et al., 2012; Schloms & Swart, 2014). As rooibos affects steroidogenic enzymes, rooibos may impact hormone driven cancers such as PCa, since PCa development is dependent on steroid hormone biosynthesis and subsequent steroid receptor activation. Particularly, the skin expresses steroidogenic enzymes, and is capable of *in situ* steroid biosynthesis (Slominski et al., 2007), with rooibos reported to slow down tumour growth and to decrease skin cancer tumour size (Marnewick et al., 2005). This anti-cancer effect may be linked to the inhibition of steroidogenic enzymes, possibly contributing to the mechanism of action. Although previous studies have investigated the inhibitory effect of rooibos on adrenal enzyme activities, the influence of rooibos on steroidogenic enzymes expressed in the prostate have not been addressed.

The aim of this study was to determine the influence of rooibos on androgen biosynthesis catalysed by 17βHSD3, AKR1C3, 17βHSD2, SRD5A1 and SRD5A2, in transiently transfected CHO-K1, HEK293 and U2OS cell systems. In this study, the effect of rooibos on the conversion of A4 and 11KA4 by 17βHSD3 and AKR1C3 was investigated with 11OHA4 not being included as the steroid is not readily metabolised by either of the two enzymes (Storbeck et al., 2013; Swart et al., 2013b). The conversion of T, 11KT and 11OHT by 17βHSD2 to their respective A4-metabolites in the presence of rooibos was also investigated. The 5α-reduction of A4 and T by SRD5A1 and SRD5A2, a prerequisite for the activation of androgens, was investigated in the presence of rooibos to determine the influence of rooibos on adrenal androgens activation. The metabolism of A4 and T was subsequently also assayed in PNT2 prostate cells in the presence of rooibos. Endogenous levels of SRD5A are greater than those of the 17βHSD isoforms, allowing analyses of 5α-reduction of androgens in normal prostate cells in the presence of competing enzymes. In contrast, BPH-1 cells express higher 17βHSD levels compared to PNT2 cells, and therefore A4 metabolism was assayed in BPH-1 cells to investigate the influence of rooibos on the metabolism of the steroid. In addition, the 17βHSD catalysed conversion of 11KA4 to 11KT was assayed in LNCaP, PC-3, BPH-1 and PNT2 prostate cells, in the presence of rooibos.

4.2 Materials and methods

4.2.1 Materials

Unfermented rooibos plant material was provided by the South African Rooibos Council (Rooibos LTD-BPK, Clanwilliam, South Africa). Steroid metabolites (T, A4, DHT, 5α-dione, AST and 3α-adiol), Dulbecco's modified Eagle's Medium (DMEM), β-glucuronidase (Type VII-A from *E.coli*; 5,292,000 units/g), Ham's F12K medium and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dutasteride was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). 11KA4, 11KT, 11OHA4 and 11OHT were purchased from Steraloids (Wilton, USA). Nucleobond® Midiprep DNA isolation kits were purchased from Macherey-Nagel (Duren, Germany) and Corning®CellBIND® Surface 24-well plates were purchased from Corning® Life Sciences (NY, USA). CHO-K1 cells were a gift from Dr. Antonio Serafin (University of Stellenbosch, Tygerberg, South Africa), U2OS cells were donated by William E Rainey (University of Michigan, Ann Harbor, USA) and HEK293 cells were obtained from the American Type tissue Culture Collection (Manassas, VA, USA). PNT2, LNCaP and PC-3 cells were obtained from the Sigma's European Collection of Cell Cultures (St. Louis, USA) and BPH-1 cells were a gift from Simon W Hayward (Vanderbilt University of Medical Center, Nashville, USA). Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA) and XtremeGene HP® DNA transfecting reagent was purchased from Roche Diagnostics (Mannheim. Germany). Penicillin-streptomycin, fetal calf serum, trypsin-EDTA and phosphate buffered saline (PBS) were obtained from Oxoid limited (Hampshire, England). Deuterated cortisol (9,11,12,12-D4-cortisol) and deuterated testosterone (Testosterone 1,2-D2, 98%) were purchased from Cambridge isotopes (Andover, MA, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL, USA) and trypan blue stain (0.4 %) together with cell count plates were purchased from Invitrogen (Eugene, USA). pH indicator strips (non-bleeding) were obtained from Merck (Darmstadt, Germany) and all other chemicals were of the finest quality and supplied by reliable scientific supply houses.

4.2.2 Plasmid constructs

17βHSD3 pcDNA3, AKR1C3 pcDNA3 and 17βHSD2 pcDNA4_mycHis_B, plasmid constructs were obtained from Prof J. Adamski (Institute of Experimental Genetics, Neuherberg, Germany). SRD5A1 and SRD5A2 pCMV7 plasmid constructs were a gift from Prof D.W Russell (Southwestern Medical School, University of Texas, Dallas, USA). The pCINeo plasmid was available for use in the laboratory.

4.2.3 Methanol extraction of unfermented rooibos

A rooibos extract was prepared by extracting 20 g unfermented plant material with chloroform (200 mL) for 8 h, followed by a methanol (200 mL) extraction for 8 h using a Soxhlet extractor fitted with a double-wall condenser as previously described (Schloms et al., 2012). The extract was dried at reduced pressure in a rotating evaporator and the vacuum released under nitrogen. The dried extract was redissolved in analytical quality deionised water (Milli-Q™ water purification system), to a final concentration of 86 mg extract/mL and centrifuged at 10 000 x g for 5 min at 4°C. The supernatant was stored in 1 mL aliquots at -20°C.

4.2.4 Identification and quantification of rooibos polyphenols

Unfermented rooibos extracts were analysed using HPLC coupled to a diode-array-detector (HPLC-DAD) (Agilent 1200 series instrument) to identify and quantify polyphenolic compounds, as previously described (Beelders et al., 2012). The following standards were included: aspalathin, nothofagin, 3, 4 dihydroxybenzoic acid, caffeine acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quercetin dehydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and p-coumaric acid. Separation was achieved using a mobile phase consisting of (A) 2% acetic acid in water (v/v) and (B) acetonitrile. The instrument was equipped with a diode-array detector (standard 13 µL flow cell, 10mm path length) controlled by Chemstation software (Agilent Technologies).

4.2.5 Enzymatic assays in transiently transfected HEK293, CHO-K1 and U2OS cells

Cells were grown in culture medium supplemented with 10% fetal bovine serum, 1.5 g $NaHCO_3$/L (pH 7), and 1% penicillin-streptomycin at 37°C, 5.0% CO_2 and 90% relative humidity. HEK293 and U2OS cells were grown in supplemented DMEM medium and CHO-K1 cells in supplemented Ham's F12K medium. After 3-5 passages confluent cells (80%) were plated into 24-well Corning® CellBIND® plates (2 x 10⁵ cells/mL, 0.5 mL/well) and incubated for 24 h. The cells were subsequently transiently transfected with the appropriate plasmid constructs, 0.5 µg, using the Mirus TransIT®-LT1/XtremeGene HP® DNA transfection reagent according to the manufacturer's instructions. pCIneo (Promega, Madison, USA), containing no insert cDNA, was included as a negative control in experiments. The cells were incubated for 72 h, after which 1 µM of the appropriate steroid substrates was added to the medium. Steroids dissolved in absolute ethanol (2 mg/mL) were diluted in culture medium to the desired concentration. Steroid substrates were also added to the cells in the presence of unfermented rooibos extracts (4.3 mg/mL). Control assays included steroid conversion assays carried out in the presence of an inhibitor, dutasteride (10 µM), for SRD5A catalysed enzyme assays. After the appropriate incubation period medium (500 µL) was removed and the steroid metabolites were extracted by liquid-liquid extraction using 10:1 volume of dichloromethane to culture medium. The samples were vortexed for 10 min, centrifuged at 3500 rpm for 5 min after which the media was aspirated and dichloromethane phase dried at

45°C under nitrogen. The dried steroid residue was redissolved in 150 μL methanol prior to analysis by UPLC-MS/MS.

4.2.6 Steroid conversion assays in PNT2, BPH-1, LNCaP and PC-3 cells

PNT2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1.5 g NaHCO$_3$/L (pH 7), 1% penicillin-streptomycin at 37°C, in an atmosphere of 90% humidity and 5% CO$_2$. After 3-4 passages confluent cells were plated into 24-well plates (2 x 10^5 cells/mL, 0.5 mL/well) and incubated for 48 h. The medium was removed and replaced with 0.5 mL RPMI-1640 medium containing steroid substrates, A4, T and 11KA4 (1 μM). Steroids in absolute ethanol (2 mg/mL) were diluted in culture medium to the desired concentration. Steroid substrates were also added to the cells in the presence of unfermented rooibos extracts (4.3 mg/mL). The cells were incubated for 48 h after which 0.5 mL aliquots were removed and steroid metabolites extracted, after β-glucuronidase treatment (4.2.9), as described above. Cortisol-D4 (15 ng) and testosterone-D2 (15 ng), were added to each sample as internal standards. Control assays included steroid conversion assays carried out in the presence of dutasteride (10 μM). BPH-1 (growth and experimental medium, RPMI-1640), PC-3 (growth and experimental medium, Ham's F12K) and LNCaP (growth and experimental medium, RPMI-1640) cells were cultured and replated using the same experimental protocols. Steroid conversion assays were also conducted in the same manner as the assays conducted in PNT2 cells.

4.2.7 Cell viability

The trypan blue exclusion assay was used in this study to assess the influence of unfermented rooibos extracts on cell viability. Polyphenolic compounds have been shown to interfere with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide in the MTT cell viability assay which is commonly used to assay cell viability (Wisman, Perkins, Jeffers, & Hagerman, 2008). In order to exclude the possible interference by the polyphenolic compounds found in the rooibos extracts, a trypan blue exclusion method was thus used which allows the analysis of live cells, non-viable cells and total cells and expresses the % of viable cells in the total number of cells counted. Following steroid conversion assays in cells trypsin-EDTA (0.5 mL) was used to collect the cells. The cells of each well were pipetted into individual micro-centrifuge tubes and centrifuged at 13,000 x g for 10 min using a bench-top centrifuge (Heraeus^{TM} Biofuge^{TM}, Thermo Scientific, USA). The trypsin-EDTA was aspirated and the cell pellet was redissolved in PBS (10 mM phosphate buffer, pH 7.4, containing 137 mM NaCl and 2.7 mM KCL) (250 μL). Cell counts were subsequently carried out, with 10 μL of cell solutions to which 10 μL trypan blue stain were added, using the Countess® automated cell counter (Invitrogen, Eugene, USA).

4.2.8 Protein determination

On the completion of steroid conversion assays, cells collected in PBS, were centrifuged at 13,000 x g for 10 min using a bench-top centrifuge (Heraeus™ Biofuge™, Thermo Scientific, USA), the PBS aspirated, the cells lysed with 50 µL passive lysis buffer (Promega, Madison, USA) and stored at -20°C overnight, prior to determining the protein concentration. Samples included the bovine serum albumin (BSA) protein standards 1 mg/mL, 0.75 mg/mL and 0.5 mg/mL prepared in PBS, together with the same BSA standards prepared with rooibos extract 1 mg/mL and 4.3 mg/mL (according to the manufacturer's instructions). PBS was added to BSA standards to compensate for the volume in which rooibos was added. Samples were analysed using the standard BSA curve generated by the Pierce BCA method (Pierce Chemical, Rockford, USA) according to the manufacturer's instructions.

4.2.9 β-glucuronidase assay

Utilization of UPLC-MS/MS to separate and detect steroids allows only the identification of unconjugated or free steroids. Therefore, in order to analyse total (free + conjugated) steroids present in the media, a β-glucuronidase assay was conducted, according to the manufacturer's instructions. Following steroid conversion assays in prostate cells, aliquots (0.5 mL) were collected and the pH adjusted to 6.5 by the addition of 1% acetic acid (± 20 µL). The samples were subsequently treated with 400 units (76 µL) of β-glucuronidase (*E.coli* Type VII-A; Sigma-Aldrich) at 37°C for 24 h to deconjugate steroids prior to liquid-liquid extraction, as described above.

4.2.10 Separation and quantification of steroid metabolites using UPLC-MS/MS

Stock solutions of A4, 11OHA4, 11KA4, T, 11OHT, 11KT, DHT, 5α-dione, AST and 3α-adiol were dissolved in ethanol (2 mg/mL). A series of standards (0.2 - 20 ng/mL) were prepared in methanol using stock solutions.

Steroid metabolites were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP column (2.1 mm x 100 mm, 2.6 µm) (Torrance, CA, USA) as previously described (Storbeck et al., 2013). Briefly, the mobile phases consisted of 1% formic acid (A) and 49%: 49%: 2% methanol: acetonitrile: isopropanol (B). Steroids were eluted at a flow rate of 0.4 mL/min and the injection volume was 5 µL and the total run time was 5 min per sample injection. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection and all steroids were analysed in multiple reaction monitoring (MRM) mode. An electrospray in the positive ionization mode (ESI+) was utilized in the MRM mode. The capillary voltage of 3.5 kV, cone voltage of 15-35 V and collision energy of 4-36 eV was set during this quantification. The following settings were used: source temperature 120°C, desolvation temperature 400°C, desolvation gas 900 L h^{-1} and cone gas 50 L h^{-1}. Calibration

curves were constructed using weighted (1/$x2$) linear least squares regression and the data was collected with the MassLynx 4.1 software program.

4.2.11 Statistical analysis

All experiments were performed in triplicate and the subsequent results are given as means ±SEM. Statistics were calculated by an unpaired *t*-test or by a one-way ANOVA, followed by a Dunnett's multiple comparison test using GraphPad Prism (version 5) software (GraphPad Software, San Diego, California). Differences were considered statistically significant at $P<0.05$.

4.3 Results

Although it has been firmly established by earlier studies that rooibos inhibits adrenal steroidogenic enzymes and modulates adrenal steroidogenesis, effects of rooibos on steroid metabolism in the prostate has not been investigated.

4.3.1 Analysis of methanolic extracts of unfermented rooibos

The methanolic extract of unfermented rooibos used in *in vitro* experiments was analysed for polyphenols using HPLC-DAD as previously described (Beelders et al., 2012). Quantification of the major polyphenolic compounds (table 4.1) showed that, per gram of unfermented rooibos plant material, the dihydrochalcones were the most abundant flavonoids present in the extract, with aspalathin being ±5-fold higher than nothofagin. Isoorientin and orientin levels were higher than vitexin and isovitexin. The rutin isomer, quercetin-3-O-robinobiocide was present at levels 2-fold higher than rutin.

Table 4.1: Major polyphenolic compounds (µg) present in the methanolic extract of unfermented rooibos plant material and in the extract administered to cells (per mL media and µM)

Flavonoid compounds	Rooibos[a]	Cells		Cells	
		1 mg/mL	µM	4.3 mg/mL	µM
Aspalathin	20 150.7 ± 424.0	68.5 ± 1.4	151.50	294.7 ± 6.2	651.46
Nothofagin	4255.5 ± 61.8	14.5 ± 0.2	33.17	62.2 ± 0.9	142.62
Isoorientin	2671.5 ± 65.7	9.1 ± 0.2	20.26	39.1 ± 1.0	87.14
Orientin	2043.2 ± 44.7	6.9 ± 0.2	15.50	29.9 ± 0.7	66.65
Quercetin-3-robinobioside	958.0 ± 17.2	3.3 ± 0.1	5.34	14.0 ± 0.3	22.95
Rutin	528.2 ± 7.8	1.8 ± 0.0	2.94	7.7 ± 0.1	12.65
Isovitexin	485.4 ± 59.5	1.7 ± 0.2	3.82	7.1 ± 0.9	16.42
Vitexin	404.9 ± 25.1	1.4 ± 0.1	3.18	5.9 ± 0.4	13.69

Values are expressed as the mean ±SEM (*n*=6).

a) Flavonoids (µg) extracted from 1 g unfermented rooibos leaves.

The final concentration of these compounds ranged from 12 µM to 652 µM in the media to which 4.3 mg/mL rooibos extract was administered to the cells.

4.3.2 The influence of unfermented rooibos extracts on cell viability

Cell viability was determined using the trypan blue exclusion method, following steroid conversion assays in CHO-K1, COS-1, HEK293 and LNCaP cells. The influence of rooibos (4.3 mg/mL) on cell viability of CHO-K1 and COS-1 cells was assayed after 3 h and 8 h, and 3 h and 6 h, respectively (fig. 4.2 a/b). The addition of rooibos did not result in a significant decrease in cell viability, with viability in both cell lines remaining at ±90% in the absence (control) and presence of rooibos extract.
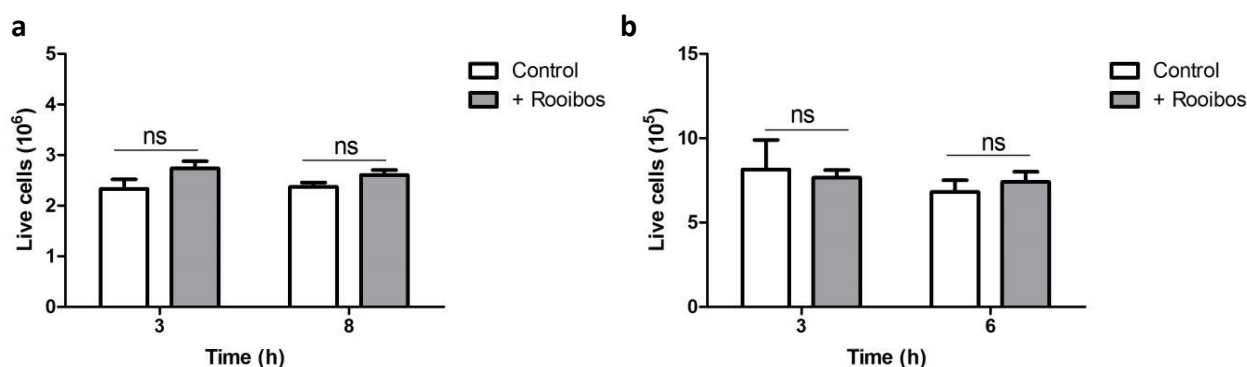

Figure 4.2: Viable cell count in the absence and presence of rooibos: (a) CHO-K1 cells at 3 h and 8 h, and (b) COS-1 cells at 3 h and 6 h, after substrate conversion assays in the absence (control) and presence of rooibos extract (4.3 mg/mL). Results are expressed as the mean ±SEM (a: n=3, b: n=2, ns=not significant).

Following the investigation in CHO-K1 and COS-1 cells, the influence of rooibos (4.3 mg/mL) on cell viability of HEK293 cells were investigated to obtain a time course analysis of a range of experimental time intervals within 48 h (fig. 4.3). At 1 h, 2 h, 4 h and 6 h, the addition of rooibos did not result in a significant decrease in cell viability, with viability detected at ±80%. At 12 h, however, rooibos resulted in a decrease in viability (±10%). Although rooibos did not result in a significant decrease in viability, compared to the control, cell viability had decreased to ±70% after 24 h and 48 h.
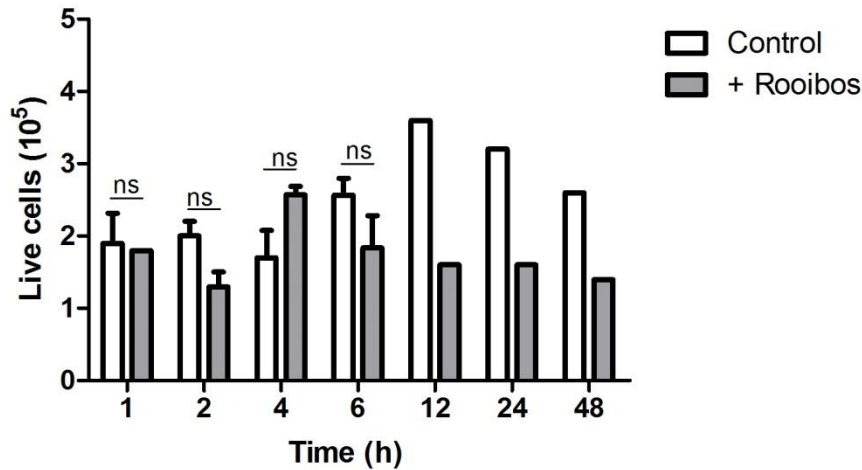
Figure 4.3: Viable HEK293 cell count in the absence and presence of rooibos. Cells were exposed for 48 h to rooibos extract (4.3 mg/mL). Results are expressed as the mean ±SEM (1 h, 4 h, 6 h: n=3, 2 h: n=2, 12 h, 24 h, 48 h: n=1, ns=not significant).

Based on the above results, assays in CHO-K1, COS-1 and HEK293 cells were thus restricted to shorter incubation periods to limit exposure to rooibos. However, steroid conversion assays in prostate cell model systems were undertaken for longer incubation periods, such as 48 h and 96 h, which are commonly used experimental periods, enabling the determination of cellular effects. Therefore, the influence of rooibos on cell viability was determined in LNCaP cells after 48 h and 96 h (fig. 4.4). The addition of rooibos extract (4.3 mg/mL) did not significantly decrease the cell viability after 48 h with viability remaining ±80%. Assays conducted for longer periods also showed that rooibos did not influence cell viability at 1 mg/mL or 4.3 mg/mL which remained at ±80% after 96 h.
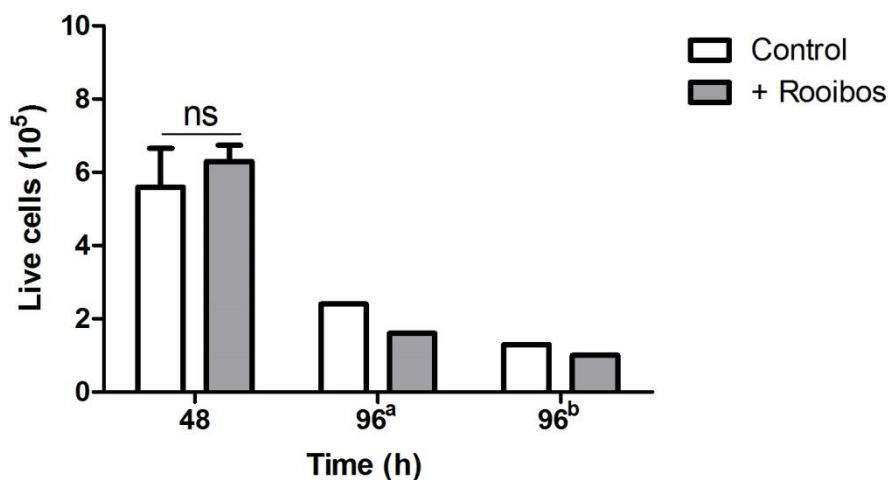

Figure 4.4: Viable LNCaP cell count in the absence and presence of rooibos. Cells were exposed for 48 h to rooibos extract (4.3 mg/mL) and for 96 h to rooibos extract (1 mg/mL[a] and 4.3 mg/mL[b]). Results are expressed as the mean ±SEM (48 h: n=3, 96 h: n=1, ns=not significant).

The influence of unfermented rooibos extracts on protein levels

A protein determination assay was completed using the Pierce BCA method following steroid conversion assays. During the duration of this study multiple protein determination assays were completed, with results showing possible interference of rooibos with the assay (data not shown). Therefore, a control analysis was designed and completed to investigate whether rooibos interferes with the Pierce BCA method. The addition of rooibos (1 mg/mL and 4.3 mg/mL) resulted in a significant increase in protein concentration determined by the method used (fig. 4.5), and rooibos at 4.3 mg/mL interfered significantly more compared to rooibos at 1 mg/mL.
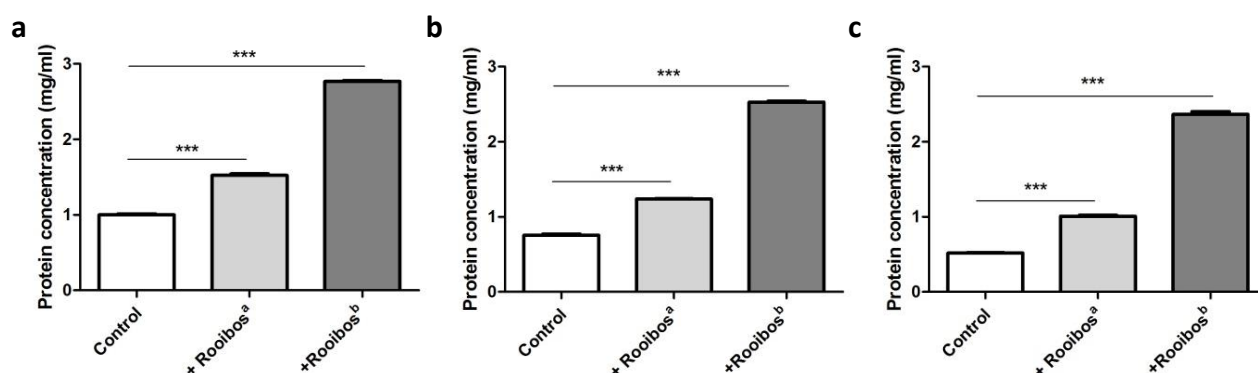

Figure 4.5: Protein determination in the absence and presence of rooibos: (a) BCA protein standard (1 mg/mL); (b) BCA protein standard (0.75 mg/mL); and (c) BCA protein standard (0.5 mg/mL) in the presence of rooibos (1 mg/mL[a] and 4.3 mg/mL[b]). The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired *t*-test. Results are expressed as the mean ±SEM (*** $P<0.001$, *n*=3).

The BSA method determines total protein levels and incorporates proteins from both live and non-viable cells. Cell viability was therefore chosen as a parameter to normalise data to and to determine the effect of rooibos on cell integrity.

Having established the effect of rooibos on cell viability, the effect of rooibos on prostate androgen metabolism, and in particular, on androgen production by 17βHSD3, AKR1C3, 17βHSD2, SRD5A1 and SRD5A2 was subsequently investigated.

4.3.3 UPLC-MS/MS analyses of steroid metabolites

The steroids of interest were analysed by UPLC-MS/MS with the C19 steroids in the conventional C19 pathway (A4, T, 5α-dione, DHT, AST and 3α-adiol) and the internal standard, D2-testosterone (fig. 4.6) and the C19 steroids in the 11OHA4 pathway (11OHA4, 11OHT, 11KA4 and 11KT) together with the internal standard, D2-testosterone (fig 4.7). The separation was achieved in a single chromatographic step, without prior derivatisation, which allowed the efficient separation and quantification of steroids.

Figure 4.6: UPLC-MS/MS chromatographic separation of A4, T, DHT, 5α-dione, AST, 3α-adiol and the internal standard, D2-testosterone are shown. Retention times are indicated on the chromatograms of steroid metabolites (5 µL of a 20 ng/mL standard solution) shown in multiple reaction monitoring (MRM) mode.

The chromatographic separation of A4 and T allowed the analyses of the 17βHSD catalysed reactions, while the separation of T, DHT, A4 and 5α-dione, allowed the analyses of the SRD5A catalysed reactions. In this study the metabolism of the C19 steroids in PNT2 and BPH-1 cells was also investigated and therefore necessitated analyses of the downstream metabolites 3α-adiol and AST produced by AKR1C2 (fig. 4.6).
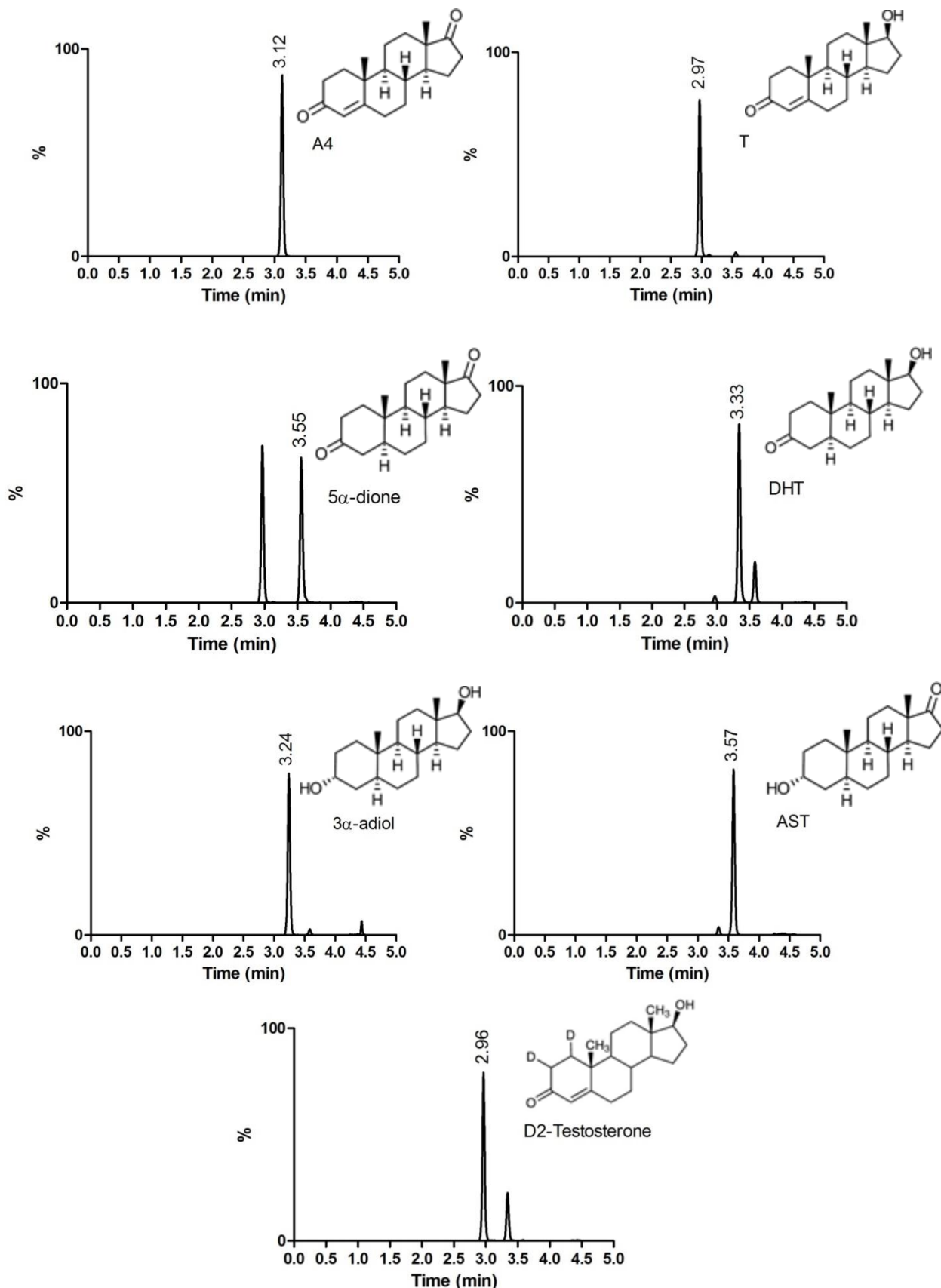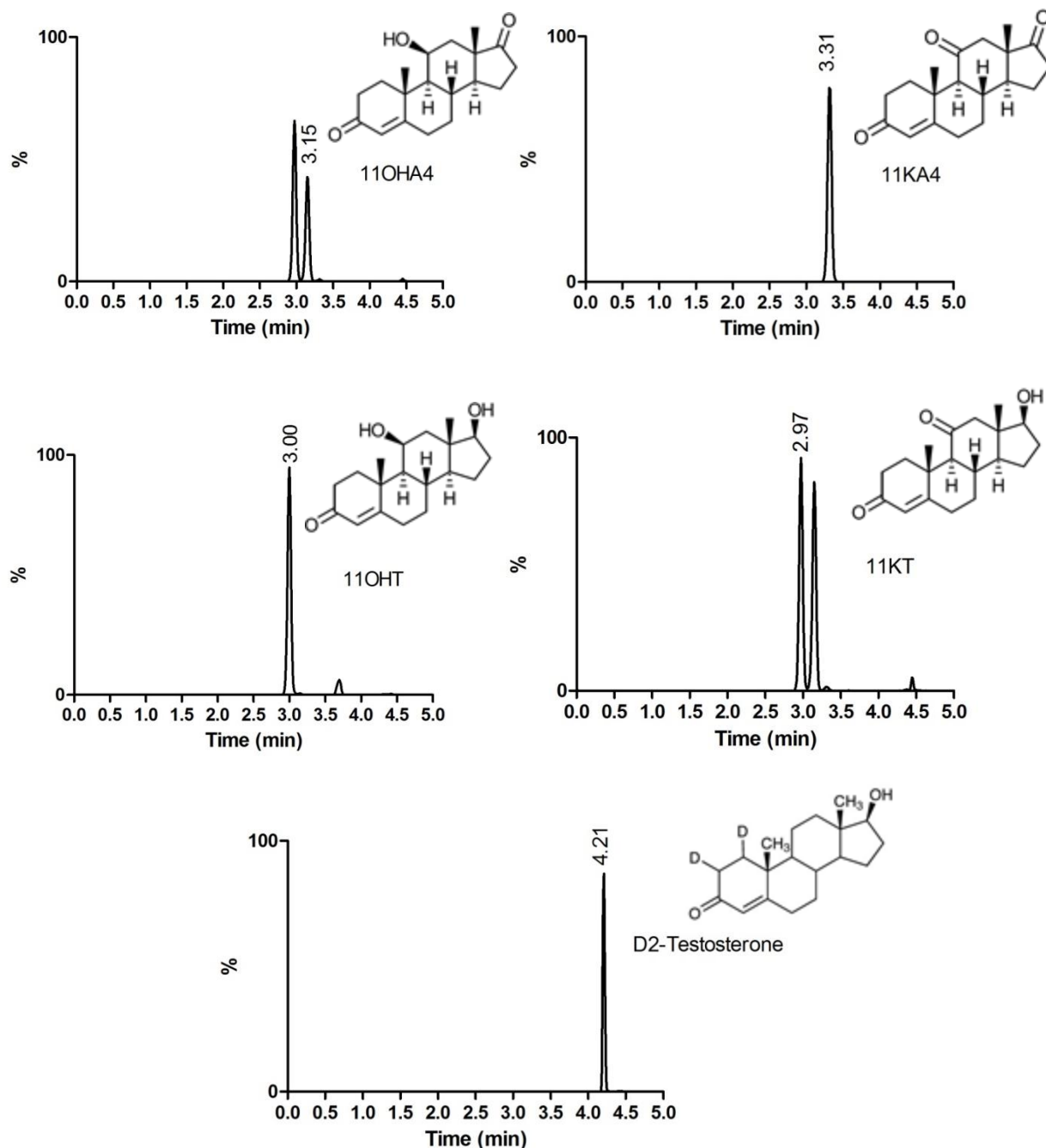

Figure 4.7: UPLC-MS/MS chromatographic separation of 11OHA4, 11KA4, 11OHT, 11KT and the internal standard, D2-testosterone are shown. Retention times are indicated on the chromatograms of steroid metabolites (5 µL of a 20 ng/mL standard solution) shown in multiple reaction monitoring (MRM) mode.

In addition, the quantification of 11OHA4, 11OHT, 11KA4 and 11KT (fig. 4.7) also enabled further analyses of the conversions of the C11-keto and C11-hydroxy derivatives of A4 and T, catalysed by both reductive and dehydrogenase activities of the 17βHSDs. In order to minimize possible cross-talk between steroids the separation of the C19 steroids in the 11OHA4 pathway were analysed on a separate method (7.2.10) to the C19 steroids in the conventional C19 pathway.

4.3.4 The influence of unfermented rooibos extracts on 17βHSD isoforms

Conversion of A4 and 11KA4 in CHO-K1 cells in the presence of rooibos

The investigation of the conversion of A4 and 11KA4 to T derivatives, by 17βHSD3 and AKRIC3 was initially examined in the presence of rooibos extracts (4.3 mg/mL) in CHO-K1 cells. While CHO-K1 cells are considered to be a non-steroidogenic cell model, 17βHSD1 and 17βHSD7 are expressed endogenously. These enzymes function primarily as reductases, converting A4 to T (Peltoketo, Luu-The, Simard, & Adamski, 1999; Y. Zhang, Word, Fesmire, Carr, & Rainey, 1996), and may therefore also catalyse the conversion of 11KA4.

The data shows that in untransfected CHO-K1 cells, 20% A4 was metabolised to T in 8 h (fig. 4.8 a/b), while the conversion of 11KA4 yielded 15% 11KT after 3 h (fig. 4.9 a/b), demonstrating the ability of endogenous 17βHSDs to metabolise the C11-keto C19 steroid. The influence of rooibos on endogenous activity of the 17βHSDs was subsequently assayed (fig. 4.8 a/b and fig. 4.9 a/b). Rooibos resulted in a significant reduction in the production of T and 11KT from A4 and 11KA4, respectively.

Having establishing the endogenous 17βHSD activity in the CHO-K1 cell line, 17βHSD3 and AKR1C3 were expressed in CHO-K1 cells and the catalytic activity towards A4 and 11KA4 assayed. The expression of 17βHSD3 resulted in 35% A4 being converted to T in 8 h (fig. 4.8 a) and 22% of 11KA4 being converted to 11KT in 3 h (fig. 4.9 a). The expression of AKR1C3 yielded comparable results when A4 was assayed and yielded 40% T (fig. 4.8 b), however, AKR1C3 demonstrated significantly greater activity towards 11KA4 than 17βHSD3, resulting in 80% 11KT being produced after 3 h (fig. 4.9 b). Interestingly, 17βHSD3 only catalysed the conversion of 20% 11KA4 (fig. 4.9 a). In the presence of rooibos, significant inhibition (25%) was detected in the levels of 11KT with only 55% 11KT being produced by AKR1C3 after 3 h (fig. 4.9 b). The catalytic activity of 17βHSD3 towards 11KA4 was low and as such the effect of rooibos was detected at negligible levels (±5% inhibition) (fig. 4.9 a). For both 17βHSD3 and AKR1C3 expressed in CHO-K1 cells, ±15% inhibition was detected when A4 was added as substrate in the presence of rooibos (fig. 4.8 a/b).

Figure 4.8: A4, 1 µM, conversion in CHO-K1 cells in the absence (control) and presence of rooibos extract (4.3 mg/mL) after 8 h: (a) expressed 17βHSD3 activity in transiently transfected cells (light grey) and endogenous 17βHSD activity (dark grey); and (b) expressed AKR1C3 activity in transiently transfected cells (light grey) and endogenous 17βHSD activity (dark grey). The experiment was performed in triplicate and the data was compared as indicated, analysed by an unpaired *t*-test. Results are expressed as the mean ±SEM (*$P<0.05$, **$P<0.01$, $n=3$).

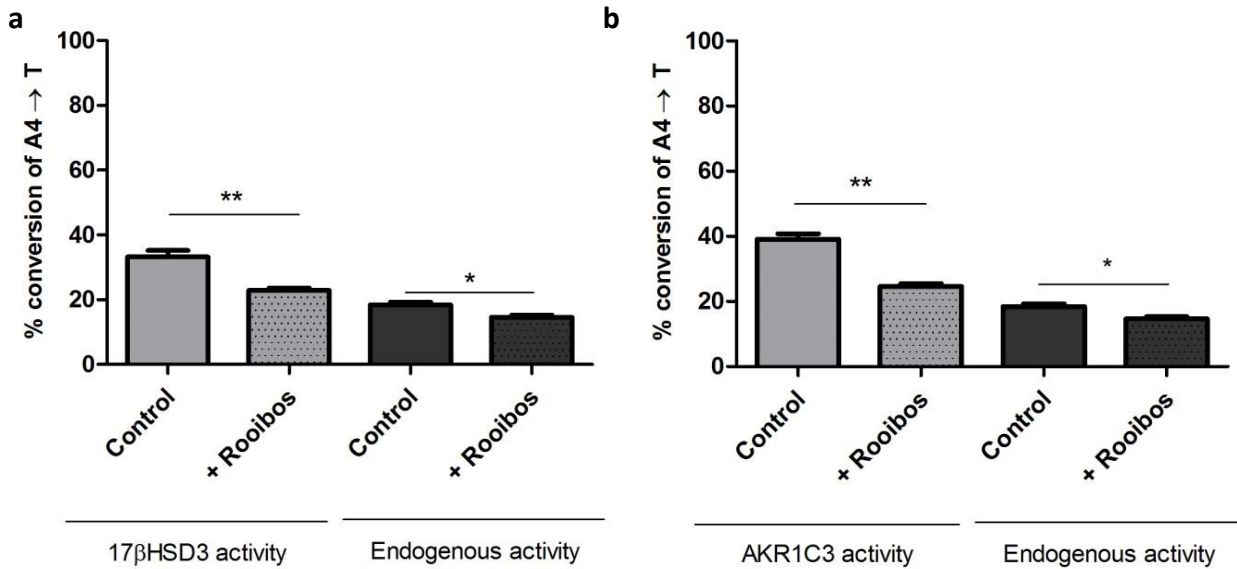**Figure 4.9:** 11KA4, 1 µM, conversion in CHO-K1 cells in the absence (control) and presence of rooibos extract (4.3 mg/mL) after 3 h: (a) expressed 17βHSD3 activity in transiently transfected cells (light grey) and endogenous 17βHSD activity (dark grey); and (b) expressed AKR1C3 activity in transiently transfected cells (light grey) and endogenous 17βHSD activity (dark grey). The experiment was performed in triplicate and the

data was compared as indicated, analysed by an unpaired *t*-test. Results are expressed as the mean ±SEM (*$P<0.05$, ***$P<0.001$, *n*=3).
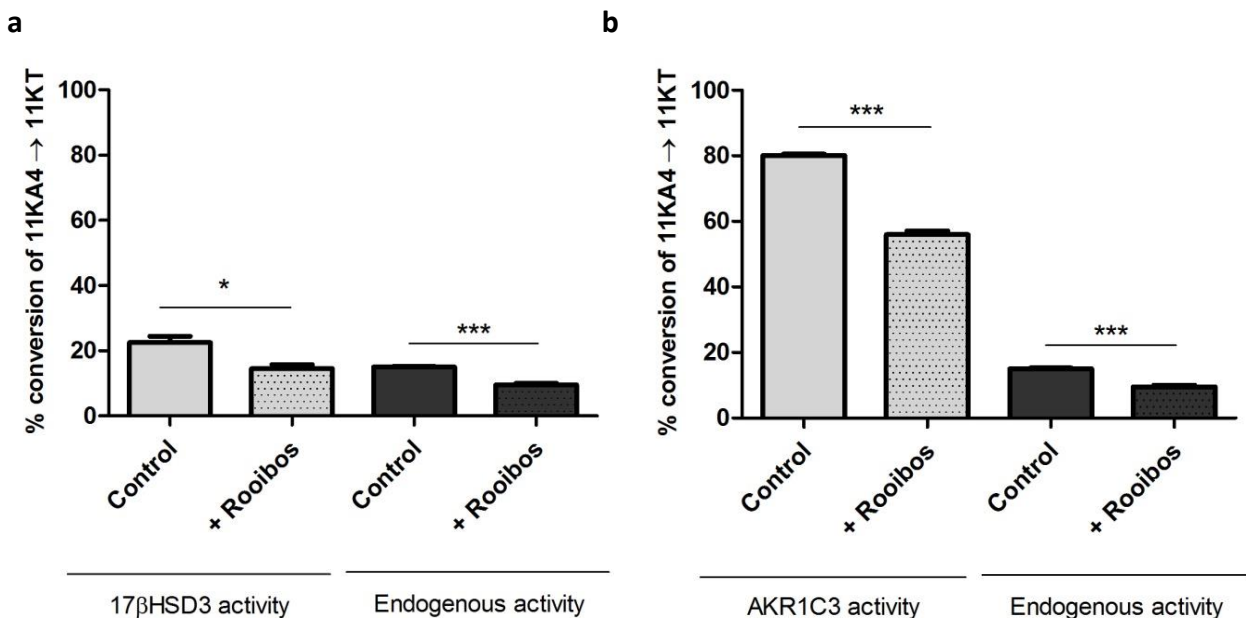
Following the initial studies into the conversion of A4 and 11KA4 to T derivatives, by endogenous and heterologously expressed 17βHSD3 and AKR1C3 in CHO-K1 cells, further investigations were conducted in HEK293 cells, which do not express endogenous 17βHSDs. In order to fully investigate the influence of rooibos on the catalytic activity of 17βHSD3 and AKR1C3 it was necessary to conduct enzymatic assays in cells in which endogenous 17βHSD activity would not interfere with the experimental parameters. Although, endogenously expressed SRD5A2 have been reported in the HEK293 cell model (Panter, Jose, & Hartmann, 2005), a time course analysis of the conversion of A4 and 11KA4 in the presence of rooibos was nevertheless completed in HEK293 cells transiently transfected with 17βHSD3 and AKR1C3. Furthermore, these assays could also indicate possible inhibition of SRD5A2 by rooibos.

<u>Conversion of A4 and 11KA4 in the presence of rooibos in HEK293 cells heterologously expressing 17βHSD3</u>

The influence of rooibos extracts (4.3 mg/mL) on the conversion of A4 and 11KA4 to T and 11KT, respectively, was investigated in HEK293 cells transiently transfected with 17βHSD3. Rooibos inhibited the conversion of A4 to T by 17βHSD3 significantly (fig. 4.10 b), with T levels only reaching 61% after 6 h, compared to 92% A4 conversion detected in the control (fig. 4.10 a). Rooibos resulted in the decreased metabolism of A4 with 50% conversion to T, detected at ±4.5 h in the presence of rooibos compared to 50% conversion to T detected at ±1.8 h in the control assay. Significant inhibition of the conversion of A4 to T by 17βHSD3 was detected over the time period showing efficient modulation of 17βHSD3 activity by rooibos.

Figure 4.10: Time course of A4, 1 µM, conversion to T by (a) 17βHSD3 expressed in transiently transfected HEK293 cells; and (b) 17βHSD3 expressed in transiently transfected HEK293 cells in the presence of rooibos extract (4.3 mg/mL). The experiment was performed in triplicate and is expressed as the mean ±SEM.

No substrate conversion was detected in untransfected HEK293 cells.

The conversion of 11KA4 to 11KT by 17βHSD3 was also significantly inhibited in the presence of rooibos (fig. 4.11 b). A ±33% inhibition resulted in a conversion only reaching 65.7% after 6 h, compared to 98.3% 11KT being detected after 6 h in the control assay (fig. 4.11 a). Rooibos decreased the rate of 11KA4 metabolism to 11KT, as 50% conversion to 11KT was detected at ±4 h in the presence of rooibos, compared to 50% conversion to 11KT detected at ± 1.5 h in the control assay. A significant inhibition of the conversion of 11KA4 to 11KT by 17βHSD3 was detected throughout the assay period, showing similar modulation of 17βHSD3 activity towards 11KA4 by rooibos.
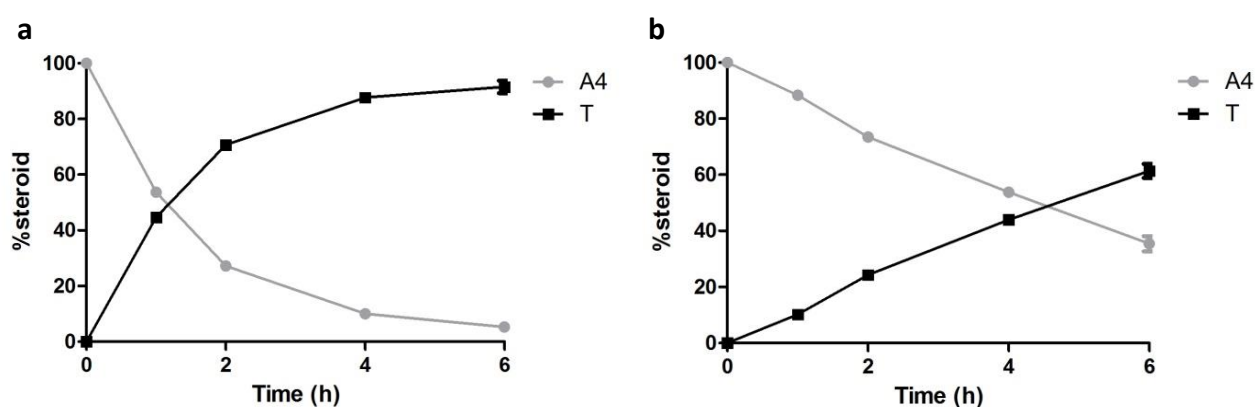

Figure 4.11: Time course of 11KA4, 1 µM, conversion to 11KT by (a) 17βHSD3 expressed in transiently transfected HEK293 cells; and (b) 17βHSD3 expressed in transiently transfected HEK293 cells in the presence of rooibos extract (4.3 mg/mL). The experiment was performed in triplicate and is expressed as the mean ±SEM.

No substrate conversion was detected in untransfected HEK293 cells.

Conversion of A4 and 11KA4 in the presence of rooibos in HEK293 cells heterologously expressing AKR1C3

The influence of rooibos extracts (4.3 mg/mL) on the conversion of A4 and 11KA4 to T and 11KT, respectively, was investigated in HEK293 cells transiently transfected with AKR1C3. In initial A4 conversion assays in HEK293 cells, low T levels were detected even after 24 h (data not shown). The conversion of A4 was therefore assayed for a longer period, up until 48 h. As the investigations above (4.3.2) showed cell viability to be compromised during long incubation periods, the data presented below were normalised. In the presence of rooibos, the conversion of A4 to T by AKR1C3 was significantly inhibited, with the levels of T formation at ±19% after 48 h in the presence of rooibos (fig. 4.12 b), compared to ±47% in the control assay without rooibos (fig. 4.12 a). These results were normalised to 70% cell viability, compensating for the effect of rooibos on cell viability, clearly indicating a significant inhibition of ±1.5%, ±6%, ±14% and ±28% of AKR1C3 by rooibos at 6 h, 12 h, 24 h and 48 h, respectively.
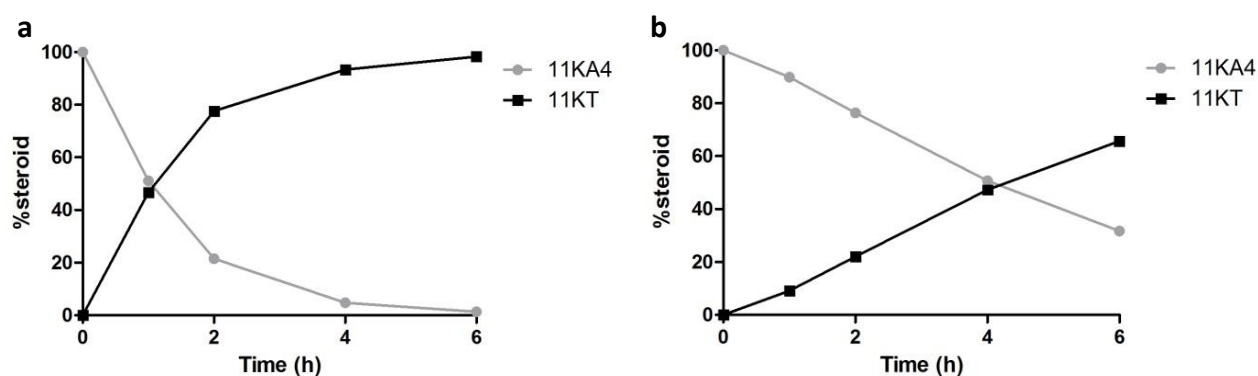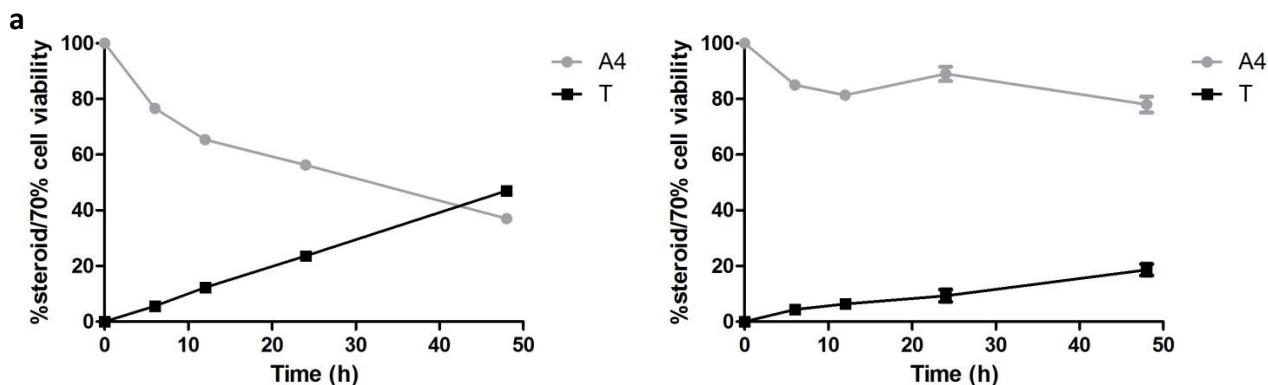
Figure 4.12: Time course of A4, 1 μM, conversion to T by (a) AKR1C3 expressed in transiently transfected HEK293 cells; and (b) AKR1C3 expressed in transiently transfected HEK293 cells in the presence of rooibos extract (4.3 mg/mL). The experiment was performed in triplicate and is expressed as the mean ±SEM (*n*=3).

No substrate conversion was detected in the assay in which HEK293 cells were transfected with pCIneo containing no insert cDNA.

The conversion of 11KA4 to 11KT by AKR1C3 was significantly inhibited (2-fold) in the presence of rooibos (fig. 4.13 b). While 77% of the substrate was converted in the absence of rooibos (fig. 4.13 a), only 36% 11KA4 was converted after 6 h when assayed in the presence of rooibos. Rooibos decreased the rate 11KA4 is metabolised to 11KT, as 50% conversion to 11KT at ±3 h was detected in the control assay, however, in the presence of rooibos 11KA4 did not reach 50% conversion to 11KT. A significant inhibition of the conversion rate of 11KA4 to 11KT by AKR1C3 was detected as observed during the assay period, showing ±11%, ±26%, ±34% and ±42% inhibition of AKR1C3 activity towards 11KA4 by rooibos at 1 h, 2 h, 4 h and 6 h, respectively.
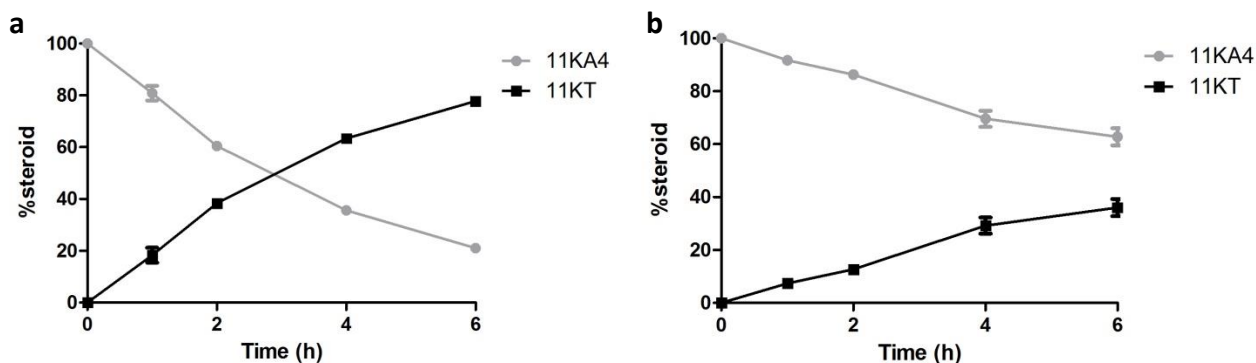
Figure 4.13: Time course of 11KA4, 1 μM, conversion to 11KT by (a) AKR1C3 expressed in transiently transfected HEK293 cells; and (b) AKR1C3 expressed in transiently transfected HEK293 cells in the presence of rooibos extract (4.3 mg/mL). The experiment was performed in triplicate and is expressed as the mean ±SEM (*n*=3).

No substrate conversion was detected in the assay in which HEK293 cells were transfected with pCIneo containing no insert cDNA.

Conversion of T, 11KT and 11OHT in the presence of rooibos in HEK293 cells heterologously expressing 17βHSD2

The influence of rooibos extract (4.3 mg/mL) on the catalytic activity of 17βHSD2 was investigated in HEK293 cells. In the presence of rooibos, the conversion of T, 11OHT and 11KT to A4, 11OHA4 and 11KA4, respectively, by 17βHSD2 was significantly inhibited (fig. 4.14). Results showed ±13% inhibition of T conversion to A4, ±27% inhibition of 11OHT conversion to 11OHA4 and ±36% inhibition of 11KT conversion to 11KA4 after 12 h. Significant inhibition of the conversion of T-metabolites to A4-metabolites by 17βHSD2 show efficient modulation of the catalytic activity of 17βHSD2 by rooibos. Interestingly, these data show that while the lowest inhibition was detected with T as substrate, inhibition of the C11-keto C19 steroid was greater than that of the C11-hydroxy C19 steroid. Furthermore, these results demonstrate the ability of 17βHSD2 to metabolise the C11-keto as well as C11-hydroxy C19 steroids, compared to 17βHSD3 and AKR1C3 which are unable to metabolise the C11-hydroxy C19 steroid, 11OHA4, while readily catalysing the conversion of 11KA4.
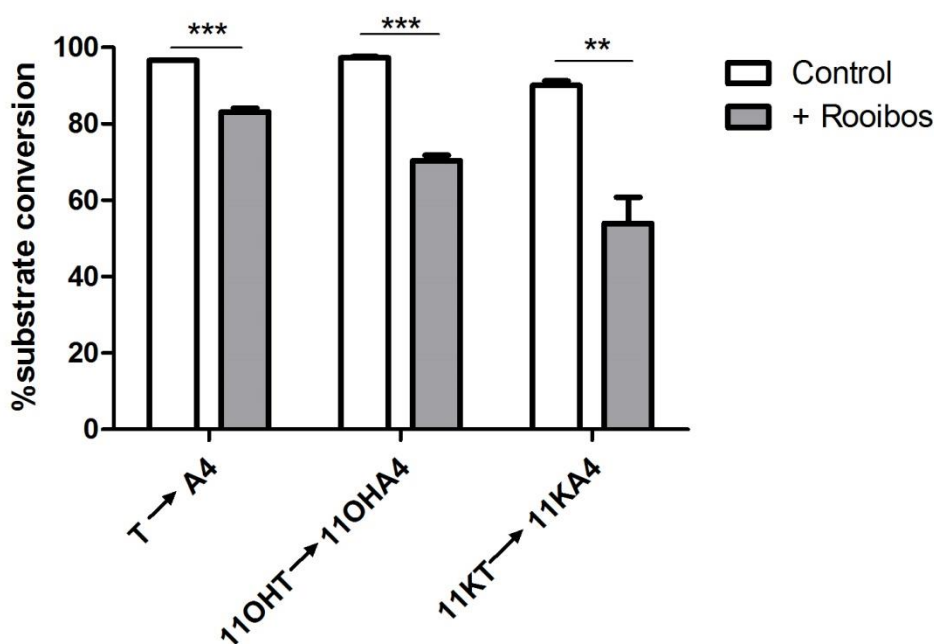
Figure 4.14: T, 11OHT and 11KT, 1 μM, conversions catalysed by 17βHSD2 in transiently transfected HEK293 cells in the absence (control) and presence of rooibos (4.3 mg/mL) after 12 h. The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired t-test. Results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, $n=3$).

No endogenous conversion of T, 11OHT and 11KT in untransfected HEK293 cells were detected.

Endogenous conversions of substrates by SRD5A in HEK293 cells were negligible, and the influence of rooibos on SRD5A could therefore not be determined. SRD5A1 was subsequently transiently transfected in U2OS cells to determine the influence of rooibos on SRD5A1.

4.3.5 The influence of unfermented rooibos extracts on SRD5A isoforms

Conversion of T to DHT in the presence of rooibos in U2OS cells heterologously expressing SRD5A1

The effect of rooibos (4.3 mg/mL) on the catalytic activity of SRD5A1 was determined and compared to that of dutasteride (10 µM), a dual inhibitor of SRD5A1 and SRD5A2. The activity of SRD5A1 towards T (1 µM) was assayed in U2OS cells transiently transfected with SRD5A1. After 4 h, inhibition of T was minimal in the presence of rooibos, with 88.4% DHT being formed compared to the control, in which 92% of the substrate was converted to DHT (fig. 4.15). No DHT was detected in the presence of dutasteride.
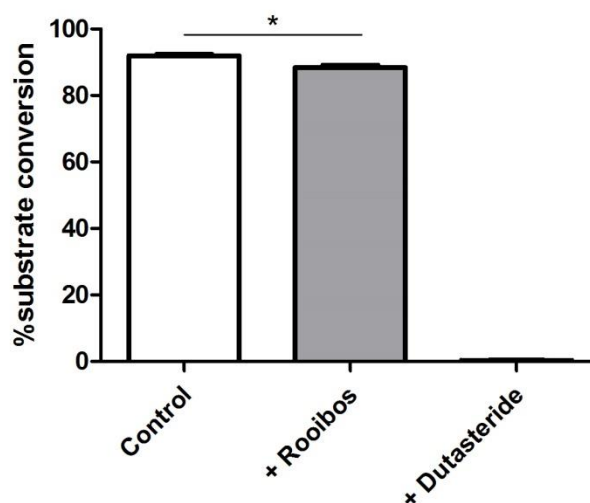
Figure 4.15: T, 1 µM, conversion by SRD5A1 in transiently transfected U2OS cells after 4 h. Assays were conducted in the presence of rooibos extract (4.3 mg/mL), and dutasteride (10 µM). The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired t-test. Results are expressed as the mean ±SEM (*$P<0.05$, $n=3$).

A control assay was included in which U2OS cells were transfected with a vector containing no insert cDNA. No conversion of T to DHT was detected, and minimal conversion of T to A4 (2%) detected. Interestingly, while U2OS cells are reported to be non-steroidogenic, data showed that these cells express endogenous 17βHSD activity, functioning primarily as reductases, converting A4 to T (data not shown).

Following the initial investigation above, the investigation was continued in HEK293 cells, expressing no endogenous 17βHSDs, however, SRD5A2 has been reported to be endogenously expressed in these cells (Panter et al., 2005). The conversion of substrates A4 and T to 5α-reduced derivatives by endogenous SRD5A2 was therefore monitored throughout. In order to investigate the influence of rooibos on the catalytic activity of SRD5As, a time course analysis, was carried out in HEK293 cells transfected with both SRD5A1 and SRD5A2.

Conversion of A4 and T in the presence of rooibos in HEK293 cells heterologously expressing SRD5A1

Enzymatic assays were carried out in transiently transfected HEK293 cells to investigate whether rooibos influences the catalytic activity of SRD5A1 towards A4 and T. After 2 h full conversion of A4 and T to 5α-dione and DHT respectively, was detected, indicative of the efficiency of this enzyme (fig. 4.16 a/b). Rooibos (4.3 mg/mL) did not inhibit SRD5A1 and had no effect on the conversion rate of these substrates over the time course, as the data show 50% substrate conversion at 20 and 30 min in the absence and presence of rooibos for A4 and T conversions, respectively.
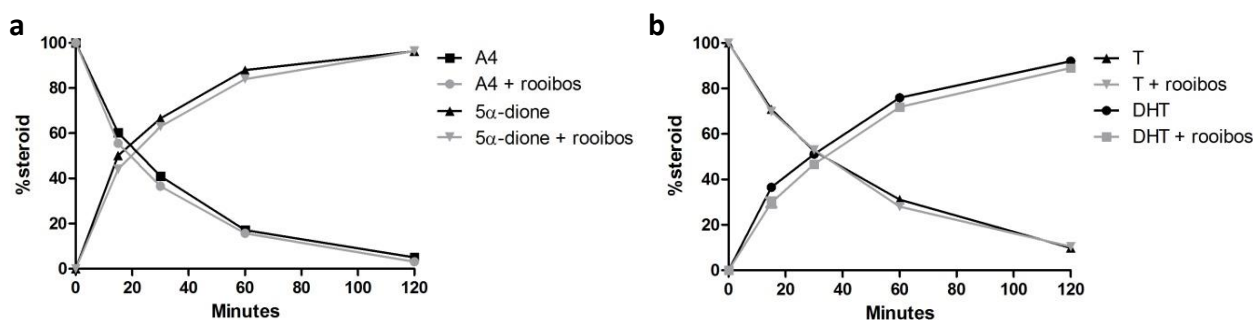
Figure 4.16: Time course of substrate conversion (1 µM) by SRD5A1 in transiently transfected HEK293 cells: (a) conversion of A4 as substrate (control) in the presence of rooibos extract (4.3 mg/mL); and (b) conversion of T (1 µM) as substrate (control) in the presence of rooibos extract (4.3 mg/mL) after 2 h. The experiment was performed in triplicate and is expressed as the mean ±SEM (*n*=3).

The production of 5α-dione and DHT by SRD5A1 in the presence of dutasteride (10 µM) was also assayed. No 5α-dione or DHT were detected after 2 h (data not shown). In untransfected HEK293 cells no significant conversions of A4 and T to 5α-reduced steroids were detected.

Conversion of T in the presence of rooibos in HEK293 cells heterologously expressing SRD5A2

The effect of rooibos was also investigated on SRD5A2 activity towards T, assayed in transiently transfected HEK293 cells. Although rooibos initially appears to inhibit the activity of SRD5A2, inhibition after 2 h is not significant (fig. 4.17 b). The trend observed suggests that rooibos may affect the rate of substrate conversion as 50% T conversion was reached after 90 min in the control assay with the substrate conversion being less than 40% in the presence of rooibos (fig. 4.17 a). This aspect will, however, need to be investigated in future studies.

b

a
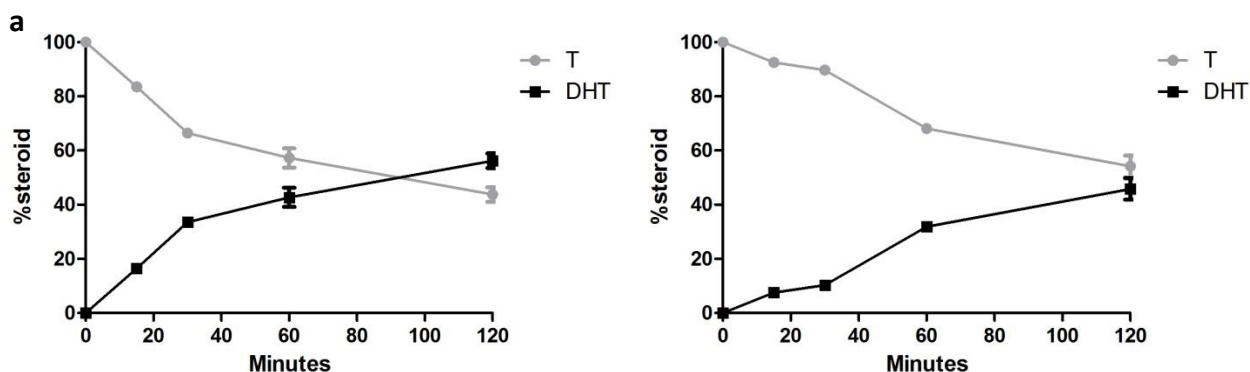
Figure 4.17: Time course of T, 1 µM, conversion to DHT by: (a) SRD5A2 expressed in transiently transfected HEK293 cells; and (b) SRD5A2 expressed in transiently transfected HEK293 cells in the presence of rooibos extract (4.3 mg/mL). The experiment was performed in triplicate and is expressed as the mean ±SEM (*n*=3).

The conversion of T to DHT was also assayed in untransfected HEK293 cells and no SRD5A activity was detected.

4.3.6 The influence of unfermented rooibos extracts on androgen metabolism in prostate cells

A4 and T metabolism in PNT2 cells in the presence of rooibos

The conversion of A4 and T was assayed in PNT2 cells in the presence of rooibos extracts (4.3 mg/mL) to determine the effect on the conversion by endogenous SRD5A in the normal cellular milieu. On completion of the assay metabolites were deconjugated after which total (free + conjugated) steroid metabolites were analysed. After 48 h, 70% of the A4 assayed had been metabolised to downstream products. The major products formed were 5α-dione (0.36 µM) and 0.09 µM AST with 0.3 µM A4 remaining (fig. 4.18). Minimal conversion of A4 to T (0.05 µM) was detected, together with negligible levels of DHT (0.02 µM) and 3α-adiol (0.008 µM) being formed (fig. 4.18). However, the levels of latter two metabolites fell below the limit of quantification (LOQ) and the influence of rooibos on these steroid metabolites could therefore not be assessed. In the presence of rooibos, A4 metabolism yielded similar levels of 5α-dione (0.33 µM), indicating that rooibos did not influence the activity of SRD5A2, with the increased A4 levels indicative of AKR1C3 inhibition. The levels of AST and T were markedly lower, with both being detected at 0.02 µM, indicating that rooibos not only inhibited AKR1C3 but may also have affected the activity of downstream AKR1C2 activity (fig. 4.18).

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Figure 4.18: A4, 1 µM, metabolism in PNT2 cells in the absence and presence of rooibos (4.3 mg/mL) after 48 h. Patterned bars represent steroid substrate. The experiment was performed in triplicate and the data compared and analysed as shown by an unpaired *t*-test. Results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, *ns*=not significant, *n*=3).

The metabolism of T (±20%) was significantly lower than that of A4. Although 0.8 µM T remained, DHT (0.07 µM) and A4 (0.0354 µM) were detected together with negligible levels of 5α-dione, AST and 3α-adiol (fig. 4.19). Interestingly, in the metabolism of A4, 50% was converted to 5α-dione while in the case of T metabolism only 17% was converted to DHT indicating that SRD5A2 preferentially catalyses the conversion of A4 over T. In addition it would appear that 17βHSD2 activity is low in PNT2 cells since only ±10% was converted to A4. In the presence of rooibos T metabolism yielded 0.05 µM DHT and negligible levels of A4 and 5α-dione, indicating that rooibos influenced the endogenous conversion of T by SRD5A2 and 17βHSD. While ±35% of the metabolised T was detected as DHT, only 25% was detected in the presence of rooibos (fig. 4.19). Similar reduced levels were also detected in the presence of rooibos in HEK293 cells transiently expressing SRD5A2 (4.3.5). The reduced conversion of T to A4 detected in the presence of rooibos, denotes the inhibition of dehydrogenase activity of 17βHSDs activity. The influence of rooibos on 3α-adiol, 5α-dione and AST levels could not be assessed, as their levels were below the LOQ.
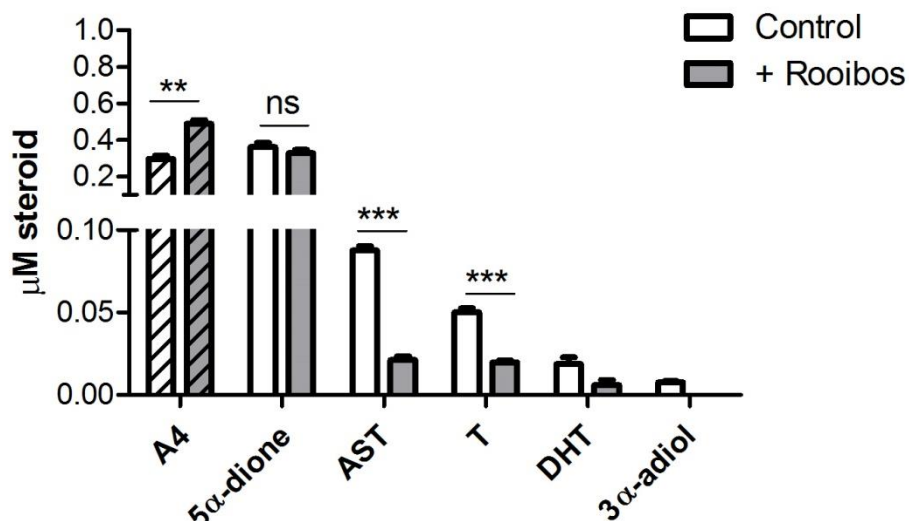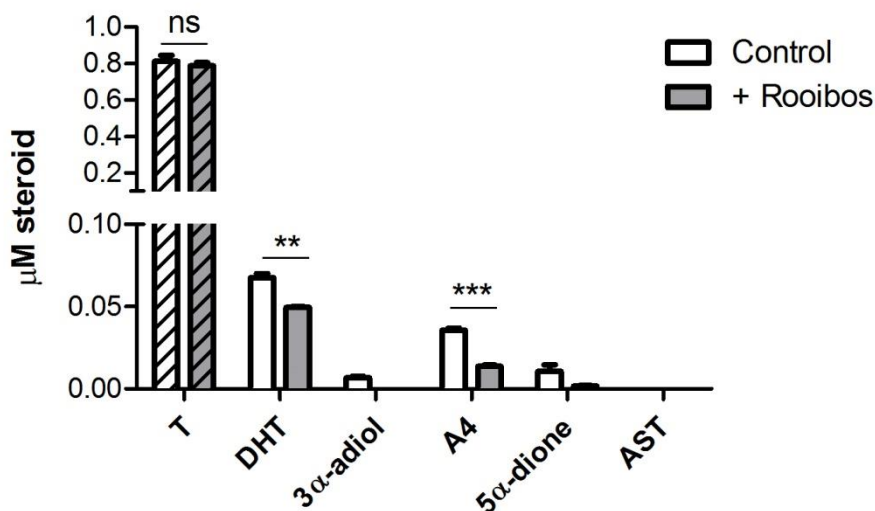
Figure 4.19: T, 1 μM, metabolism in PNT2 cells in the absence and presence of rooibos (4.3 mg/mL) after 48 h. Patterned bars represent steroid substrate. The experiment was performed in triplicate and the data compared and analysed as shown by an unpaired *t*-test. Results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, *ns*=not significant, *n*=3).

In this study unconjugated and total steroids were analysed, following the deconjugation by β-glucuronidase of steroid metabolites. The data showed no difference in the levels detected, indicating that steroids were not conjugated. Therefore, UGT activity is not prominent in PNT2 cells.

The metabolism of A4 and T in PNT2 cells was subsequently assayed in the presence of rooibos and dutasteride to further analyse the inhibition of SRD5A by the 5α-reductase inhibitor and rooibos. The production of 5α-dione in the presence of dutasteride (10 μM) was inhibited with no 5α-dione detected after 48 h (fig. 4.20). Increased substrate level, A4 (0.97 μM) was detected in the presence of dutasteride, and the product of 17βHSD, T, was reduced in the presence of rooibos.
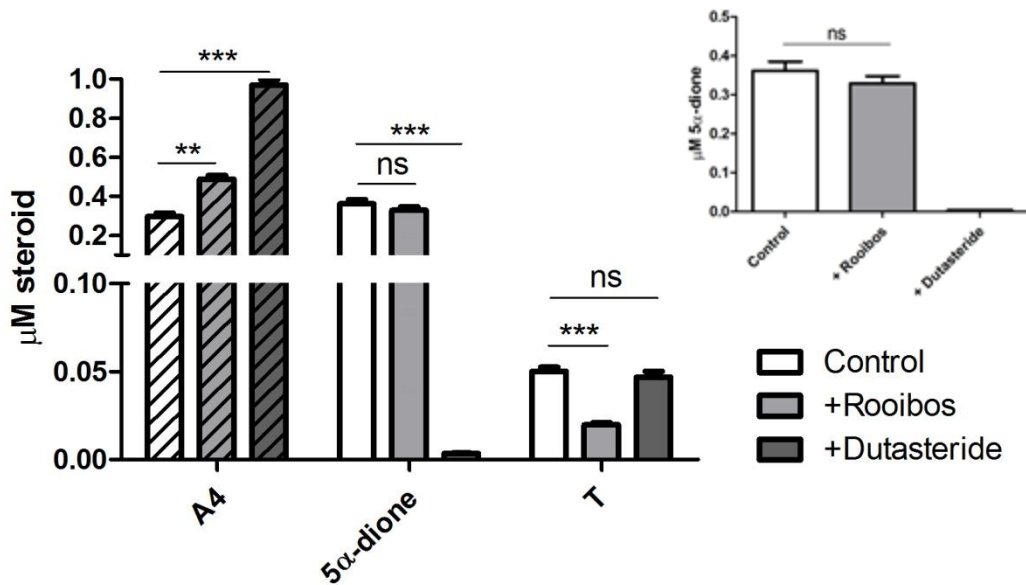
Figure 4.20: A4, 1 µM, conversion to 5α-dione by SRD5A in PNT2 cells in the absence (control) and presence of rooibos (4.3 mg/mL) and dutasteride (10 µM) after 48 h. Patterned bars represent steroid substrate. The experiment was performed in triplicate and data was compared to the control groups and analysed by one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, *ns*=not significant, *n*=3).

The production of DHT in the presence of dutasteride (10 µM) was inhibited with no DHT being detected after 48 h (fig. 4.21). Although an increased substrate level of T could not be detected in either the presence of rooibos or dutasteride, A4 levels together with DHT levels were reduced, in the presence of rooibos.
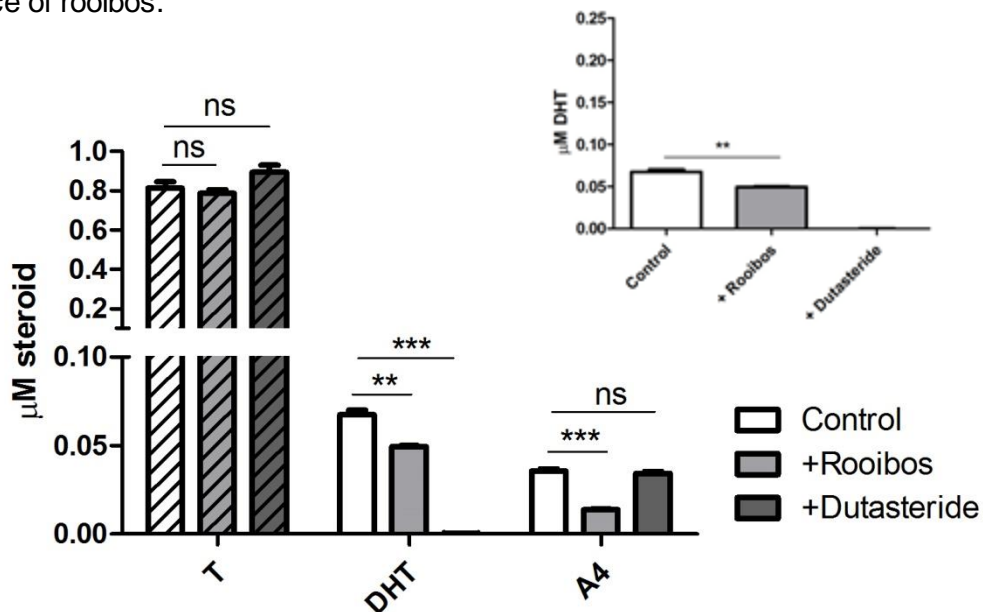
Figure 4.21: T, 1 µM, conversion to DHT by SRD5A in PNT2 cells in the absence (control) and presence of rooibos (4.3 mg/mL) and dutasteride (10 µM) after 48 h. Patterned bars represent steroid substrate. The experiment was performed in triplicate and data was compared to the control groups and analysed by one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, *ns*=not significant, *n*=3).

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A4 metabolism in BPH-1 cells in the presence of rooibos

The conversion of A4 was assayed in BPH-1 cells, in the presence of rooibos extracts (4.3 mg/mL) to determine the influence of rooibos on endogenous A4 conversion to T via reductive 17βHSDs. On completion of the assay metabolites were deconjugated after which total steroids were analysed. A4 was primarily metabolised to T (±0.75 μM) with high levels of 5α-dione (±0.12 μM) also being detected, denoting preferred reductive 17βHSD activity as well as that of SRD5A, respectively (fig. 4.22). Levels of 3α-adiol were also significantly higher than AST levels which may be attributed to higher levels of substrate being available for the AKR1C2 reductive reactions. Rooibos resulted in a significant reduction (±43.5%) of T production, indicating inhibition of the catalytic activity of reductive 17βHSD towards A4. Reduced T levels in the presence of rooibos subsequently led to decreased levels of DHT (±47%) and 3α-adiol (±70%). Of interest with the decreased conversion of A4 to T, more A4 was channelled to produce 5α-dione (±46%) via SRD5A1/2 and AST (±26%) via AKR1C2.
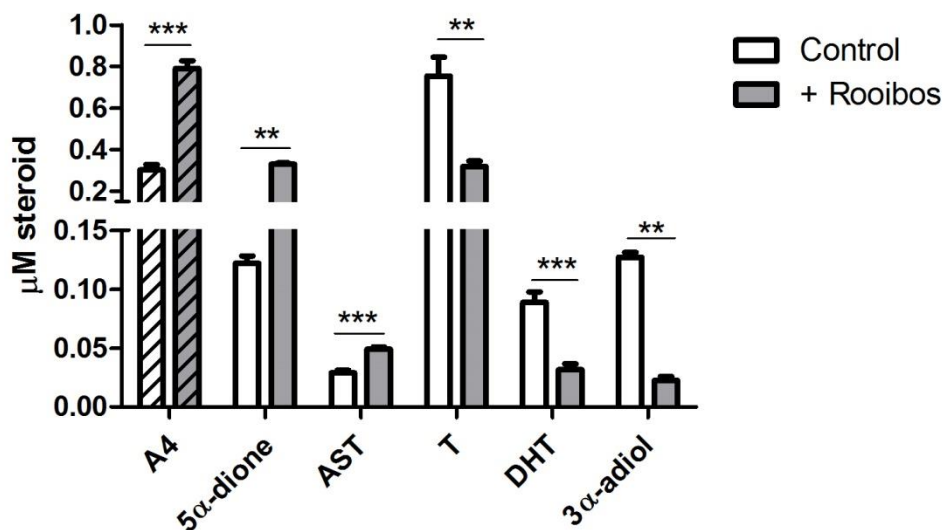
Figure 4.22: A4, 1 μM, conversion in BPH-1 cells in the absence; and in the presence of rooibos (4.3 mg/mL) after 48 h. Patterned bars represent steroid substrate. The experiment was performed in triplicate and results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, n=3).

In addition unconjugated and total steroids were also analysed, following a β-glucuronidase treatment of culture media from BPH-1 cells, and showed no difference in the levels of steroids were detected. UGT activity is therefore also not prominent in BPH-1 cells.

11KA4 conversion to 11KT in prostate cells in the presence of rooibos

The conversion of 11KA4 to 11KT via reductive 17βHSDs endogenous to prostate cells was also investigated in the presence of rooibos (fig. 4.23). Rooibos resulted in a significantly reduced conversion of 11KA4 in LNCaP (±11.3% inhibition), PC-3 (±26% inhibition) and BPH-1 (±17.6% inhibition) cells, however, the difference was insignificant in PNT2 cells. PNT2 cells did not readily convert 11KA4 to 11KT, and therefore the influence of rooibos on the 17βHSD catalysed reaction regarding the C11-keto substrate could not be established. It is interesting to note that while A4 was readily metabolised to T in PNT2 cells, the conversion of A4 and 11KA4 were both efficiently catalysed in BPH-1 cells. The conversion of 11KA4 to 11KT was catalysed more readily in BPH-1 cells than by PC-3 cells, followed by LNCaP cells. These results suggests higher expression levels of reductive 17βHSD in BPH-1>PC-3>LNCaP cells, regarding the metabolism of the C11 keto-steroid. Downstream metabolites of 11OHA4 due to SRD5A and AKR1C2 are also able to form in the aforementioned prostate cell models, however, the levels of these metabolites were negligible and as such are not shown.
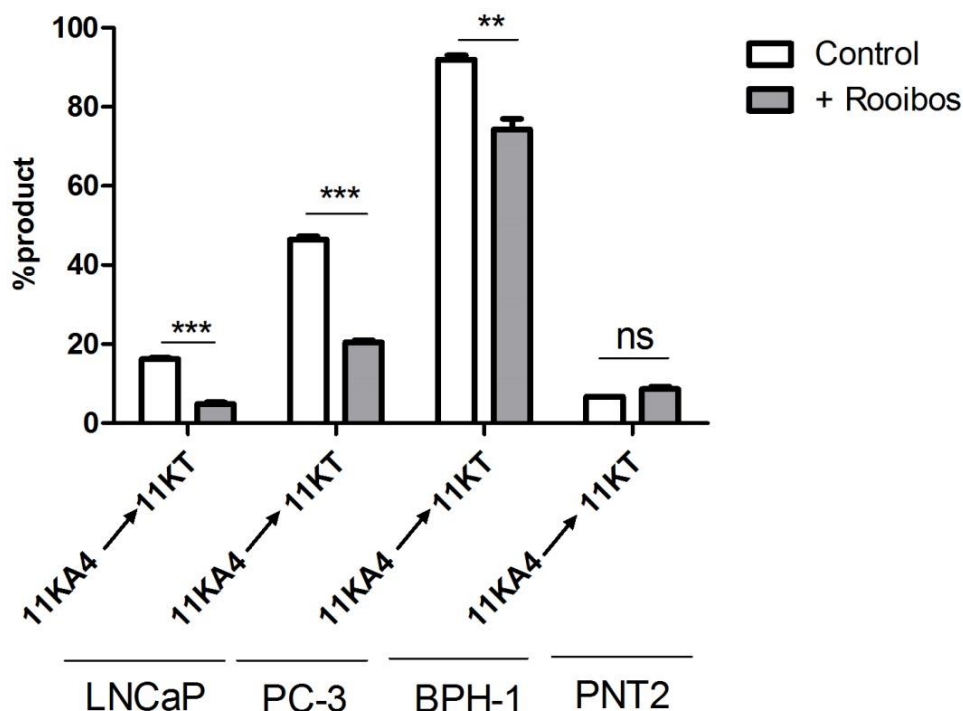

Figure 4.23: 11KA4, 1 µM, conversion to 11KT in LNCaP, PC-3, BPH-1 and PNT2 cells in the absence and in the presence of rooibos (4.3 mg/mL) after 48 h. The experiment was performed in triplicate and results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, ns=not significant, n=3).

4.4 Discussion

The physiological role of rooibos has, to date, been widely investigated *in vivo* and *in vitro,* with this polyphenol-rich extract exhibiting anti-mutagenic, antioxidant and anti-carcinogenic properties (Marnewick et al., 2000; Sasaki et al., 1993; Shimoi et al., 1996). Increasing evidence of the modulation of steroidogenic enzymes by rooibos as a possible mechanism of action has been reported, specifically regarding enzymes involved in adrenal steroidogenesis (Perold, 2009; Schloms et al., 2012; Schloms & Swart, 2014). These findings led to the present study and the hypothesis that rooibos may also modulate steroid metabolism in the prostate. The enzymes investigated in the present study are expressed in the prostate, with AKR1C3 and SRD5A1 up-regulated in PCa. Both the 17βHSDs and SRD5As are intricately involved in the three prostate androgen pathways, the conventional C19 pathway, the 5α-dione pathway and the 11OHA4 pathway.

Cell viability assays in the presence of rooibos showed that cell viability was not affected in the experimental protocols employed in this study. The inhibitory effects detected are therefore due to rooibos-enzyme interactions with cell viability not being compromised. The data shows that the viability in LNCaP cells, after 96 h incubation period in the presence of the highest concentration was not affected by rooibos. However, in HEK293 cells, a decrease in cell viability was detected after the 12 h incubation period with rooibos, which necessitated normalising data to cell numbers.

Another aspect which was addressed was the endogenous expression of steroidogenic enzymes in the cell model systems. Analyses of metabolites produced in experimental assays would be confounded by endogenous enzyme expression, catalysing either the added substrate or the formed product. In this study, assays were therefore firstly conducted in three different cell models transfected with the relevant enzymes to allow the analyses of substrate conversion by endogenous enzymes. HEK293 cells ultimately presented the best cell model for the investigation into the influence of rooibos on the steroidogenic enzymes as these cells exhibited negligible endogenous interference by 17βHSD or SRD5A.

Investigations into the catalytic activity of 17βHSD3 and AKR1C3 towards A4 and 11KA4, were conducted since their products, T and 11KT, represent active androgens (Rege et al., 2013; Storbeck et al., 2013). The potential inhibition of the catalytic activity of the 17βHSD enzymes by rooibos may result in a decrease in the biosynthesis of active androgens. 17βHSD3 plays a central role in androgen biosynthesis, specifically in T production, in the testis and in prostatic tissue (Mindnich et al., 2004; Montgomery et al., 2008). Similarly AKR1C3 converts A4 metabolites to T metabolites, and while it is not expressed in the testis, AKR1C3 is expressed in prostatic tissue (Luu-The, 2001).

The data presented in this chapter clearly shows that rooibos inhibits the catalytic activity of 17βHSD3 and AKR1C3, blocking the biosynthesis of active androgens, suggesting a possible role

for rooibos in the prostate and possibly in PCa. Considering the inhibition of endogenously expressed 17βHSDs and transfected 17βHSDs in CHO-K1 cells by rooibos, the data shows rooibos not only inhibiting the catalytic activity of the expressed 17βHSD3 and AKR1C3 enzymes but also 17βHSD1 and 17βHSD7 activity endogenous to these cells. These results suggest that since rooibos inhibits the biosynthesis of T and 11KT, less ligand will be available to bind and activate the AR. In a recent study, the influence of rooibos was shown to decrease T production in mouse Leydig cells. TM3 cells were treated with unfermented and fermented rooibos (0.25, 0.5 and 1 mg/mL) for 24 h and a significant reduction in T production was detected, suggestive of the inhibitory effect of rooibos on the catalytic activity of 17βHSD (Opuwari & Monsees, 2014).

The inhibition by rooibos of 17βHSD3 and AKR1C3 detected may likely be attributed to the polyphenol compounds present in rooibos, with the flavonoid concentrations within the rooibos extract ranging between 12 μM and 652 μM. Quercetin, a flavonoid-derivate of rutin, present in rooibos, has been shown to inhibit AKR1C3 at concentrations ranging from 100 nM to 20 μM (Krazeisen et al., 2001). Rutin has also been suggested to inhibit both 17βHSD3 and AKR1C3, with a recent pharmacophore-based virtual screening study depicting the crystal structure of 17βHSD3 and AKR1C3 in which rutin was bound at the base of the substrate binding domain (Schuster et al., 2011). Structural differences regarding the number and position of hydroxyl and glucosyl moieties as well as the structural flexibility of flavonoids have been implicated to contribute to their enzyme modulating properties. It was suggested that inhibitor potency increased with increasing hydroxyl groups (Krazeisen et al., 2001; Schloms & Swart, 2014). It is possible that the hydroxyl groups of the flavonol are involved in the binding of these compounds to the active site of 17βHSD enzymes, with the formation of hydrogen bonds stabilizing the orientation of flavonoid compounds in the active site. In the present study 11KA4 was assayed in HEK293 cells transiently transfected AKR1C3, in the presence of rutin (10 μM) (quercetin-3-O-rutinoside). No significant inhibition was detected after 4 h (data not shown). It is possible that the rutinoside disaccharide moiety may interfere with the binding of the rutin in the active pocket. Quercetin, which is the aglycone of rutin, is present in trace amounts in unfermented rooibos, while isoquercetin and hyperoside, with C3 glucosyl and galactosyl moieties, respectively, are also present at low levels. Quercetin-3-O-robinoside and rutin are, however, present at markedly higher levels. In addition, gut flora allows the metabolism of quercetin-3-O-robinoside and rutin (Erlund et al., 2000; Hollman, 2004 and references therein), with the subsequent bioavailability of quercetin at detectable levels in the body, suggesting possible cellular effects (Manach et al., 1995).

Previous studies have reported that 17βHSD3 and AKR1C3 convert A4 to T, and that 17βHSD3 is substantially more efficient (Dufort et al., 1999; Storbeck et al., 2013). Steroid conversion assays in HEK293 cells clearly show that 17βHSD3 is more efficient in metabolising A4 in comparison to AKR1C3. However, in CHO-K1 cells ±35-40% conversion of A4 by both enzymes was detected. AKR1C3 readily catalysed the conversion of 11KA4, with 11KT being 2-fold higher than T, which is

in agreement with previous studies (Storbeck et al., 2013). In addition, AKR1C3 catalysed the conversion of 11KA4 more efficiently than 17βHSD3, with a 4-fold greater production of 11KT. In contrast, 17βHSD3 metabolised A4 and 11KA4 to their respective products equally efficiently.

Analyses of the data obtained in the two cell model systems, CHO-K1 and HEK293 cells transiently transfected with 17βHSD3 and AKR1C3 showed marked differences in the catalytic conversion of the substrates assayed. In CHO-K1 cells the conversion rate of the substrates assayed was not efficient, regardless of the incubation period, with the exception of the heterologously expressed AKR1C3 converting 11KA4. In the subsequent assay in HEK293 cells, in which substrate conversions ranged from 50%-95%, the conversion rates were dependent on the incubation period. The variance obtained in the data set could be due to many factors. It has been reported, as previously mentioned, that CHO-K1 cells express 17βHSD1 and 7 endogenously, while HEK293 cells express low levels of SRD5A2. It is therefore possible that oxidative/reductive enzymes, which may also be substrate specific, are expressed in these cell lines that will accept C19 steroids as substrates, unfortunately influencing the outcome of the data set. In addition it is likely that the presence of NAD(P)+/NADP(H) and regenerating systems may differ in these cell models which would also affect the catalytic activities of the enzymes. Nevertheless, this study set out to investigate the possible inhibition of 17βHSD3 and AKR1C3 by rooibos, and even though the conversion rate was low, analyses of substrate and product levels indicated that rooibos, in CHO-K1 cells elicited a ±50% inhibition of both enzymes.

In the prostate 17βHSD2 metabolises T, 11OHT and 11KT to their inactive A4 derivatives and in this manner modulates levels of available AR ligands. The data obtained in this study showed that rooibos inhibited the catalytic activity of 17βHSD2 towards these substrates with the highest inhibition observed being towards C11 derivatives, 11KT>11OHT>T (40%, 20% and 15%). Interestingly, the inhibition of CYP11B1 by flavonoids, vitexin, orientin and rutin, was recently reported to be substrate specific. In the presence of the aforementioned flavonoids the inhibition of DOC conversion was shown to be negligible, while the inhibition of deoxycortisol conversion was significantly higher (Schloms & Swart, 2014), supporting substrate specific inhibition of rooibos and its flavonoids.

The above findings, together with reports that show SRD5As produce steroids that are capable of agonist activity towards the AR (Bruchovsky & Wilson, 1968), led to further investigation into the influence of rooibos on the conversion of A4 and T by SRD5A1 and SRD5A2. Possible modulation of SRD5A activity towards A4 and T could lead to decreased active androgen formation and decreased activation of AR-driven genes, as both SRD5A1 and SRD5A2 play a central role in prostate androgen metabolism.

The inhibition of SRD5As by phytoestrogens and flavonoids has not been extensively researched to date, specifically considering the prostate as peripheral target tissue. Considerable research into

the development of inhibitors of SRD5A activity has been conducted, with the present study reporting for the first time on the influence of rooibos on SRD5A1 and SRD5A2. Rooibos inhibited the conversion of T to DHT by SRD5A1 in U2OS cells, but was insignificant in comparison to the inhibition by dutasteride. In addition, these assays also showed, for the first time, that endogenous 17βHSD in U2OS cells, a non-steroidogenic cell model, exhibited reductive 17βHSD activity, which necessitated SRD5A assays to be completed in HEK293 cells. Rooibos did not inhibit the catalytic activity of SRD5A1 in the HEK293 cell system as the 5α-reduction of A4 and T was not influenced. It, however, appears that rooibos may influence the catalytic activity of SRD5A2 towards T, affecting the rate at which T is converted to DHT.

It is reported that SRD5A1 preferentially converts A4 over T, and in the present study, assays with SRD5A1 confirm that this enzyme preferably metabolises A4, while also metabolising T more readily than SRD5A2. This study also show the preference of the endogenous expressed SRD5A in PNT2 cells towards the conversion of A4 to 5α-dione (±45%), 5-fold more than the conversion of T to DHT (±7%), possibly denoting the reduction of A4 by PNT2 cells to SRD5A2, reflecting the expression of SRD5A2 reported to be expressed in normal epithelial cells of the prostate (Luu-The et al., 2008), although both SRD5A1 and SRD5A2 has been reported in normal prostatic epithelial cells (Iehle et al., 1999).

Further analysis of A4 and T metabolism in PNT2 cells, showed that while it appears that rooibos did not inhibit the catalytic activity of SRD5A towards A4, the conversion of T to DHT in this prostate cell model was inhibited significantly, confirming an inhibitory effect by rooibos of SRD5A2, and corroborates the inhibitory effect of rooibos on SRD5A2 activity towards T reported in HEK293 cells. The inhibitory effect of rooibos on SRD5A2 in PNT2 cells was also confirmed in additional conversion assays conducted in the presence of dutasteride, showing significant reduced conversion of T to DHT. A study by Evans et al. (1995), also reported on dietary lignans and isoflavonoids inhibiting SRD5A in BPH tissue homogenates. However, both isoforms were inhibited with SRD5A1 being more sensitive to inhibition compared to SRD5A2 (Evans et al., 1995). This aforementioned study therefore supports the possible interference of flavonoids and rooibos on the catalytic activity of SRD5A, but also of 17βHSD, as this study showed the inhibition of 17βHSD by the compounds tested. However, none of the compounds assayed have been shown to be present in rooibos.

The analysis of the influence of rooibos on the metabolism of A4 and T in PNT2 cells also showed that while SRD5A metabolised A4 more readily than T, both reductive and oxidative 17βHSD activities were detected, albeit it at low levels, evident in the low levels of T and A4, respectively. Rooibos nevertheless inhibited both significantly. In addition, AKR1C2 activity was evident, converting 5α-dione to AST, with findings also suggesting that rooibos inhibited the production of AST significantly, as negligible levels were detected in the presence of extracts. The inhibitory

effect of rooibos on AKR1C2 could, however, not be corroborated with a significant increase in 5α-dione levels and warrants further investigations.

While SRD5A, 17βHSD and AKR1C2 enzyme activities towards the steroid substrates above were clearly shown, no UGT activity was detected. It is interesting to note that PNT2 cells represent epithelial cells derived from normal prostate tissue and it has been shown that UGT2B17 is expressed exclusively in the basal epithelial cells and UGT2B15 in the epithelial luminal cells (Bélanger et al., 2003). UGTs expressed in prostate cells, would conjugate T, DHT, 3α-adiol and AST. However, in these assays described above, no UGT activity was detected in this cell line upon analyses of the free steroids in the media. The assays completed in PNT2 cells were conducted concurrently with assays in LNCaP cells, with UGT activity being detected in LNCaP cells, also indicating efficient deconjugation of steroids following a β-glucuronidase assay.

Although UGT activity was also not detected in BPH-1 cells, these cells were an ideal prostate cell model for the investigation into the influence of rooibos on the reductive activity of 17βHSD which was more prominent than that of SRD5A towards A4. Rooibos resulted in a significantly reduced conversion of A4 to T, and the subsequent conversion to metabolites, DHT and 3α-adiol. This inhibition, however, shunted the increased A4 substrate to 5α-dione and AST. Analyses of the conversion of 11KA4 to 11KT in BPH-1, LNCaP, PC-3 and PNT2 cells in the presence of rooibos show that while higher levels of product were detected in BPH-1 cells, inhibition by rooibos was greater in LNCaP and PC-3 cells. While the metabolism of C11-hydroxy- and C11-keto A4 and T metabolites have been shown in the 11OHA4 pathway in LNCaP cells (Storbeck et al., 2013; Swart et al., 2013a), this study also shows for the first time that the C11-keto steroid is metabolised in PNT2 , BPH-1 and PC-3 prostate cells, suggesting that the 11OHA4 pathway may be relevant in normal, benign and cancerous prostatic cells.

Taken together, rooibos inhibited the catalytic activity of 17βHSD3, AKR1C3 and 17βHSD2 and appears to affect the rate at which SRD5A2 metabolises T in transiently transfected cell models. In addition rooibos inhibited the conversion of T to DHT, suggestive of endogenous SRD5A activity modulation, as well as the conversion of A4 to T, suggesting rooibos inhibits endogenous reductive 17βHSD in PNT2 and in BPH-1 cells. Furthermore rooibos appears to inhibit AKR1C2 activity, with a reduced conversion of 5α-dione to AST being detected in PNT2 cells. Rooibos also inhibited the 17βHSD catalysed conversion of 11KA4 to 11KT in LNCaP, BPH-1 and PC-3 prostate cells. As such, rooibos may have significant implications in PCa, as well as in CRPC - perhaps even more so the inhibition of reductive 17βHSDs, such as AKR1C3, as this enzyme is up-regulated as PCa progresses. Inhibition of the catalytic activity of reductive 17βHSDs will impede the production of active androgens, and precursor steroids for SRD5A. Decreased production of active androgens will lead to reduced AR activation and subsequently reduced transcription of AR-regulated genes, impacting favourably on cell growth and proliferation. The progression of PCa is dependent on

androgen biosynthesis catalysed by an intricate system of enzymes and the modulation of these enzymes by rooibos may therefore aid treatment strategies. In combination with general treatment regimes against PCa, including ADT and the use of AR antagonists, using rooibos as a natural herbal supplement could have beneficial anti-carcinogenic effects in hormone driven diseases. However, the inhibitory effect of $17\beta HSD2$ and AKR1C2 by rooibos cannot be ignored, as the inhibition of these enzymes would result in increased levels of active androgens with rooibos thus having deleterious effects.

This line of investigation and the influence of rooibos on prostate androgen metabolism in LNCaP cells will be further developed in chapter 5.

Chapter 5

An investigation into the influence of rooibos on androgen metabolism in LNCaP cells

5.1 Introduction

Diseases of the prostate such as BPH and PCa are prevalent in the aging male, and are androgen dependent at the time when circulating gonadal T declines, suggesting that the local or intracrine production of androgens may drive these diseases (Labrie et al., 2000; Labrie et al., 1995). Indeed both BPH and PCa are considered hormone-dependent diseases and require androgens for their secretory function with cancerous cells retaining this sensitivity to androgens (Gianfrilli et al., 2014). It has been reported that a complement of androgen-producing enzymes are expressed in human PCas, and are as such involved in the local production of the potent androgen, DHT, playing important roles in biological behaviour of prostate carcinoma cells (Nakamura et al., 2005). Androgen metabolism pathways, which include the conventional C19 (3.4.1), the 'alternative' 5α-dione (3.4.2) and the 11OHA4 pathways (3.4.3), are indeed intricately involved in active androgen production. Androgens from testicular and adrenal origin are utilized in these pathways given the expression of steroid-metabolizing enzymes, *viz*.: 3βHSDs, 17βHSDs, 11βHSDs, SRD5As, 3αHSDs and UGTs.

The balance between androgen induced cell proliferation and apoptosis is proposed to be a major regulator of growth of the normal and cancerous prostate. In normal conditions a steady state exists between synthesis and inactivation of active androgens, however, tissue transformation can be associated with an alteration of this balance. In both prostate disease, BPH and PCa, there is an imbalance between prostate cell growth and apoptosis, possibly because certain factors minimize cell apoptosis while others stimulate proliferation. Intrinsic (in particular growth factors) and extrinsic (in particular steroid hormones) factors directly and indirectly regulate prostate tissue growth and differentiation (Nelson et al., 2003).

Increasing evidence suggests that PCa cells alter local and paracrine steroid hormone metabolism (Gianfrilli et al., 2014). In the past decade, a growing number of studies have explored the role of local androgen production in cancer progression and transformation into CRPC. Widely accepted is the hypothesis that the progression of PCa during ADT is not due to the development of an androgen insensitive tumour clone but rather to the fact that cancer cells acquire mechanisms to escape systemic androgen deprivation while still taking advantage of signalling through the AR (Harkonen et al., 2003; Locke et al., 2008; Risbridger, Davis et al., 2010; Scher et al., 2004). Importantly, the AR can undergo point mutations, becoming more promiscuous, allowing activation by non-androgenic ligands (Scher & Sawyers, 2005). In addition, ERβ once bound to its ligand, acts via the AR regulating AR-driven genes in PCa cells (Muthusamy et al., 2011).

It is estimated that 233,000 new cases of PCa will be diagnosed annually in the USA and that 29,480 deaths, as a result of PCa, will occur per year (American Cancer Society, 2014). In BPH and PCa treatment by androgen deprivation, to deprive the AR of its ligand, include inhibitors of steroidogenesis and AR antagonists. The former approach requires a detailed knowledge of the extra-gonadal biosynthesis and metabolism of androgens and the discrete enzymes involved (Penning, 2010). A recent study considered both benign and malignant intact prostatic tissue to investigate individual profiling of prostate intracrinology, *ex vivo*, with the specific focus on 17βHSDs. The study showed 17βHSD3 is expressed in both BPH and PCa tissues at similar levels, and while 17βHSD2 was also expressed in BPH samples, 17βHSD2 was not expressed in PCa tissues. PCa samples expressed more reductive 17βHSD activity, such as AKR1C3 and 17βHSD7 compared to BPH samples, but decreased reductive 17βHSD activity compared to oxidative 17βHSD activity, such as 17βHSD type 4/8/10 was, however, detected in PCa samples. Furthermore, BPH samples showed decreased *de novo* enzymatic production of androgens, suggestive of predominant oxidative 17βHSD activity (Gianfrilli et al., 2014).

In prostate androgen metabolism, 3α-reduction of steroids entails steroid potency modulation, similar to 17βHSDs, through modifications between hydroxy and ketone groups at C3 of the four carbon ring backbone structure common to all steroids. Following this first step of inactivation, phase II metabolism is characterised by a conjugation reaction of a charged moiety with the hydroxy-group at the C3 and C17, and is almost universally associated with a decrease in steroid potency (Gosetti et al., 2013). The addition of glucuronidated moieties to androgens has the net effect of lowering receptor activation through both decreased potency and increased secretion (Barry et al., 1952; Klyne et al., 1945; Nozaki, 2001). Of interest, sulfation of androgens can also occur and is also classified under phase II metabolism of steroids. Glucuronidated steroids, in contrast to sulfated steroids, have been studied in the context of the downstream metabolism of active androgens with glucuronidated steroids derived from DHT being detectable in the serum of men (Bélanger et al., 1998; Moghissi et al., 1984). The enzymes responsible for the glucuronidation of androgens in prostatic tissue are UGT2B15 and UGT2B17 (Barbier & Bélanger, 2008), with the expression of *UGT2B17* reported to be up-regulated while *UGT2B15* expression is down-regulated in the progression of cancer from benign disease to lymph node metastasis (Paquet et al., 2012). Characterizing phase II metabolism of steroid derivatives are, however, still understudied and could possibly be a future target for CRPC (McNamara et al., 2013).

In a study by Byrns et al., (2012), the quantification of the downstream metabolites of A4 in AKR1C3 transfected LNCaP cells showed that AKR1C3 redirects the metabolism of A4 to testosterone-glucuronide (T-G). In addition, the cells became resistant to the growth inhibitory effects of finasteride, a specific SRD5A1 inhibitor. Importantly, the conversion of A4 to T-G was blocked by indomethacin, a selective AKR1C3 inhibitor, indicating the inhibition of the formation of active androgens in PCa cells by this drug. The authors concluded that the beneficial effects of

SRD5A inhibitors in the treatment of PCa would depend on whether AKR1C3 is expressed or not, and that an AKR1C3 inhibitor would be beneficial for the treatment of PCa to inhibit the conversion of A4 and 5α-dione, to T and DHT, respectively. This study therefore highlights the potential role of AKR1C3 inhibitors in PCa, as well as the intricate catalyses of androgen metabolism by 17βHSDs and UGTs in prostate cell systems (Byrns et al., 2012).

Many treatment strategies, such as castration and ADT, are currently employed against PCa, but irrespective of the treatment regimen, in ±30–40% of cases the cancer can re-emerge (2–5 years later) with a tell-tale increase in PSA. PSA is an important clinical marker, which is commonly used for early diagnosis and to monitor treatment response, prognosis, and progression in patients with PCa (Lieberman, 2004). In a recent study, PSA levels for enrolled subjects with BPH were approximately ±4 ng/mL compared to ±10 ng/mL for those with PCa (Gianfrilli et al., 2014). Although PSA levels can be distinguished between BPH and PCa patients, the PSA index (free PSA: total PSA) would increase the screening accuracy of PSA and the subsequent discrimination between BPH and PCa. However, it has been suggested that the application of this classical monitoring factor of PCa is less effective in the attempts to distinguish among multiple stages of the disease. In the on-going fight against PCa, there exist economic and socioeconomic costs of overtreatment, as well as complications associated with the current available treatments (including ADT). Together with these factors, the overall quality of life and life expectancy of affected subjects should be considered (Gianfrilli et al., 2014), advocating new treatment approaches against PCa progression.

In recent years, the shift by the general public towards natural and herbal medicinal products, away from mainstream pharmaceuticals, has led to increased interest in natural plant products. An investigation in LNCaP and PC-3 cell lines, showed polyphenol compounds exhibiting phytoestrogenic activity inhibiting cell growth in both cell lines, suggesting a diet rich in polyphenols as a possible approach in the treatment of PCa (Mitchell et al., 2009). However, the relationship between diets rich in polyphenol compounds and cancer incidence is controversial with some studies indicating positive correlation while others indicate no association. Rooibos which is rich in polyphenol compounds has, however, been shown to possess anti-carcinogenic and anti-mutagenic properties in skin tumours (Marnewick et al., 2005). Rooibos may therefore play a role in the modulation of the development and progression of hormone-dependent cancers, given that rooibos has also been shown to influence steroidogenic enzymes (Perold, 2009; Schloms & Swart, 2014; Schloms et al., 2013).

The aim of this study was to determine steroid conversion as it occurs in the C19 pathway, as well as in the 11OHA4 pathway in LNCaP cells, through steroid quantification using UPLC-MS/MS. The metabolism of A4, T, DHT and AST in the presence of rooibos was subsequently assayed in order to determine the influence of rooibos on endogenous 17βHSDs, SRD5As and 3αHSDs expressed

in LNCaP cells. The metabolism of 11OHA4 in the presence of rooibos, and two of its flavonoids, isoorientin and rutin, was also assayed. Since evidence of possible modulation of UGT activity in LNCaP cells was detected, the influence of rooibos on endogenous UGTs expressed in LNCaP cells was further investigated. In this approach T, DHT and AST metabolism was assayed in the presence of rooibos. The possible modulation of cancer markers in prostate cells by rooibos was subsequently investigated and PSA levels in the absence and presence of DHT in LNCaP cells were assayed in the presence of rooibos.

5.2 Materials and methods

5.2.1 Materials

Unfermented rooibos plant material was provided by the South African Rooibos Council (Rooibos LTD-BPK, Clanwilliam, South Africa). Steroid metabolites (T, A4, DHT, 5α-dione, 3α-adiol and AST), D-(+)-Glucose, β-glucuronidase (Type VII-A from *E.coli*; 5,292,000 units/g), RPMI-1640, phenol free RPMII-1640 medium and rutin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isoorientin was purchased from Extrasynthese (Genay, France) and indomethacin and dutasteride were purchased from Sequoia Research Products Ltd (Pangbourne, UK). HEPES buffer (1 M) and sodium pyruvate (100 mM) were purchased from Biochrom (Berlin, Germany). 11OHA4, 11OHT, 11KA4, 11KT and DHT-G were purchased from Steraloids (Wilton, USA) and Corning®CellBIND® Surface 24-well plates were purchased from Corning® Life Sciences (Corning, NY, USA). LNCaP cells were obtained from the Sigma's European Collection of Cell Cultures (St. Louis, MO, USA). Penicillin-streptomycin, fetal calf serum and trypsin-EDTA were obtained from Gibco BRL (Gaithersburg, MD, USA). Charcoal-treated fetal calf serum was obtained from Highveld Biologicals (Lyndhurst, RSA). Deuterated cortisol (9,11,12,12-D4-cortisol) and deuterated testosterone (Testosterone 1,2-D2, 98%) were purchased from Cambridge isotopes (Andover, MA, USA). Dimethyl sulfoxide and pH indicator strips (non-bleeding) was obtained from Merck (Darmstadt, Germany). All other chemicals were of the finest quality and supplied by reliable scientific supply houses.

5.2.2 Methanol extraction of unfermented rooibos

Unfermented rooibos plant material was sequentially extracted with chloroform and methanol using a glass soxhlet extractor and aliquots (1 mL) prepared and stored as previously described (4.2.3). Rooibos polyphenols were subsequently identified and quantified using HPLC as previously described (4.2.4).

5.2.3 Steroid conversion assays in LNCaP cells

LNCaP cells were cultured in RPMI-1640 media, pH 7, supplemented with 10% fetal bovine serum, 1.5 g NaHCO$_3$/L, 2.5 g D-(+)-Glucose, 1% penicillin-streptomycin, 1% HEPES and 1% sodium pyruvate using Corning® CellBIND® surface plates at 37°C, in an atmosphere of 90% humidity and

5% CO_2. Cells were plated into 24-well Corning® CellBIND® surface plates (2 x 10^5 cells/mL, 0.5 mL/well) and incubated for 48 h. The medium was removed and replaced with 0.5 mL RPMI-1640 medium containing the following steroid substrates (1 µM): A4, T, DHT, AST, 3α-adiol and 11OHA4. Steroids (2 mg/mL) were diluted in culture medium to the desired concentration after which cell were exposed to steroid substrates added to the cells, without and in the presence of unfermented rooibos extracts (4.3 mg/mL). Duplicate aliquots (0.5 mL) were removed at 6 h, 24 h, 36 h and 48 h and steroid metabolites extracted, after β-glucuronidase treatment (5.2.4). Steroid metabolites were extracted by liquid-liquid extraction using 10:1 volume of dichloromethane to culture medium. The samples were vortexed for 10 min, centrifuged at 3500 rpm for 5 min after which the media was aspirated and dichloromethane phase dried at 45°C under nitrogen. The dried steroid residue was redissolved in 150 µL methanol prior to analysis by UPLC-MS/MS. Cortisol-D4 (15 ng) and testosterone-D2 (15 ng), were added to each samples prior to extraction. Control assays included steroid conversion assays carried out in the presence of inhibitors, indomethacin (20 µM; in DMSO), a 17βHSD inhibitor, dutasteride (10 µM; in ethanol), a SRD5A inhibitor and DMSO. The latter was shown to have no effect on steroid conversion assays at the experimental concentration.

5.2.4 β-glucuronidase assay

Analyses of total (free + conjugated) steroids was conducted following steroid conversion assays in prostate cells by treating the steroids in the collected aliquots with β-glucuronidase, as previously described (4.2.9), prior to steroid extraction.

5.2.5 PSA chemiluminescent immunoassay

LNCaP cells were cultured as described in 5.2.3. Cells were plated into 24-well Corning® CellBIND® surface plates (4 x 10^4 cells/mL, 0.5 mL/well) in phenol free RPMI-1640 media supplemented with 5% charcoal stripped fetal calf serum and 1% penicillin-streptomycin and incubated for 48 h. The medium was removed and replaced with 0.4 mL/well phenol free RPMI-1640 supplemented with 1% penicillin-streptomycin only. After a 24 h incubation period, phenol free RPMI-1640 media containing DHT ranging from 0.001, 0.01, 0.1 and 1 µM was added in 0.1 mL/well. Cells were also incubated with DHT in the presence of rooibos extracts (1 mg/mL and 4.3 mg/mL). The cells were incubated for 96 h after which medium (0.5 mL) aliquots were removed and analysed for PSA levels using an Access Hybritech PSA chemiluminescent immunoassay (Coulter, 2010).

5.2.6 Separation and quantification of steroid metabolites using UPLC-MS/MS

Stock solutions of A4, T, DHT, DHT-G, 5α-dione, 3α-adiol, AST, 11OHA4, 11OHT, 11KA4 and 11KT were dissolved in ethanol (2 mg/mL) and a series of standards (0.2 - 20 ng/mL) were prepared in methanol.

Steroid metabolites were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP column (2.1 mm x 100 mm, 2.6 μm) (Torrance, CA, USA) as previously described (4.2.10). All experiments were performed in triplicate and the subsequent results are given as means ± SEM, and statistics were calculated as previously described (4.2.11).

5.3 Results

5.3.1 Analysis of polyphenols in methanolic extracts of unfermented rooibos

In this study LNCaP cells were exposed to rooibos extracts at two concentrations, 1 mg/mL and 4.3 mg/mL. Analyses of the major polyphenol compounds show that the flavonoids ranged from 2.9 μM to 152 μM for 1 mg/mL rooibos extract and 12.6 μM to 652 μM for 4.3 mg/mL rooibos extract (table 4.1). The dihydrochalcones were present at the highest concentrations (794.08 μM), 4-fold higher than the flavones (183.9 μM) and 22-fold higher than the flavonols (35.6 μM).

5.3.2 Analyses of steroid metabolism in LNCaP cells

An investigation into steroid metabolism in LNCaP cells was conducted and the steroid metabolites quantified using UPLC-MS/MS. The endogenous pathways were thus assessed to determine the steroid flux prior to investigating the effect of rooibos on steroid metabolism.

5.3.2.1 Analyses of steroid metabolism in the C19 pathway

The metabolism of A4, T, DHT, AST and 3α-adiol was assayed in LNCaP cells and the steroid metabolites analysed using UPLC-MS/MS (fig. 5.1). Analyses of the conversion of A4 in LNCaP cells showed that, in the conversion by 17βHSD and SRD5A, the latter was the preferred route as higher levels of 5α-dione than T being detected (fig. 5.1 a). In addition, AST was also a major product indicating the 3αHSD reduction to be the preferred metabolic route compared to the reduction to DHT by 17βHSD.
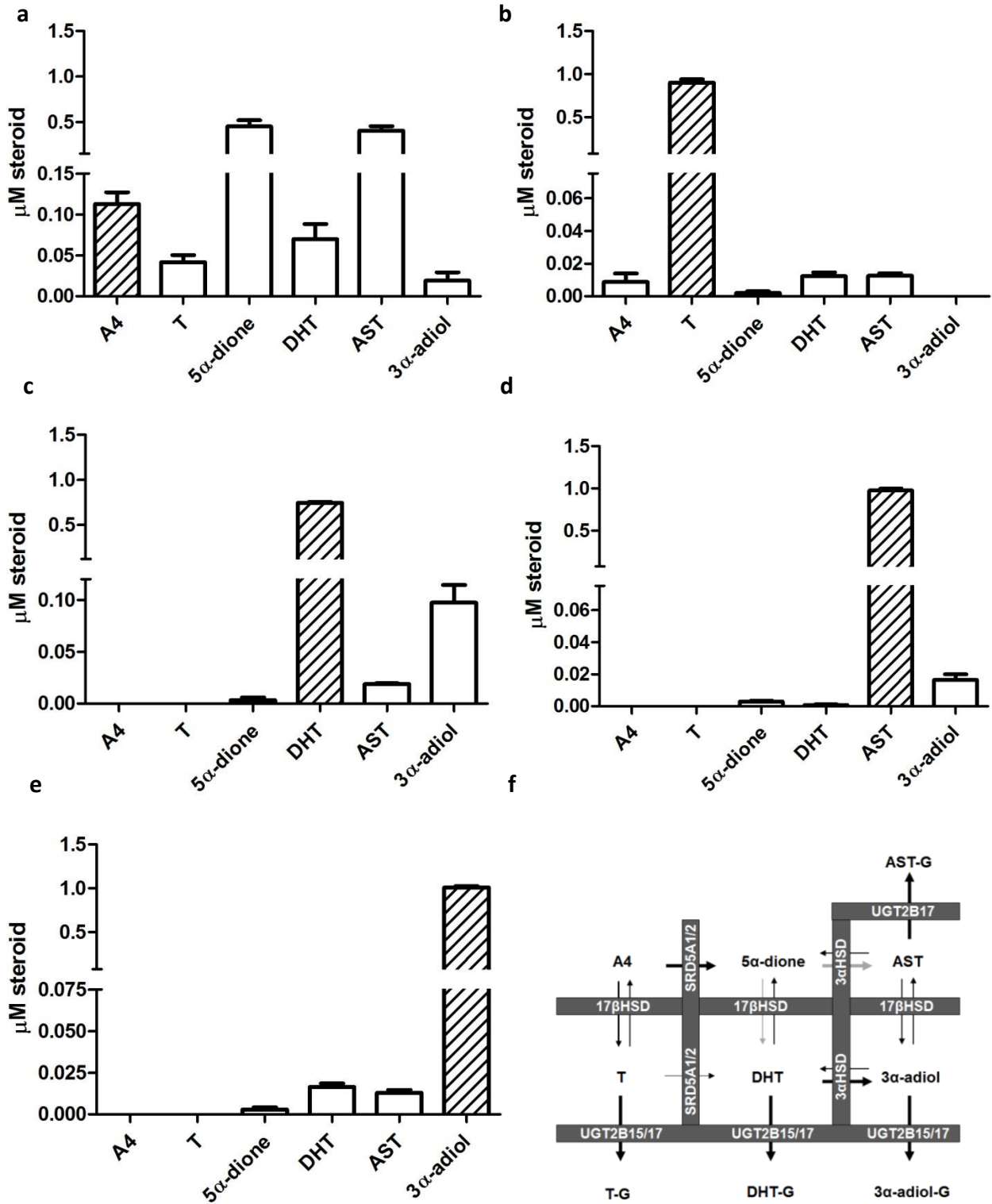
Figure 5.1: Steroid substrate (1 µM) conversions in LNCaP cells after 48 h. Steroid metabolites shown as total (free + conjugated): (a) A4 metabolism, (b) T metabolism, (c) DHT metabolism, (d) AST metabolism, (e) 3α-adiol metabolism and (f) diagram representing androgen metabolism in LNCaP cells. Substrates assayed represented in patterned bars; black arrows, conversions assayed; bold black arrows, preferred conversion; bold and grey arrows, preferred 5α-dione conversion within androgen metabolism.

Subsequent analyses of T conversion (fig. 5.1 b) showed that most of the steroid was in the conjugated form after 48 h, with very low levels of A4 being detected. The data thus shows that the conversion of A4 to T is more efficient compared to the conversion of T to A4 in LNCaP cells, indicating a predominant reductive 17βHSD activity (fig. 5.1 a/b). The minor oxidative activity is also evident considering the conversion of DHT, as negligible 5α-dione levels were detected after 48 h (fig. 5.1 c). While ±50% DHT was conjugated low levels of 3α-adiol were also detected showing further conversion by the reductive activity of AKR1C2. The analyses of the conversion of AST and 3α-adiol (fig. 5.1 d, fig 5.1 e) clearly indicate that these steroids are in the conjugated form after 48 h with negligible levels of other metabolites being detected, 3α-adiol in the case of AST conversion and AST and DHT in the case of 3α-adiol conversion. The production of DHT suggests dehydrogenase activity, possibly 17βHSD6, catalysing the conversion of 3α-adiol (fig. 5.1 e). UGT activity by UGT2B15 and UGT2B17 was prominent in LNCaP cells and showed conjugation of T, DHT, 3α-adiol and AST. Conjugated steroid levels were as follows: 3α-adiol (±0.99 μM) > AST (±0.97 μM) > T (±0.89 μM) > DHT (±0.75 μM). Steroid levels below the LOQ were not shown.

5.3.2.2 Analyses of 11OHA4 metabolism in LNCaP cells

In this study the conversion of 11OHA4 by 11βHSD2 and 17βHSD was analysed. 11βHSD2 is endogenously expressed in LNCaP cells and it has been shown in our laboratory that 11βHSD2 preferentially catalyses 11OHA4 since negligible levels of 11OH-5α-dione (produced by SRD5A activity) was detected after 72 h (unpublished data). In addition, it has been shown that the conversion of 11OHA4 by 17βHSD is negligible (Storbeck et al., 2013). Analyses of 11OHA4 metabolism in LNCaP cells (fig. 5.2), showed that 11OHA4 is preferentially converted to 11KA4 by 11βHSD2, which is subsequently converted to 11KT by reductive 17βHSD. Negligible levels of 11OHT were detected as 11OHA4 was not readily reduced by 17βHSD.
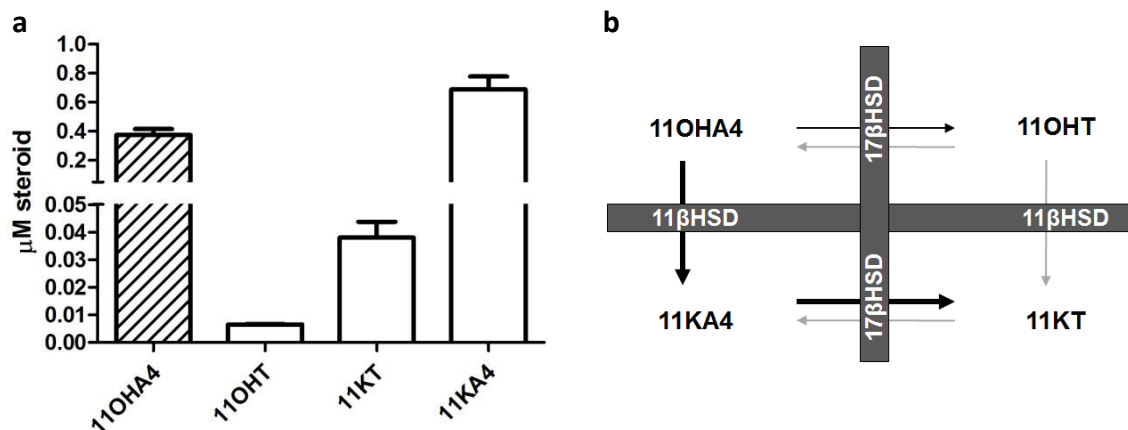
Figure 5.2: 11OHA4, 1 µM, metabolism in LNCaP cells after 36 h; (a) 11OHA4 metabolism and its metabolites, and (b) diagram representing androgen metabolism in LNCaP cells. Substrate assayed represented in patterned bar; black arrows, conversions assayed; bold black arrows, preferred conversion; and grey arrows, conversions occurring within androgen metabolism.

5.3.3 The influence of unfermented rooibos on steroidogenic enzymes catalysing androgen metabolism in LNCaP cells

5.3.3.1 The analyses of the metabolism of A4, T, DHT and AST in the presence of rooibos extracts

The metabolism of A4, T, DHT and AST was assayed in the presence of rooibos extracts (4.3 mg/mL) in LNCaP cells. In this study it was shown that rooibos inhibits 17βHSD3 and AKR1C3 activity heterologously expressed in CHO-K1 and HEK293 cells (4.3.4). In order to confirm these data in a prostate cell model, the conversion of A4 was assayed. Analyses of the steroid metabolites showed that the presence of rooibos resulted in a significant increase in A4 levels (1.9-fold, ±0.11 µM). While rooibos did not inhibit SRD5A activity lower levels of AST were detected. It appears therefore that rooibos inhibited AKR1C2 activity, however, the influence of rooibos on AKR1C2 needs to be investigated in isolation in order to draw accurate conclusions. T, DHT and 3α-adiol levels were below LOQ, and as such the influence of rooibos could not be accurately established.

Figure 5.3: A4, 1 µM, metabolism in LNCaP cells in the presence of rooibos (4.3 mg/mL) after 48 h. Steroids are shown as total (free + conjugated) steroids. Patterned bars represent steroid substrate. The experiment was performed in triplicate and results are expressed as the mean ±SEM (*$P<0.05$, **$P<0.01$, $n=3$).

Further investigations into the influence of rooibos on reductive 17βHSD expressed in LNCaP cells were subsequently conducted. Rooibos (4.3 mg/mL) resulted in A4 conversion being reduced in LNCaP cells, after 24 h (fig. 5.4). In the presence of rooibos a ±30.7% inhibition of A4 conversion was detected, similar to the inhibition detected in the presence of indomethacin (±29.7%), a known AKR1C3 inhibitor (Byrns et al., 2012). Taken together these results therefore denote the inhibitory effect of rooibos on reductive 17βHSD and AKR1C3 activity endogenous to LNCaP cells.
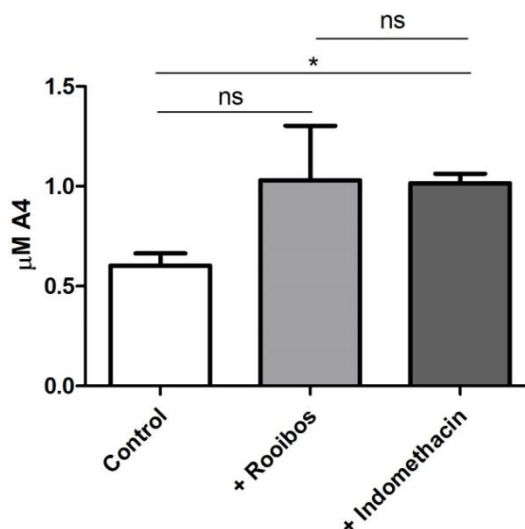
Figure 5.4: Analysis of A4 levels in LNCaP cells after 24 h. A4, 1 µM, was assayed in the presence of rooibos (4.3 mg/mL) and indomethacin (20 µM). The experiment was performed in triplicate and results are expressed as the mean ±SEM (*$P<0.05$, ns=not significant, $n=2$).

The analysis of T metabolism in the presence of rooibos (fig. 5.5) showed that the levels of T were reduced significantly (1.2-fold, ±0.2 µM), however, due to the low levels of downstream steroid metabolites detected, being below the LOQ, the influence of rooibos could not be accurately assessed. These results suggest rooibos possibly inhibits the glucuronidation of T, and as such more T is channelled to A4 and DHT, as in the presence of rooibos T levels are reduced, but A4 and DHT levels are increased. These data suggest an amount of T possible to override any inhibition that may occur on 17βHSD2 and SRD5A (4.3.4, 4.3.5).
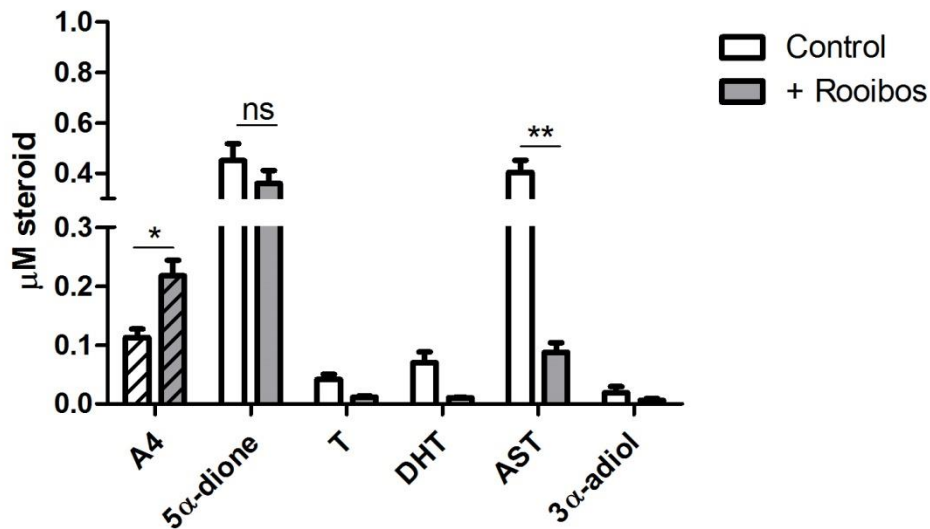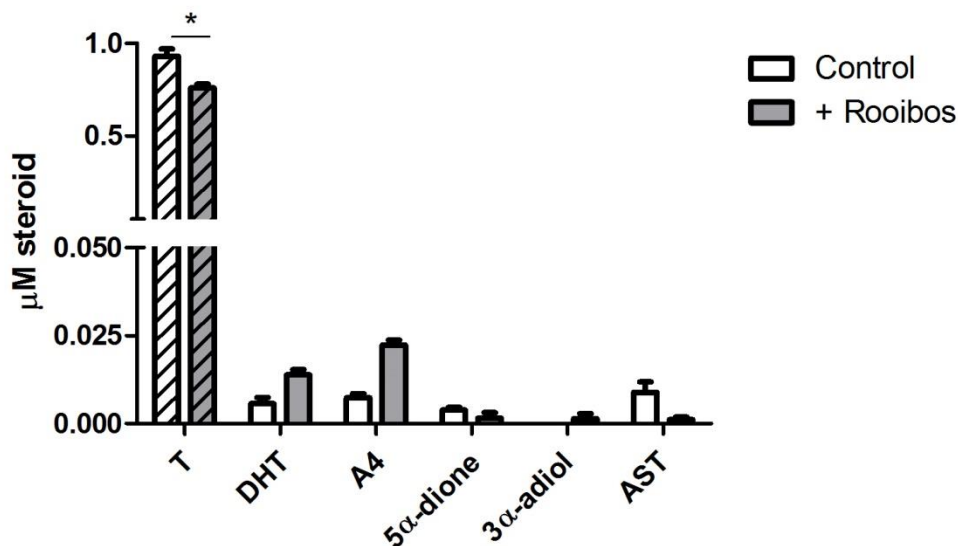

Figure 5.5: T, 1 µM, metabolism in LNCaP cells in the presence of rooibos (4.3 mg/mL) after 48 h. Steroid metabolites are shown as total (free + conjugated) steroids. Patterned bars represent steroid substrate. The experiment was performed in triplicate and results are expressed as the mean ±SEM (*P<0.05, n=3).

DHT and AST metabolism was also assayed to determine the effect of rooibos on downstream metabolism in LNCaP cells. DHT levels increased significantly (1.9-fold, ± 0.5 µM) in the presence of rooibos while a significant decrease (± 0.03 µM) was detected in 3α-adiol levels (fig. 5.6), suggesting inhibition of AKR1C2 activity by rooibos. The increased DHT level may allow more DHT to be glucuronidated, or inactivated to form 5α-dione[1] via 17βHSD2. This incubation period of 6 h, however, might be too soon to allow speculation and a 48 h incubation period might be more accurate in assessing the influence of rooibos on DHT metabolism.
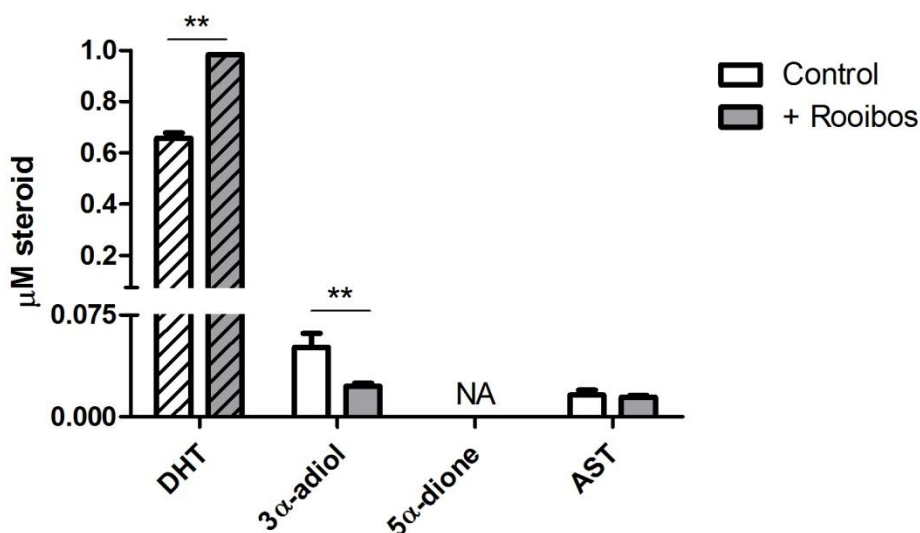
Figure 5.6: DHT, 1 µM, conversions in LNCaP cells in the absence and in the presence of rooibos (4.3 mg/mL) after 6 h. Steroids are shown as total (free + conjugated) steroids. Patterned bars represent steroid substrate. The experiment was performed in triplicate and results are expressed as the mean ±SEM (**$P<0.01$, *NA*=not assayed, *n*=3).

Since the biosynthesis of AST appeared to be inhibited by rooibos the metabolism of AST was also assayed. Rooibos decreased the levels of AST significantly (1.1-fold, ± 0.1 µM), while increased 3α-adiol (± 0.02 µM) was detected (fig 5.7). If, as with the case of T, rooibos inhibited the glucuronidation of AST, more substrate would be available to be converted to form 3α-adiol via 17βHSD, especially, if the rate of reduction by 17βHSD trumps the rate of AST-G formation. The influence of rooibos on the catalytic activity of 17βHSD towards AST was not investigated in this study and it is possible that the effect of rooibos on the enzyme's activity towards AST is minimal, as in the case of T shown above.

[1] At the time of this study, the steroid was unavailable for analyses.
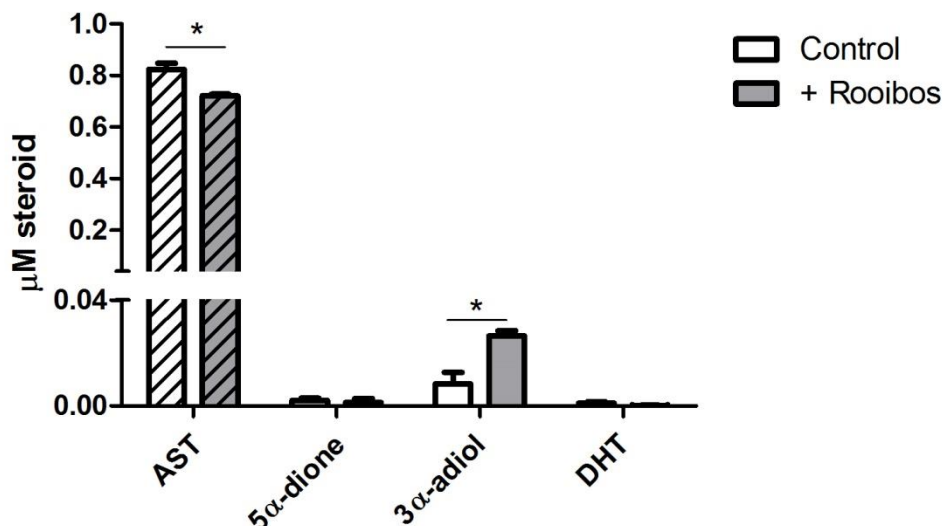
Figure 5.7: AST, 1 µM, conversions in LNCaP cells in the absence and in the presence of rooibos (4.3 mg/mL) after 48 h. Steroids are shown as total (free + conjugated) steroids. Patterned bars represent steroid substrate. The experiment was performed in triplicate and results are expressed as the mean ±SEM (*$P<0.05$, *n*=3).

5.3.3.2 The analyses of the metabolism of 11OHA4 in the presence of rooibos extracts

The metabolism of 11OHA4 in the presence of rooibos allowed further analyses of not only the modulation by rooibos of 17βHSD3 and AKR1C3 which catalyses the conversion of 11KA4 to 11KT but also that of 11βHSD2 which catalyse the conversion of 11OHA4 to 11KA4, thus supplying the substrate for the 17βHSD isoforms expressed in LNCaP cells. In the presence of rooibos no significant modulation of 11OHA4 or 11KA4 levels were detected, denoting no inhibition of 11βHSD2 (fig. 5.8). A reduction in 11KT levels (3.7-fold, ± 0.03 µM) were detected, corroborating the modulation of reductive 17βHSD by rooibos, as was also shown in HEK293 cells (4.3.4). It should be noted that increased 11KA4 levels are expected in the presence of rooibos, however, this was not clearly detected and warrants further investigation. Isoorientin, a flavone and rutin, a flavonol, did not decrease the levels of 11OHA4 or 11KT significantly. In contrast, increased 11KA4 levels (1.5-fold, ± 0.25 µM) were detected in the presence of rutin, denoting possible modulation of 11βHSD2 by this flavonoid (fig. 5.8). It should be noted that the metabolism by SRD5A of 11OHA4 and its metabolites as well as the subsequent catalyses by downstream steroidogenic enzymes may contribute to the steroid levels detected in these assays.

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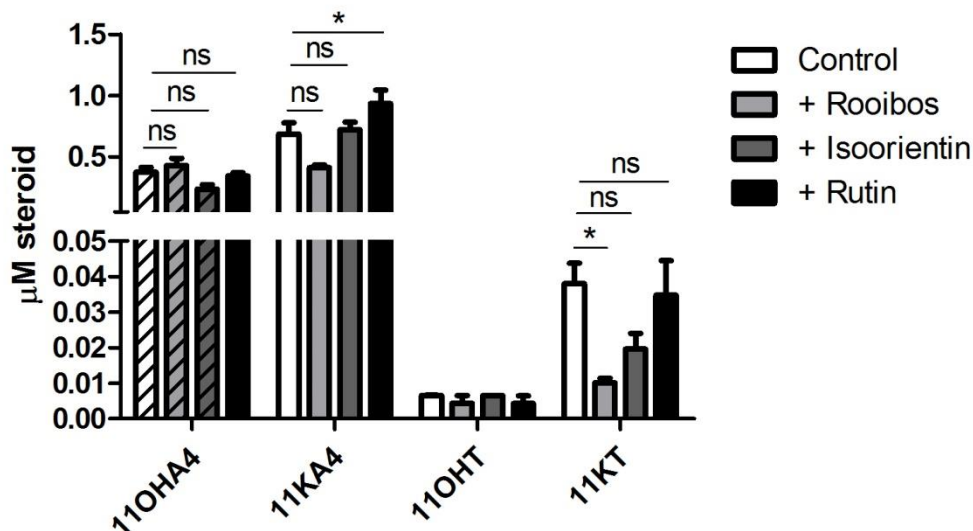
Figure 5.8: 11OHA4, 1 μM, metabolism in LNCaP cells in the presence of rooibos (4.3 mg/mL), isoorientin (10 μM) and rutin (10 μM) after 36 h. Patterned bars represent steroid substrate. The experiment was performed in triplicate and results are expressed as the mean ±SEM (*$P<0.05$, *ns*=not significant, *n*=3).

The data shown above suggests possible modulation of the inactivation and conjugation of T, DHT and AST in LNCaP cells by rooibos. The influence of rooibos on the conjugating activity of UGTs in LNCaP cells was therefore assayed.

5.3.3.3 Rooibos inhibits the conjugation of T, DHT and AST by UGT2B15 and UGT2B17 in LNCaP cells

The investigation of unfermented rooibos extract (4.3 mg/mL) on the conjugation of DHT, T and AST by endogenously expressed UGT2B15 and UGT2B17 was investigated in LNCaP cells. While UGT2B15 catalyses the conjugation of glucuronic acid at C17 of the steroid molecule, UGT2B17 catalyses conjugation at both the C3 and C17, enabling thus the conjugation of the downstream A4 metabolite, AST. The decreased levels of T and AST shown above suggested that rooibos influenced the catalytic activity of the UGTs. However, increased DHT levels were detected, which could not be attributed solely to the decrease in 3α-adiol levels (fig. 5.6).

UGT activity was thus investigated by analysing both free and total steroids in the medium with the latter analyses carried out following deconjugation using a β-glucuronidase assay. β-glucuronidase deconjugates conjugated steroids, to allow total (free + conjugated) steroid analysis using UPLC-MS/MS. Free steroid levels were determined and quantified by UPLC-MS/MS and following a β-glucuronidase assay, total steroid levels were also quantified. Analyses of the free and total steroid levels in the metabolism of DHT showed that ±27.4% of DHT was conjugated with ±0.6 μM remaining in the free form after 6 h (fig. 5.9). In the presence of rooibos extracts, free DHT levels being significantly increased (±0.4 μM) with the levels of total DHT being similar to the levels of free DHT. This result shows that rooibos inhibited the conjugation of DHT by UGT2B15 and UGT2B17 in LNCaP cells.
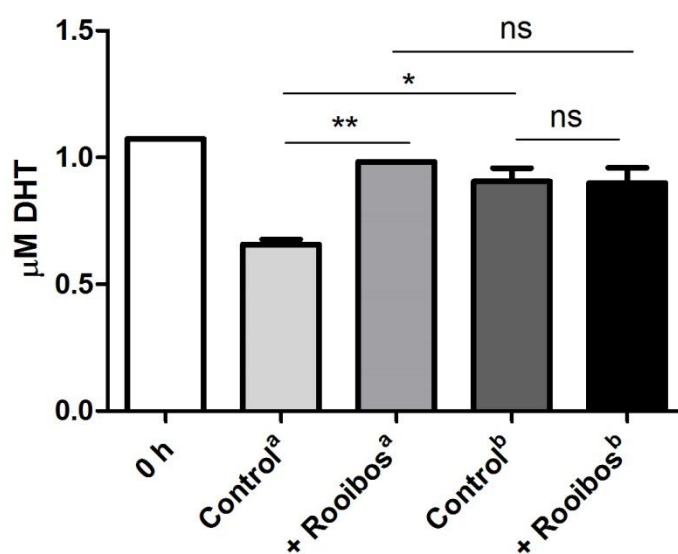
Figure 5.9: DHT, 1 μM, conversion in LNCaP cells in the absence (control) and presence of rooibos (4.3 mg/mL) after 6 h. Steroids are shown as free steroid[a] and total (free + conjugated) steroid[b]. The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired t-test. Results are expressed as the mean ±SEM (*$P<0.05$, **$P<0.01$, *ns*=not significant, *n=3*).

An investigation into the influence of rooibos on the glucuronidation of T catalysed by both UGT2B15 and UGT2B17 and the glucuronidation of AST catalysed by UGT2B17 only, was subsequently conducted. The metabolism of T and AST in LNCaP cells was assayed in the presence of rooibos resulted in increased levels of free T and AST indicating a decrease in the glucuronidation of T (fig. 5.10 a) and AST (fig. 5.10 b) in LNCaP cells. In the absence of rooibos both T and AST were fully metabolised, with no detectable level of either steroid remaining. Following a β-glucuronidase assay, the steroid levels detected, T (± 0.89 µM) and AST (± 0.99 µM), indicated that both T and AST are conjugated after 48 h with negligible levels of substrate remaining. In the presence of rooibos, the levels of free T and AST are significantly increased, with ±0.2 µM T and ±0.4 µM AST being detected after 48 h. Analyses of the total T and AST suggests rooibos modulates the conjugating activity of UGT2B15 and UGT2B17 towards T and UGT2B17 towards AST in LNCaP cells, since ±84.4% T and ±49.3% AST were in the conjugated form after 48 h.
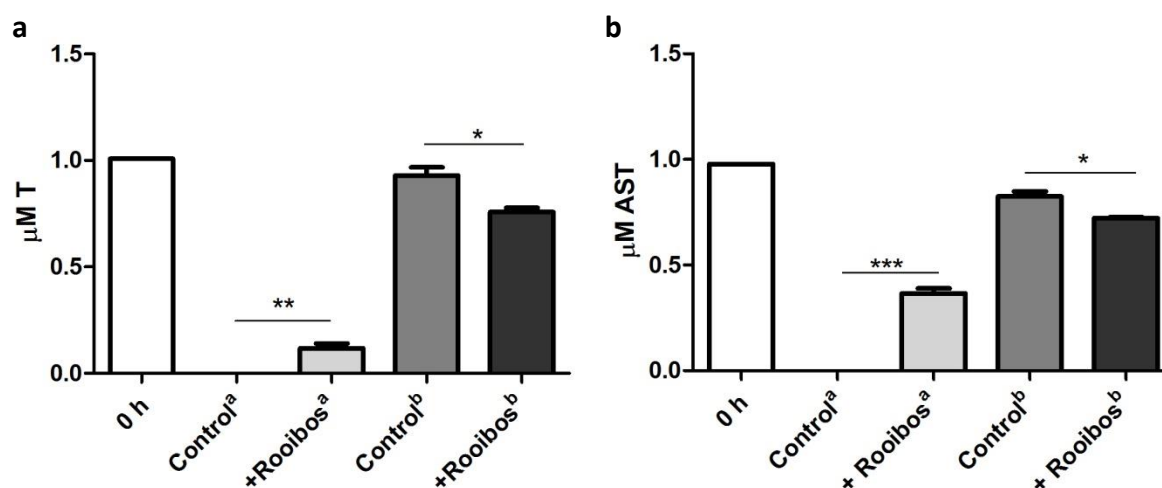
Figure 5.10: Influence of rooibos on steroid conjugation in LNCaP cells after 48 h: (a) T, 1 µM, conversion; and (b) AST, 1 µM, conversion. Assays were conducted in the absence (control) and in the presence of rooibos (4.3 mg/mL) and steroids analysed by UPLC-MS/MS following a β-glucuronidase assay. Steroids are shown as unconjugated steroid[a] and total steroid[b]. The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired t-test. Results are expressed as the mean ±SEM (*$P<0.05$, **$P<0.01$, $n=3$).

Upon closer inspection comparisons of the detected levels of T and AST (total steroid) in the assays conducted in the presence of rooibos, following the β-glucuronidase assay indicated a possible interference of rooibos with the β-glucuronidase assay. A β-glucuronidase assay using DHT and the glucuronidated DHT (DHT-G) as substrates was subsequently conducted in the presence of rooibos (4.3 mg/mL). The addition of rooibos at 4.3 mg/mL did not result in interference with the assay as the levels of steroids detected in the case of DHT (fig. 5.11 a) and DHT-G (fig. 5.11 b) were not statistically different. Furthermore, the presence of rooibos did not result in a reduction in steroid recovery following the β-glucuronidase assay (fig. 5.11 b).
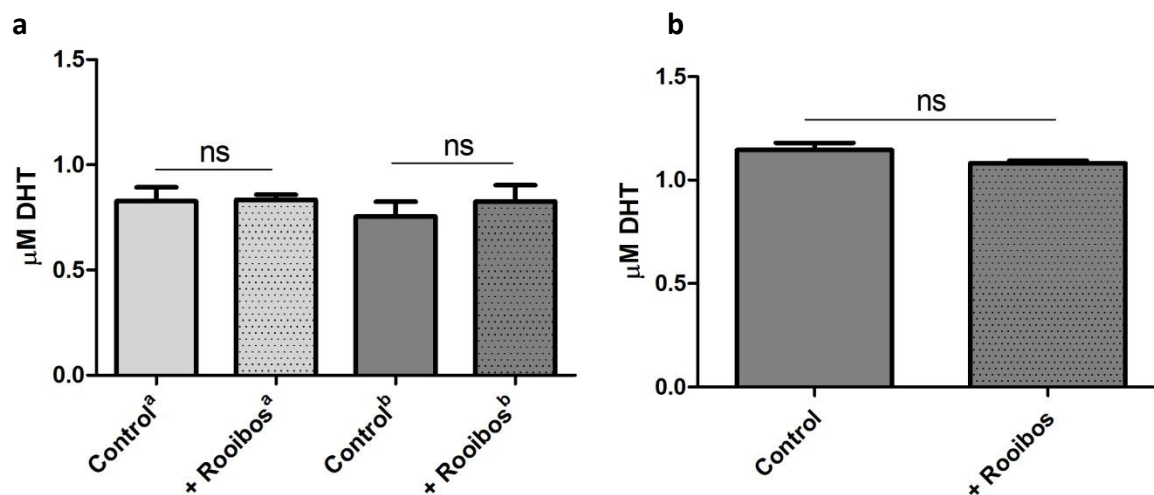
Figure 5.11: Influence of rooibos on steroid recovery: (a) DHT, 1 µM; and (b) DHT-G, 1 µM. Assays were conducted in the absence (control) and in the presence of rooibos (4.3 mg/mL) and steroids analysed by UPLC-MS/MS following a β-glucuronidase assay. Steroids are shown as free steroid[a] and total steroid[b]. The experiment was performed in triplicate and results are expressed as the mean ±SEM (*ns*=not significant, *n=3*).

5.3.4 Rooibos inhibits PSA levels in LNCaP cells

Further investigations were conducted to determine whether rooibos influences PSA levels which may subsequently modulate downstream cellular processes.

Stimulation of PSA levels by DHT in LNCaP cells

A preliminary investigation was conducted in which LNCaP cells were incubated in the presence of DHT, 0.1 and 1 µM, for 96 h, after which PSA levels were determined. PSA levels were increased, at both concentrations of DHT (fig. 5.12). However, at 1 µM, DHT resulted in PSA levels reaching the upper limit (150 ng/mL) of the immunoassay. The influence of rooibos on PSA levels was therefore investigated using DHT concentrations lower than 1 µM.
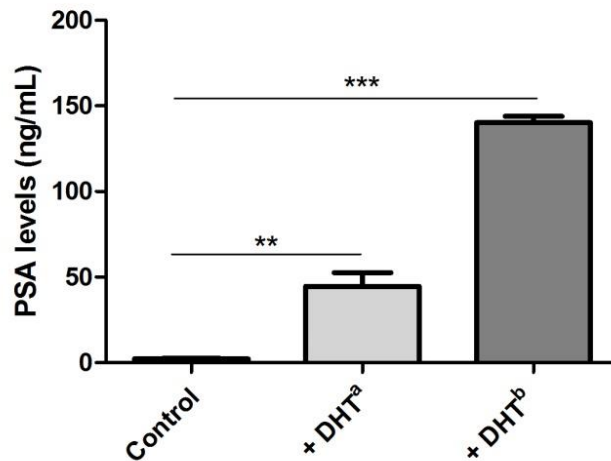
Figure 5.12: PSA levels in LNCaP cells after 96 h. Control, culture media without DHT; culture media with DHT[a], 0.1 μM; and culture media with DHT[b], 1 μM. The experiment was performed in triplicate and compared as shown by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM (**$P<0.01$, ***$P<0.001$, $n=3$).

Inhibition of PSA levels by rooibos in LNCaP cells

The influence of rooibos extracts was firstly assessed on basal PSA levels in LNCaP cells prior to determining the effect of DHT-stimulated production of PSA. Incubating LNCaP cells in the presence of rooibos (4.3 mg/mL) for 96 h resulted in significant decreases in basal PSA levels. While PSA levels decreased significantly ($P<0.01$) when cells were incubated in the presence of the higher concentration of rooibos (4.3 mg/mL), at a lower concentration (1 mg/mL), the decrease in PSA levels was less apparent with the 2-fold decrease approaching significance ($P=0.0517$) (fig. 5.13).
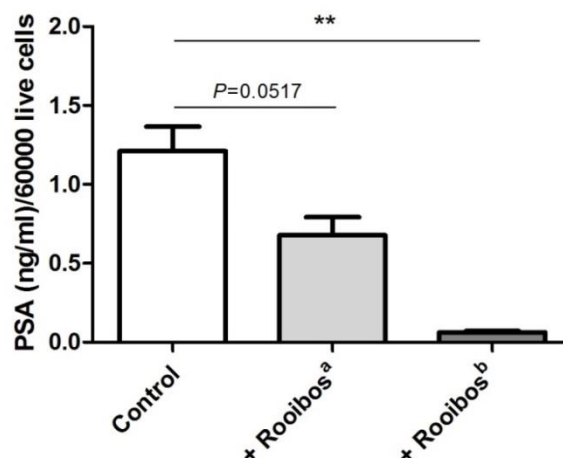
Figure 5.13: Influence of rooibos on basal PSA serum levels in LNCaP cells after 96 h. Control, culture media without rooibos; rooibos[a], 1 mg/mL; and rooibos[b], 4.3 mg/mL. PSA normalised to 60 000 live cells per mL. The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired t-test. Results are expressed as the mean ±SEM (**$P<0.01$, approaching significance $P=0.0517$, $n=3$).

The influence of rooibos (4.3 mg/mL) on DHT-stimulated PSA levels was subsequently determined in LNCaP cells, assayed in the presence of DHT, 0.001, 0.01 and 0.1 µM after 96 h. The data shows that while DHT stimulated PSA levels in a dose-dependent manner rooibos (4.3 mg/mL) decreased the levels significantly in the case of, 0.001 µM DHT ($P<0.01$) (fig. 5.14 a) and in the case of 0.01 µM DHT ($P<0.05$) (fig. 5.14 b). In contrast rooibos (4.3 mg/mL) did not result in a significant decrease in PSA levels at 0.1 µM DHT (fig. 5.14 c). In addition, rooibos (1 mg/mL) did not decrease PSA levels significantly in the presence of 0.001, 0.01 or 0.1 µM DHT (data not shown).
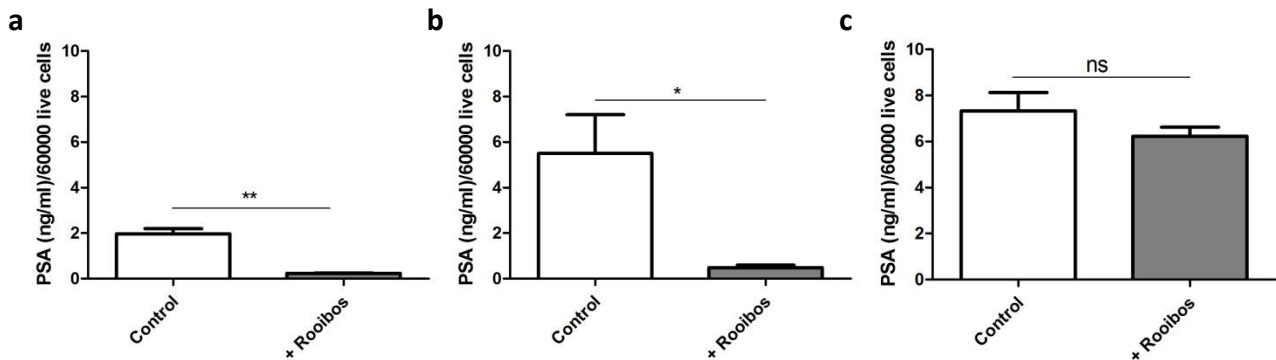
Figure 5.14: Influence of rooibos on PSA levels in LNCaP cells after 96 h. Assays were conducted in the absence (control) and in the presence of rooibos (4.3 mg/mL): (a) DHT, 0.001 µM; (b) DHT, 0.01 µM; and (c) DHT, 0.1 µM. PSA was normalised to 60 000 live cells per mL. The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired *t*-test. Results are expressed as the mean ±SEM (*$P<0.05$, **$P<0.01$, *ns*=not significant, *n*=3).

The influence of rooibos (4.3 mg/mL) on DHT-stimulated PSA levels was subsequently calculated as a ratio, represented by the PSA levels in the control assay: the PSA levels in the presence of rooibos (fig. 5.15). Rooibos reduced PSA levels at 0.001 µM and 0.01 µM DHT concentrations, resulting in a ratio of ±7. This ratio decreased dramatically, ±7 fold, at the highest concentration assayed.
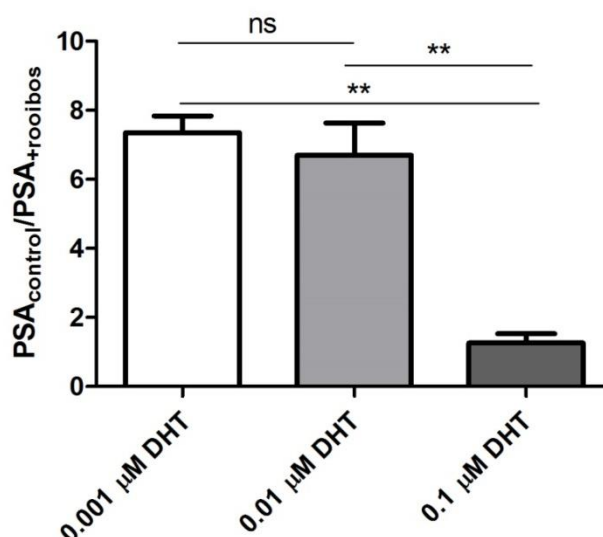
Figure 5.15: Ratios depicting the influence of rooibos on PSA concentrations in LNCaP cells assayed after 96 h without and in the presence of rooibos. Assays were conducted in the absence (control) and in the presence of rooibos (4.3 mg/mL) at DHT concentrations 0.001 µM, 0.01 µM and 0.1 µM. PSA was normalised to 60 000 live cells per mL. The experiment was performed in triplicate and data was compared as shown and analysed by an unpaired t-test. Results are expressed as the mean ±SEM (**$P<0.01$, ns=not significant, n=3).

5.4 Discussion

The determination of androgen pathways and enzymatic conversions were undertaken in LNCaP cells as it is important to examine in detail the status of *in situ* androgen metabolism and/or synthesis before modulation thereof by rooibos can be ascertained. This study showed that A4 is readily converted to 5α-dione, indicative of the preferred conversion of A4 by SRD5A in the 'alternative' 5α-dione pathway within LNCaP cells, corroborating previous reports (Chang et al., 2011; Sharifi, 2012). In addition, the data show that predominant conjugation of T, DHT, AST and 3α-adiol occurs in LNCaP cells, and will contribute to androgen production in these cells. The 11OHA4 pathway describing the metabolism of 11OHA4 has been reported (Storbeck et al., 2013; Swart et al., 2013b), with this study confirming that 11OHA4 is readily metabolised by 11βHSD2, but not by reductive 17βHSD. As mentioned previously the catalytic activity of UGT towards the C19 steroids within the 11OHA4 pathway has not, to date, been determined. It is possible that the hydroxy- or keto-groups on C11 hinders conjugation of these steroids. The glucuronidation of both 11KA4 and 11KT was investigated in this study and upon analyses of free and total steroid metabolites it was found that neither steroid was conjugated (data not shown), which indicates the inability of the UGTs present in LNCaP cells to conjugate these C11 steroids.

Evidence of an inhibitory effect on expressed 17βHSD in cells systems by rooibos (4.3.4) led to the subsequent investigation into the possible attenuation of endogenously expressed 17βHSD in LNCaP cells, by rooibos. Investigations into preferred oxidative or reductive 17βHSD activity in LNCaP cells showed predominant reductive 17βHSD activity to be present. 17βHSD expression has been reported in LNCaP cells, as 17βHSDs are intricately involved in the favoured activation or inactivation of androgen ligands. Predominant AKR1C3 mRNA expression in LNCaP cells have been reported, with minimal 17βHSD3 mRNA expression also reported (Luu-The et al., 2008). A recent study also reported on AKR1C3 and 17βHSD3 expression in PCa tissue, together with the presence of reductive 17βHSD7 (Gianfrilli et al., 2014). Another study, however, reported that the expression of AKR1C3 was minimal in LNCaP cells (Byrns et al., 2012; Fung et al., 2006), with others suggesting the presence of 17βHSD1 and 17βHSD7 (Laplante & Poirier, 2008). 17βHSD1 has been shown to exhibit negligible reductive activity towards A4 (Nokelainen et al., 1996; Puranen et al., 1997). Reductive 17βHSDs, specifically AKR1C3, are responsible for the increased T levels in PCa tissue, resulting in the progression of PCa (Nakamura et al., 2005). Possible modulation of reductive 17βHSD activity, such as AKR1C3, in prostate cells by rooibos therefore may indicate a potential beneficial effect in androgen-dependent diseases.

In this study, evidence of the inhibition of the catalytic activity of endogenous reductive 17βHSD in LNCaP cells by rooibos was shown with the metabolism of A4 significantly inhibited by rooibos. Indomethacin, a selective AKR1C3 inhibitor, blocked A4 conversion to the same extent as that obtained with rooibos, denoting the specific inhibition of AKR1C3 in LNCaP cells. This finding suggests that rooibos inhibited endogenously expressed AKR1C3 in LNCaP cells, and confirms results obtained in heterologously expressed AKR1C3 in CHO-K1 and HEK293 cells (4.3.4). Upon analyses of total steroids in the metabolism of A4 in the presence of rooibos in LNCaP cells, an increase in A4 levels were detected together with a decrease in T levels, however, the latter was not significant. In the presence of rooibos the change in 5α-dione levels were not significant, suggesting that rooibos did not modulate SRD5A activity. Higher levels of SRD5A1 compared to SRD5A2 have been reported to be present in LNCaP cells, denoting the conversion of A4 to 5α-dione to be catalysed by SRD5A1 (Iehle et al., 1999). Interestingly, a decrease in AST was detected suggesting rooibos inhibited endogenous AKR1C2 activity in LNCaP cells. The decrease in AST formation may, however, yield 5α-dione as substrate for 17βHSD resulting in the formation of DHT via 5α-dione pathway. Interestingly, no increase in DHT levels was detected in the presence of rooibos, showing that the metabolism of A4 in LNCaP cells yields steroids which exhibit minimal agonist activity towards the AR.

In addition to the increased A4 levels in the presence of rooibos being comparable to those in the presence of indomethacin, reduced conversion of 11KA4 to 11KT was also detected, suggesting the inhibition of reductive 17βHSD. Reports on the expression of 17βHSD isoforms in prostate cells are contradictory and therefore the endogenously expressed 17βHSDs in LNCaP cells may

include 2 or more of the 14 human isoforms identified to date (Penning & Byrns, 2009; Persson et al., 2009; Prehn, Möller, & Adamski, 2009; Schuster et al., 2011). The influence of rooibos on a specific isoform in LNCaP cells can therefore only be assumed at this time and conclusions can only be drawn in terms of what has been published, specifically in terms of the reductive or oxidative capacity of the 17βHSD isoforms.

The inhibition by rooibos of 17βHSDs can most likely be attributed to the polyphenols present in rooibos. As previously mentioned both quercetin and rutin has been suggested to alter the reductive activity of AKR1C3 and 17βHSD3 activity, respectively, however, in this study rutin exhibited no inhibitory effect (data not shown). Aspalathin and isoorientin were also assayed and were shown to have no inhibitory effect at 10 µM. It should be noted that the flavonoid concentrations of the extracts ranged between 12 µM and 652 µM and as such the inhibitory effect may be due to flavonoids present at higher concentrations. Interestingly, in the presence of rooibos no inhibition of 11βHSD2, converting 11OHA4 to 11KA4, was detected. This result was expected as it has been reported that rooibos inhibits the catalytic activity of 11βHSD1 only, while having no effect on 11βHSD2 (Schloms et al., 2013). Furthermore, in the presence of rutin, increased conversion of 11OHA4 to 11KA4 occurred, suggesting stimulation of 11βHSD2 activity, prompting further investigation.

Having shown the inhibitory effects of 17βHSDs by rooibos and negligible inhibition of SRD5A2 while SRD5A1 activity was unaffected, AKR1C2 and UGTs were additional downstream enzymes considered in the C19 pathway of prostate androgen metabolism (fig. 5.1 f). Data does suggest that rooibos attenuates AKR1C2 activity - rooibos decreased the conversion of DHT to 3α-adiol, however, the influence of rooibos on the catalytic activity of AKR1C2 was not the focus of this study.

In this study it was reported for the first time that rooibos inhibits the conjugation of T, DHT and AST in LNCaP cells. The inhibition of T-G, DHT-G and AST-G formation in the presence of rooibos suggests interference with both UGT2B15 and UGT2B17 activity. Rooibos would therefore result in higher levels of T, DHT and AST within prostatic tissue. Although AST is considered to be an inactive AR ligand, T once converted to DHT becomes a potent AR ligand. Rooibos hinders phase II inactivation of steroids, therefore placing rooibos in an unfavourable position in terms of aiding in treatment regimes against PCa progression. PCa is driven by androgens, and reduced inactivation of AR ligands will lead to subsequent increased cell proliferation and growth. It is possible that the polyphenol compounds in rooibos may hamper the conjugation of androgens with UGT2B15 and UGT2B17 binding flavonoids and conjugating these compounds rather than steroids, resulting in reduced steroid conjugation. Phase II enzymes, such as UGT1A1, UGT1A8 and UGT1A9 have been suggested to be involved in the glucuronidation of green tea catechins (Lu et al., 2003), however, UGT2B15 and UGT2B17 have only been reported to catalyse androgen conjugation.

Although flavonoids are also methylated and sulphonated, it has, however, been suggested that tea polyphenols are preferentially glucuronidated. The levels of phase II enzymes and their activities towards tea polyphenols therefore determine the metabolic profile *in vivo* (Lambert & Yang, 2003; Lu et al., 2003). It has been shown in Japanese males that the main circulating glucuronidated steroids, AST-G and 3α-adiol-G, were present at lower levels than in Western males. This finding led to the suggestion that lower peripheral steroid biosynthesis could account for the lower PCa risk in Japanese males (Ross et al., 1992). This begs the question as to whether diet and lifestyle differences could account for the lower peripheral steroid biosynthesis, since Western diets include high fat foods and Japanese diets are more soy based foods (Messina & Barnes, 1991; Messina et al., 1994; Nair et al., 1984). It is possible that the soy based diets, high in polyphenols, could result in decreased peripheral steroid biosynthesis, in that these compounds may serve either as substrates for, or inhibitors of steroidogenic enzymes. The aforementioned study, however, did not consider UGTs specifically. Although this study showed that rooibos inhibited the glucuronidation of T, DHT and AST further downstream metabolism of these steroids to 3α-adiol, could yield a metabolite which may be glucuronidated more efficiently, as results have shown. The levels of 3α-adiol in the presence of rooibos were not specifically analysed in this study. The modulation by rooibos of the UGTs expressed in the prostate is the focus of future studies in which the possible glucuronidation of rooibos flavonoids will also be investigated.

In this study the influence of rooibos on PSA levels were also investigated, in order to elucidate the possible effects of rooibos' inhibitory action of steroidogenic enzymes on cellular processes. Rooibos resulted in a significant reduction in basal PSA levels, which appears to be dose dependent, however, further studies will be conducted. While rooibos resulted in a significant reduction in PSA levels in DHT-stimulated PSA production in LNCaP cells at concentrations below 0.01 μM, the inhibitory effect of rooibos dissipated at the higher DHT levels (0.1 μM). The inhibitory effect of rooibos suggests less activation of the AR by DHT and subsequently less AR-activated PSA secretion. DHT can be metabolised by 17βHSD (to 5α-dione), AKR1C2 (to 3α-adiol) and UGT2B15/17 (to DHT-G) (fig. 5.16). Reduced PSA levels suggest reduced DHT levels in the presence of rooibos, however, this study shows rooibos reduced UGT2B15 and UGT2B17 activity, oxidative 17βHSD (in HEK293 cells) and AKR1C2 activity. The PSA levels therefore suggest antagonist activity towards the AR in LNCaP cells, specifically as the down-regulation of basal PSA secretion was also detected in the absence of DHT.
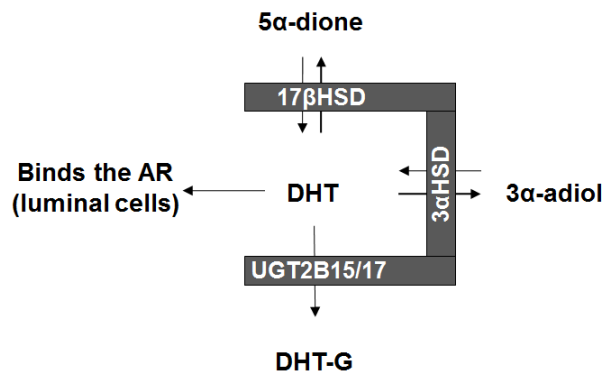
Figure 5.16: DHT metabolism by 17βHSD, AKR1C2 and UGT2B15/17. DHT is also able to bind the AR.

The potential role of phytoestrogens and AR interaction has been reported, with the mutated AR present in LNCaP cells suggestive of promiscuous ligand specificity, allowing other ligands such as phytoestrogens to bind (Mitchell et al., 2009). It has been shown that the T877A mutation in the AR ligand binding domain (Veldscholte et al., 1990) present in LNCaP cells is a common mutation that occurs in about 18% of PCas (Heinlein & Chang, 2004; Shi et al., 2002). In addition, flavonoids have been reported to inhibit PSA levels in breast cancer and PCa cells, the latter transfected with human AR (Zand et al., 2001, 2000). Although mutations of the AR occur in PCa progression, increased ER expression occurs concurrently. It has therefore been suggested that the action of phytoestrogens were mediated through AR as well as ER dependent pathways (Kumar et al., 2011). Indeed in LNCaP cells, steroid hormone-induced PSA secretion was decreased by blocking AR or ERβ, suggesting interaction or "cross-talk" between the mutant AR and ERβ in the regulation of PSA mRNA expression in these cells (Arnold et al., 2007). Phytoestrogens have been reported to be ERβ agonist, which act through the AR and thereby down regulate AR expression and AR-dependent gene expression (Kumar et al., 2011). The possibility therefore exists that rooibos may have either AR antagonist or ERβ agonist activity in LNCaP cells. Furthermore, rooibos significantly reduces PSA levels at lower DHT concentrations, an effect that, however, diminishes at higher concentrations, signifying an androgen level of 0.1 μM that activates the AR and AR-driven genes overriding the inhibitory effect of rooibos.

Attributing the effects of rooibos on PSA levels, in terms of receptor interaction, to flavonoids present in the extract would be purely speculative as specific rooibos flavonoids were not assayed in this study. Quercetin, however, has been shown to inhibit AR protein expression in a dose-dependent manner, as well as inhibiting the secretion of PSA and human glandular kallikrein. The study showed PSA mRNA levels were down-regulated, with quercetin also inhibited AR-mediated PSA expression at the transcriptional level (Xing et al., 2001). Interestingly, quercetin has also been shown to down-regulate ER expression (Miodini et al., 1999). Rooibos has been reported to contain quercetin (Beelders et al., 2012; Beltrán-Debón et al., 2011) and together with quercetin-3-

robinobioside and rutin, which may be metabolised to quercetin (Kim et al., 1998), could therefore possibly contribute toward the effect of rooibos on PSA levels.

While rooibos decreased glucuronidation of T and decreased DHT-stimulated PSA secretion in LNCaP cells, researchers have shown with the exposure of LNCaP cells to biochanin A, an isoflavone, glucuronidation of T is increased and PSA levels decreased. In this study PSA levels were determined after an incubation period of 6 days in media containing 5 µM biochanin A (Sun et al., 1998). It should be noted that in the current study rooibos, contains more than 47 polyphenolic compounds with a ranges of flavonoids present at appreciably higher concentrations. These compounds may exhibit different levels of bioactivity, with some being inhibitory while others may have stimulatory effects, possibly accounting for the decreased glucuronidation of T detected in this study. Both this study and the study above detected reduced PSA secretion, with Sun et al., (1998), suggesting indirect and direct mechanisms, at transcription level, by which biochanin A mediated its effects. The comparative study also reported on preliminary studies detecting minor changes in AR binding sites and AR protein levels, as to account for the reduction in PSA levels (Sun et al., 1998).

In summary, whether rooibos may impact positively on PCa and impede the progression of PCa remains uncertain. Inhibition of reductive 17βHSD activity in prostate cells will reduce active androgen production, however, inhibition of UGTs and AKR1C2 will lead to increased active androgens. Homeostasis between proliferative and apoptotic pathways is maintained in prostate cells, with a disruption of this balance leading to PCa initiation. Progression of PCa is androgen dependent, with ADT treatment strategies employed to reduce active androgen production. Rooibos would have to reduce potent androgen production, with no toxic effects for rooibos to be used as a possible herbal supplement in PCa. This study highlights a possible role for rooibos in PCa – inhibition of reductive 17βHSD activity, reducing the production of steroids such as T while potentially binding the ERβ and/or the AR, thereby modulating AR activation. Together these results indicate a role for rooibos in aiding treatment strategies employed in PCa. This study, however, also shows altered inactivation and phase II metabolism of steroids in LNCaP cells in the presence of rooibos. Although rooibos resulted in reduced glucuronidation of metabolites assayed, together with increased DHT levels due to the inhibition of AKR1C2 activity, PSA levels were decreased both basally and in DHT-stimulated LNCaP cells, indicating positive effects at cellular level.

Chapter 6

Conclusion

The central aim of this study was to investigate whether rooibos influences the catalytic activity of enzymes involved in prostate androgen metabolism. Prostate androgen metabolism involves intricate enzyme pathways that interconvert inactive and active androgens, with the latter driving prostate cell growth and proliferation. These same pathways drive PCa, and progression to CPRC. Treatment regimes include, ADT (chemical or medical castration), together with AR antagonists as well as enzyme inhibitors. However, with all these treatments at hand, and on-going research being conducted into new possible treatment strategies, PCa is still a devastating disease. In this scenario, herbal supplements aiding therapeutic approaches may yet have a role to play. Although natural plant products are considered by many to be unscientific and untested, there is growing interest and support for natural approaches in the treatment of diseases. This study therefore investigated the effects of rooibos on PCa, seeking scientific evidence for medicinal uses supporting a potential role for rooibos in supplementing clinical strategies.

This study reports on the inhibitory effect of rooibos on the catalytic activity of 17βHSD3, AKR1C3, 17βHSD2 and modulation of SRD5A2, heterologously expressed in cell systems. The mode of action by which rooibos inhibited the catalytic activity of these enzymes was not the focus of this study and will, as such be discussed briefly. Muto et al., (2001) reported the inhibition of CYP450 enzymes by green tea catechins, which was achieved by the inhibition of CYP450 together with the inhibition of POR (Muto et al., 2001). CYP450 enzymes form a complex with NADPH-POR which enables P450 enzymes to receive electrons from NADPH via POR (Miller, 2005). Middleton et al., (2000), also suggested enzymes of importance as targets of polyphenols are enzymes with NADPH as a cofactor, as would be the case for CYP450 enzymes as well as reductive 17βHSDs and SRD5As (Middleton Jr. et al., 2000). The possibility therefore exists for rooibos to not only influence the catalytic activity of 17βHSD, but to also influence the complex that forms when 17βHSDs receive electrons from NADPH. Therefore, modulation of the catalytic activity of 17βHSD by rooibos would depend on the levels of 17βHSD expressed in the cell system, the compounds present in rooibos, and the expression and availability of relevant cofactors.

This study also reported on the inhibitory effect of indomethacin on the catalytic activity of reductive 17βHSD towards A4 endogenously expressed in LNCaP cells, to the same extent as in the presence of rooibos. Indeed, it has been proposed that indomethacin, a known AKR1C3 inhibitor, forms an inhibitory complex with AKR1C3, and the redox-partner (NADPH) (fig. 6.1). The reverse reaction may also be inhibited by indomethacin. The mode of action by which rooibos inhibits 17βHSDs, and by which rooibos modulates SRD5A2, may be similar to that of indomethacin.

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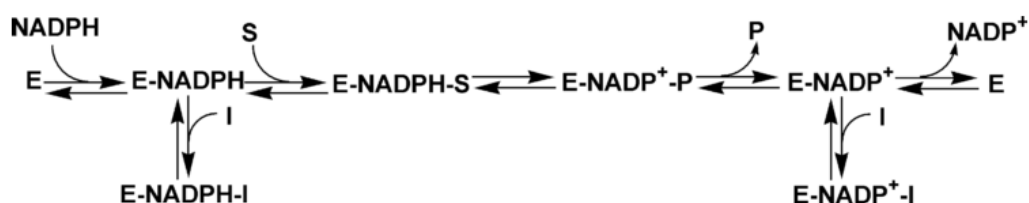
Figure 6.1: Proposed formation of inhibitory complexes by indomethacin during the ordered bi bi reaction catalysed by AKR1C3. Abbreviations as follows: E, enzyme; I, inhibitor; S, substrate; and P, product. Reproduced with permission from (Byrns, Steckelbroeck, & Penning, 2008).

The aforementioned mode of action is further supported as rooibos inhibits the catalytic activity of 11βHSD1, but not that of 11βHSD2 (Schloms et al., 2013), as previously mentioned. The reduction reaction catalysed by 11βHSD1 utilizes the co-factor NADPH, compared to 11βHSD2 which utilizes NAD$^+$ (Miller & Auchus, 2011). In contrast, however, this study also reports on the inhibitory effect of rooibos on the catalytic activity of 17βHSD2, catalysing oxidative reactions dependent on NAD$^+$. This suggests a more complicated role for rooibos - especially if substrate, as well as the aforementioned co-factor specificities, contribute to the inhibitory mode of action of rooibos. Indeed substrate specific inhibition by rooibos of CYP21A2 and CYP11B1, enzymes involved in adrenal steroidogenesis, have been reported (Schloms & Swart, 2014), suggesting that the manner in which rooibos would inhibit the enzyme in question would be dependent on the availability of a specific substrate.

Although the influence of rooibos on androgen metabolism in the prostate cannot be attributed to a single flavonoid, the data presented in this study may indicate synergistic effects by the polyphenolic compounds present in rooibos. In order to determine whether a specific flavonoid compound was contributing to the inhibitory effect of rooibos on steroidogenic enzymes catalysing androgen metabolism in transfected cell systems and in prostate cell models, selected flavonoids were investigated. These included rutin, aspalathin and isoorientin, which, however, exhibited no inhibitory effects. These results demonstrates either that the specific flavonoid compounds were not the contributing compounds, or that the concentrations at which these compounds were assayed, were ineffectual as a compound such as aspalathin was only assayed at 10 µM, while being present at a significantly higher concentration in the extract. It should, however, be considered that the inhibition elicited by rooibos may be due to synergistic effects of the polyphenols present in rooibos. Kumar et al., (2011), showed a significant increase in the bioactivity of three polyphenolic compounds once used in combination. Genistein, quercetin together with biochanin A inhibited the growth of PCa cells, more so than compared to growth in the presence of individual compounds or compounds assayed in dual combinations (Kumar et al., 2011). Rooibos compounds may therefore act synergistically to inhibit the catalytic activity of steroidogenic enzymes in prostate cells.

Although studies reporting on the effects of flavonoid compounds present in rooibos on PCa are limited, one flavonoid compound which has been the subject of many investigations is quercetin. From the results of this study, it appears as if quercetin may be one of the flavonoids involved in the inhibitory effects that rooibos exhibits towards the 17βHSDs as well as the possible AR interaction resulting in reduced PSA levels. It has been suggested that quercetin interferes with the binding of 17βHSDs in the active pocket as well as with AR binding. It should be noted that aglycones of the flavonoids present in rooibos *viz*.: quercetin (from rutin) and apigenin (from vitexin and isovitexin) have been shown to have adverse side-effects (Singh et al., 2012; van der Woude et al., 2003), and therefore not all polyphenol compounds present in rooibos are necessarily beneficial. In this study, however, rutin and vitexin together with isovitexin were not present in high concentrations. Cells to which rooibos was administered received ±12.65 μM rutin and a cumulative ±30.11 μM vitexin and isovitexin. In the consumption of rooibos, rutin may be metabolised to quercetin with vitexin and isovitexin being metabolised to apigenin, and considering further *in vivo* metabolism and conjugation, compounds reaching target tissue may be present at markedly lower levels. It has been suggested in literature that the concentrations of flavonoids used in experimental protocols in cell culture systems far exceed the plasma concentrations that would be reached in human studies (Lambert & Yang, 2003), questioning the effectiveness of supplementation of rooibos and/or individual flavonoids. Furthermore, the levels of these flavonoid compounds pale in comparison to the dihydrochalcones, which accounted for ±794.08 μM to which cells were exposed and to which the bioactivity could be attributed. None the less, it is not to say that rutin, vitexin and isovitexin might not play a role in the inhibitory effects of rooibos observed in this study, as studies have reported cellular effects in the presence of 10 μM of these flavonoids (Schloms & Swart, 2014; Schloms et al., 2013). Considering rooibos as a herbal supplement is tempting, however, dosage amount of flavonoid levels should be considered. Although high levels of flavonoids in rooibos have been reported, these are affected by manufacturing processes which leads to discrepancies in end product and flavonoid levels. Processing together with polyphenol levels being influenced by climate, soil and harvesting methods of the plant, results in marked variations between rooibos production batches (Breiter et al., 2011; Stalmach et al., 2009).

This study reported on the inhibition of PSA levels by rooibos in the absence and presence of DHT. The data suggested that rooibos may act as an AR antagonist or ERβ agonist. ERβ once bound to its ligand, acts via the AR and regulates AR-dependent genes in prostatic tissue (Arnold et al., 2007). In the prostate, ERβ is activated by 3β-adiol and exerts its anti-proliferative activity, while the proliferative action of the AR is driven by DHT. Prostate growth is thus governed by the balance between AR and ERβ activation. 3β-adiol, a product of DHT catalysed by 3-keto reductase, is a high-affinity ligand and agonist of ERβ. Muthusamy et al., (2011), showed that 17βHSD6 converts DHT to 3β-adiol which activates the ERβ reporter in cell models, expressing an estrogen response element-luciferase reporter constructs. This study, however, also showed that

in PCa tissue (Gleason grade higher than 3), both ERβ and 17βHSD6 are undetectable. They concluded therefore that the formation of 3β-adiol via 17βHSD6 from DHT is an important growth regulatory pathway that is lost in PCa (Muthusamy et al., 2011). This raises issues regarding the ERβ agonist role rooibos may play in PCa, to when and if the ERβ is down-regulated in PCa and that the interaction of rooibos and ERβ may not be relevant. It may be that rooibos interacts with the AR and ER in normal prostatic tissue and in BPH, but it is the interaction of rooibos with the AR that would contribute to the effect of rooibos in PCa.

In a recent report emanating from our laboratory, investigating the physiological role of 11OHA4 in PCa, it was shown that the androgenic activity of the C11-keto forms of A4, T and DHT were more androgenic than their respective C11-hydroxy forms (Storbeck et al., 2013). In this study it was shown that 11KT was not conjugated, compared to T which is readily conjugated in LNCaP cells, suggesting 11KT, once formed, would be efficiently channelled to 11KDHT. As previously mentioned 11KDHT possesses agonist activity towards the AR comparable to DHT and may thus have implications in CRPC. As the glucuronidation of C11-keto and C11-hydroxy steroids have, to date, not been elicited, the findings of this study prompt further investigations into the inactivation and conjugation of these compounds since the hydroxyl and keto moieties at the C11 of the steroid carbon backbone may hinder conjugation of these C19 steroids.

It is worth mentioning that the preferred conversions of specific substrates by enzymes were also reported in this study. SRD5A1 was shown to preferentially convert A4 to 5α-dione, compared to the conversion of T to DHT, with the preferential conversion of A4 also being observed in the prostate cell models, *viz.*: PNT2 (4.3.6), LNCaP (5.3.2.1) and BPH-1 and PC-3 (data not shown). As such the 'alternative' 5α-dione pathway is supported, and places this pathway as a dominant pathway in prostate androgen metabolism in these cells.

This study reports on the inhibition of the catalytic activity of 17βHSD3, AKR1C3, 17βHSD2, UGT2B15 and UGT2B17 by rooibos, together with reduced PSA secretion, possibly mediated via AR and/or ER involvement. Although this study could not establish an inhibitory effect on SRD5A1 by rooibos, it appears as if rooibos inhibited SRD5A2 activity towards T, together with possibly inhibiting AKR1C2. This study also showed the modulation of prostate androgen metabolism in PNT2, BPH-1, PC-3 and LNCaP prostate cell models, regarding the C19 steroids involved in both the conventional C19 and in the 11OHA4 pathways. This study therefore provides insight into the influence of rooibos on androgen metabolism in normal prostate cells and in PCa cells, and provides evidence of prostate androgen metabolism modulation and enzymatic inhibition by rooibos extracts.

As reported in this study, the presence of rooibos inhibited the catalytic activity of AKR1C2 in PCa cells, however, this was not tested in an isolated system away from competing enzymes. Future studies would include investigating the effect of rooibos on the catalytic activity of AKR1C2 as well

as that of UGT2B15 and UGT2B17 in transfected cell systems. In addition the ability of the UGTs to conjugated flavonoids compounds in rooibos will also be studied. The ability of rooibos and flavonoid compounds to interfere with the transfer of electrons between redox partners and the enzymes catalysing androgen metabolism will also be explored. Since rooibos interferes with the catalytic activity of steroidogenic enzymes in the prostate cell models, it is possible that it may also interfere with the expression of relevant enzymes. Further investigations can therefore be conducted utilizing both qPCR and Western blot, in order to elucidate the effects of rooibos. Within this approach, 17βHSDs and SRD5As could be included together with AKR1C2 and UGTs. Further investigations would also be included to determine interactions with the AR, AR binding sites and ERβ to fully characterise the influence of rooibos at molecular and cellular levels.

Chapter 7

<u>Research protocols</u>

7.1 Introduction

The completion of this study relied on multiple experimental procedures together with tissue culture techniques, UPLC-MS/MS analysis and data analysis for steroid quantification. This chapter will discuss experimental activities and procedures conducted in the investigation into the influence of rooibos on normal and PCa cells.

7.2 Experimental procedures

7.2.1 Methanol extraction of unfermented rooibos

A rooibos extract was prepared by extracting 20 g unfermented plant material with chloroform (200 mL) for 8 h, followed by a methanol (200 mL) extraction for 8 h using a Soxhlet extractor fitted with a double-wall condenser as previously described (Schloms et al., 2012). Unfermented rooibos plant material, 20 g, was placed in a glass Soxhlet extractor fitted with a double-wall condenser and a drying tube packed with granular calcium chloride. The extractor was fitted to a round-bottom flask containing 200 mL chloroform. The plant material was extracted for approximately 8 h at ±61.15°C, until the effluent from the Soxhlet apparatus was clear. The plant material was subsequently extracted with 200 ml methanol for 8 h at ±64.7°C. The extract was dried at reduced pressure in a rotating evaporator and the vacuum released under nitrogen. The dried extract was redissolved in analytical quality deionised water (Milli-Q^{TM} water purification system), to a final concentration of 86 mg extract/mL and centrifuged at 10 000 x g for 5 min at 4°C. The supernatant was stored in 1 mL aliquots at -20°C. The extract was protected from light and oxygen at all times to avoid any compositional changes. All extracts were centrifuged with a bench-top centrifuge (Heraeus^{TM} Biofuge^{TM}, Thermo Scientific, USA) prior to use, to separate any undissolved matter.

7.2.2 Identification and quantification of rooibos polyphenols

Unfermented rooibos extracts were analysed using HPLC coupled to a diode-array-detector (HPLC-DAD) to identify and quantify polyphenolic compounds, as previously described (Beelders et al., 2012). Aspalathin, nothofagin, 3, 4 dihydroxybenzoic acid, caffeine acid, luteolin, vitexin, quercetin 3-β-D-glucoside, quercetin dehydrate, ferulic acid, rutin hydrate, syringic acid, vanillic acid and p-coumaric acid standards were prepared in methanol. Retention times were determined and UV spectra were recorded. All analyses were performed using a mobile phase consisting of (A) 2% acetic acid in water (v/v) and (B) acetonitrile. HPLC analyses were conducted on an Agilent 1200 series instrument (maximum pressure 400bar) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat and diode-array detector (standard 13 µL flow cell, 10 mm

path length) controlled by Chemostation software (Agilent Technologies). The dwell volume of this system was measured as 0.82 mL.

7.2.3 Plasmid preparation

Luria-Bertani (LB) medium (100 mL), containing ampicillin (100μg/mL), was inoculated with freezer stocks of plasmid-containing *E.coli* cells, 100 μL, overnight at 37°C at 230 rpm (Innova shaking incubator, New Brunswick). The plasmid DNA was isolated using the Nucleobond® Midiprep DNA isolation kit, according to the manufacturer's instructions (Machery-Nagal). After precipitation, the DNA pellet was redissolved in nuclease free water, 200 μL, and the plasmid yield was calculated by UV spectrophotometry (Cary 60 UV-VIS; Agilent technologies), 260/280 nm. The average yield ranged from 100 μg - 200 μg per 100 mL.

7.2.4 Enzymatic assays in transiently transfected HEK293, CHO-K1 and U2OS cells

HEK293 cells were cultured in growth medium, DMEM containing 10% fetal bovine serum, 1.5 g NaHCO$_3$/L (pH 7), and 1% penicillin-streptomycin at 37°C, 5.0% CO$_2$ and 90% relative humidity for 2 to 3 days until 80% confluent. Cells were collected by trypsinization (0.1% trypsin and 0.04% EDTA, 1mL/100mm plate) and centrifuged at 2000 rpm for 5 min, after which the supernatant was discarded and cells pellet resuspended in DMEM. After centrifugation the cells were resuspended in DMEM and replated in a Corning® CellBIND® surface 75 cm^2 flask. This process was repeated over a period of two weeks. After 3-5 passages, cells were replated into 24-well Corning® CellBIND® surface plates at a concentration of 2 x 10^5 cells/mL, 0.5 mL/well and incubated for 24 h. Stocks were also prepared and stored in liquid nitrogen until further use according to ATCC instructions. The replated cells were subsequently transiently transfected with the appropriate plasmid constructs, 1 μg/μL, using the Mirus TransIT®-LT1/XtremeGene HP® DNA transfection reagents, according to the manufacturer's instructions. Cells were transfected by adding transfection reaction mix, 50μL culture media without fetal calf serum or penicillin-streptomycin, which contained plasmid DNA, 0.5 μL, and transfection reagent, 1.5 μL. pClneo (Promega, Madison, USA), containing no insert cDNA, was included as a negative control in experiments. The cells were incubated for 72 h, after which 1 μM of the appropriate steroid substrates was added to the medium. Steroid stock solutions dissolved in absolute ethanol (2 mg/mL) were diluted in culture medium to the desired concentration. In all assays conducted, ethanol added to cells did not exceed 0.01%. Steroid substrates were also added to the cells in the presence of unfermented rooibos extracts (4.3 mg/mL). After the appropriate incubation period medium (500 μL) was removed and the steroid metabolites were extracted by liquid-liquid extraction using 10:1 volume of dichloromethane to culture medium. The samples were vortexed for 10 min, centrifuged at 3500 rpm for 5 min after which the media was aspirated and dichloromethane phase dried at 45°C under nitrogen. The dried steroid residue was redissolved in 150 μL methanol prior to analysis by UPLC-MS/MS. Protocols in which rooibos was assessed included assays in the presence of the

inhibitor, dutasteride (10 μM) in the case of SRD5A catalysed reactions. Experimental volume changes with the addition of rooibos extract were compensated for with the addition of equal volume of deionised water. CHO-K1 (growth and experimental medium, Ham's F12K) and U2OS (growth and experimental medium, DMEM) cells were cultured, replated and transfected using the same experimental protocols. Steroid conversion assays were also conducted in the same manner as the assays conducted in HEK293 cells.

7.2.5 Steroid conversion assays in PNT2, BPH-1, PC-3 and LNCaP cells

PNT2 and BPH-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1.5 g $NaHCO_3$/L (pH 7), 1% penicillin-streptomycin at 37°C, in an atmosphere of 90% humidity and 5% CO_2. PC-3 cells were grown in supplemented Ham's F12K medium. Cells were cultured and maintained as described in the aforementioned section. After 3-4 passages confluent cells were plated into 24-well plates (1 x 10^5 cells/mL, 0.5 mL/well) and incubated for 48 h. The medium was removed and replaced with 0.5 mL culture medium containing steroid substrates (1 μM). Steroid stock solutions in absolute ethanol (2 mg/mL) were diluted in culture medium to the desired concentration. In all assays conducted, ethanol added to cells did not exceed 0.01%. Steroid substrates were also added to the cells in the presence of unfermented rooibos extracts (4.3 mg/mL). The cells were incubated for 48 h after which 0.5 mL aliquots were removed and steroid metabolites extracted as described above. Cortisol-D4 (15 ng) and testosterone-D2 (15 ng), were added to each sample as internal standards. Protocols included assays carried out in the presence of dutasteride (10 μM). Experimental volume changes with the addition of rooibos extract was compensated for with the addition of equal volume of deionised water.

LNCaP cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 1.5 g $NaHCO_3$/L (pH 7), 2.5 g D-(+)-Glucose, 1% penicillin-streptomycin, 1% HEPES and 1% sodium pyruvate using Corning® CellBIND® surface plates at 37°C, in an atmosphere of 90% humidity and 5% CO_2. Cells were plated into 24-well Corning® CellBIND® surface plates (2 x 10^5 cells/mL, 0.5 mL/well) and incubated for 48 h. The medium was removed and replaced with 0.5 mL RPMI-1640 medium containing the following steroid substrates (1 μM): A4, 11OHA4, 11KA4, T, DHT, AST, 3α-adiol. Steroid stock solutions dissolved in absolute ethanol (2 mg/mL) were diluted in culture medium to the desired concentration. In all assays conducted, ethanol added to cells did not exceed 0.01%. Steroid substrates were also added to the cells in the presence of unfermented rooibos extracts (4.3 mg/mL). The cells were incubated for 6 h, 12 h, 24 h and 48 h after which duplicate aliquots (0.5 mL) were removed. Steroids were extracted from one of the aliquots as described above, while steroid metabolites extracted from the other extracted as described above after β-glucuronidase treatment (7.2.8). Cortisol-D4 (15 ng) and testosterone-D2 (15 ng), were added to each sample as internal standards prior to extraction. Protocols included assays in which steroid conversions were assessed in the presence of inhibitors, indomethacin (20 μM; in DMSO), a 17βHSD inhibitor and dutasteride (10 μM; in ethanol), a SRD5A inhibitor. Experimental volume

changes with the addition of rooibos extract was compensated for with the addition of equal volume of deionised water.

7.2.6 Cell viability

The trypan blue exclusion assay was used in this study to assess the influence of unfermented methanolic rooibos extracts on cell viability. Polyphenolic compounds have been shown to interfere with reagents (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) used in the MTT cell viability assay which is commonly used to assess cell viability (Wisman et al., 2008). It is possible to assess cell viability with the MTT assay even if polyphenolic compounds are added to the cell media, controls are, however, required to be in place. In order to assess cell viability and exclude possible interference by the polyphenolic compounds in the rooibos extracts, the trypan blue exclusion method was the preferred method in this study. This method will allow the assessment of the total number of cells present and will enable analysis of live cells and non-viable cells. Viable cells are expressed as a % of the total number of cells counted. Following steroid conversion assays in cells trypsin-EDTA (0.5 mL) was used to collect the cells. The cells of each well were pipetted into individual micro-centrifuge tubes and centrifuged at 13,000 x g for 10 min using a bench-top centrifuge (Heraeus™ Biofuge™, Thermo Scientific, USA). The trypsin-EDTA was aspirated and the cell pellet was redissolved in PBS (250 µL). Cell counts were subsequently carried out, with 10 µL of cell solutions to which 10 µL trypan blue stain were added, using the Countess® automated cell counter (Invitrogen, Eugene, USA).

7.2.7 Protein determination

On completion of steroid conversion assays cells, collected in PBS, were centrifuged at 13,000 x g for 10 min using a bench-top centrifuge (Heraeus™ Biofuge™, Thermo Scientific, USA), the PBS aspirated, the cells lysed with 50 µL passive lysis buffer (Promega, Madison, USA) and stored at -20°C overnight, prior to determining the protein concentration using the BCA method (Pierce Chemical, Rockford, USA) according to the manufacturer's instructions. Control analysis included the BSA standards, 2 mg/mL, 1.5 mg/mL and 1 mg/mL prepared in PBS (according to the manufacturer's instructions), together with rooibos 1 mg/mL (5.815 µL) and 4.3 mg/mL (25 µL) also prepared in 0.5 mL PBS. BSA standards 2 mg/mL, 1.5 mg/mL and 1 mg/mL (20 µL), in the presence of rooibos extracts (20 µL), using 20 µL for the assay, were compared to the standard BSA curve using the BCA protein determination kit according to the manufacturer's instructions. Experimental volume changes with the addition of rooibos extract was compensated for with the addition of equal volume of PBS (20 µL) to the BSA standards.

7.2.8 β-glucuronidase assay

The liquid-liquid steroid extraction described allows only for the isolation of free unconjugated steroids. In order to analyse the conjugated steroid metabolites present in the media, a β-glucuronidase protocol was conducted, according to the manufacturer's instructions to deconjugate steroids. Subsequent UPLC-MS/MS analyses will enable the quantification of all the metabolites present (free and conjugated) in these samples.

Following steroid conversion assays in prostate cells, the pH of the media aliquot (0.5 mL) was adjusted to 6.5 by the addition of 1% acetic acid (± 20 μL) using pH indicator strips (Merck Millipore, Germany). The samples were subsequently treated with 400 units (76 μL) of β-glucuronidase (*E.coli* Type VII-A; Sigma-Aldrich) at 37°C for 24 h to deconjugate glucuronidated steroids prior to liquid-liquid extraction, as described above. β-glucuronidase was prepared in analytical quality deionised water (1 mg/mL) and stored at -20°C prior to assay. Control analysis included DHT and DHT-G (1 μM) added to culture media, prior to a β-glucuronidase treatment followed by steroid extraction. Both DHT and DHT-G were subjected to a β-glucuronidase treatment, and DHT quantified using UPLC-MS/MS. Recovery of 1 μM DHT and unconjugated DHT from DHT-G (fig. 7.1) was detected, denoting complete deconjugation of conjugated steroids. The amount of conjugated steroids were determined by subtracting the amount of free steroids from the amount of total steroids detected using UPLC-MS/MS, and presenting the difference as a % over the total (free + conjugated) amount of steroids.
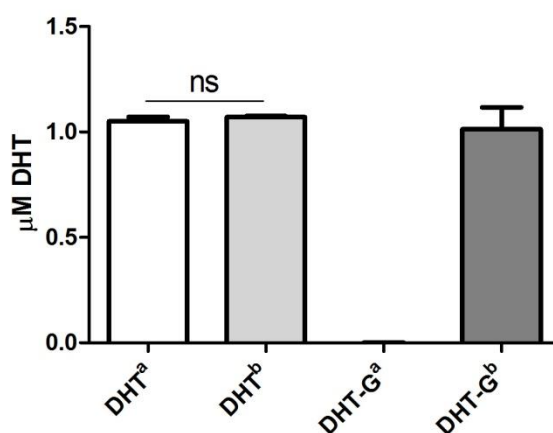
Figure 7.1: DHT and DHT-G (1 μM) analysis representing free steroid[a] analysis by UPLC-MS/MS, and total steroid[b] (free + conjugated) analysis by UPLC-MS/MS following a β-glucuronidase treatment. The experiment was performed in triplicate and results are expressed as the mean ±SEM (*ns*=not significant).

Further control analysis included the aforementioned control analysis in the presence of rooibos (1 mg/mL and 4.3 mg/mL), to determine whether rooibos interfered with the β-glucuronidase assay.

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7.2.9 PSA chemiluminescent immunoassay

LNCaP cells were cultured as described in 7.2.5. Cells were plated into 24-well Corning® CellBIND® surface plates (4 x 10^4 cells/mL, 0.5 mL/well) in phenol free RPMI-1640 media supplemented with 5% charcoal stripped FCS and 1% penicillin-streptomycin. After a 48 h incubation period, the medium was removed and replaced with 0.4 mL/well phenol free RPMI-1640 supplemented with 1% penicillin-streptomycin only. After a 24 h incubation period, phenol free RPMI-1640 media containing steroid substrate DHT (0.001, 0.01, 0.1, 1 μM; 0.1 mL/well) was added to make up 0.5 mL/well. DHT stock solution dissolved in absolute ethanol (2 mg/mL) was diluted in culture medium to the desired concentration. DHT was also added to LNCaP cells in the presence of unfermented rooibos extracts (1 mg/mL and 4.3 mg/mL). The cells were incubated for 96 h after which medium (0.5 mL) was removed and PSA levels analysed using an Access Hybritech PSA chemiluminescent immunoassay, for the quantitative determination of total PSA levels (Access Immunoasay Systems). Briefly, the assay is a two-site immunoenzymatic 'sandwich' assay, using mouse monoclonal anti-PSA alkaline phosphatase conjugate and paramagnetic particles coated with a second mouse monoclonal anti-PSA antibody. The PSA in the sample binds to the monoclonal anti-PSA on the solid phase, while the monoclonal anti-PSA alkaline phosphatase conjugate reacts with a different antigenic site on the bound PSA. After an incubation period, bound PSA are held in a magnetic field and unbound materials are washed away. Light is subsequently emitted upon addition of the chemiluminescent substrate, Lumi-Phos*530, which reacts with the bound PSA. Emission is measured with a luminometer, and is proportional to the concentration of PSA in the sample. The amount of PSA in the sample is determined from a stored, multi-point calibration curve, with detection limits between 0.008 ng/mL and 150 ng/mL (Coulter, 2010). Experimental volume changes with the addition of rooibos extract was compensated for with the addition of equal volume of deionised water. Culture media spiked with rooibos equivalent to the concentration used in the assay was shown not to interfere with the PSA immunoassay.

7.2.10 Separation and quantification of steroid metabolites using UPLC-MS/MS

Stock solutions of A4, 11OHA4, 11OHT, 11KA4, 11KT, T, DHT, DHT-G, 5α-dione, 3α-adiol and AST were dissolved in ethanol (2 mg/mL). A series of standards (0.2 - 20 ng/mL) were prepared in methanol. In the analyses the limit of quantification (LOQ) was set at the lowest standard (0.2 ng/mL) μM value (± 0.017 – 0.025 μM).

Steroid metabolites were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP column (2.1 mm x 100 mm, 2.6 μm) (Torrance, CA, USA) as previously described (Storbeck et al., 2013). The mobile phases consisted of 1% formic acid (A) and 49%: 49%: 2% methanol: acetonitrile: isopropanol (B). As mentioned previously the separation of the C19 steroids in the 11OHA4 pathway were analyses on a separate method to the C19

steroids in the conventional C19 pathway. C19 steroids in the conventional pathway were eluted at a flow rate of 0.4 mL/min, using a linear gradient from 15% B to 40% B in 1.0 min, followed by a linear gradient to 55% B in 3.5 min and a subsequent linear gradient to 100% B in 0.1 min. C19 steroids in the 11OHA4 pathway were eluted at a flow rate of 0.4 mL/min, using a linear gradient from 15% B to 38.5% B in 3.5 min, followed by a linear gradient to 100% B in 0.1 min. The injection volume was 5 µL and the total run time was 5 min per sample injection for both separation methods. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection and all steroids were analysed in multiple reaction monitoring (MRM) mode. An electrospray in the positive ionization mode (ESI+) was utilized in the MRM mode. The capillary voltage of 3.5 kV, cone voltage of 15-35 V and collision energy of 4-36 eV was set during this quantification (table 7.1). The following settings were used: source temperature 120°C, desolvation temperature 400°C, desolvation gas 900 L h^{-1} and cone gas 50 L h^{-1}. Calibration curves were constructed using weighted (1/$x2$) linear least squares regression and the data was collected with the MassLynx 4.1 software program.

Table 7.1: Parameters for the detection and quantification of C19 steroids by UPLC-MS/MS: retention times (RT, min), cone voltages (CV) and collision energy (CE).

Steroid metabolite	RT (min)	Precursor ion	CV	Product ion A	CE	Product ion B	CV	Product ion C	CE
conventional C19 method									
A4	3.12	287.2	30	96.9	15	108.8	15	-	-
T	2.97	289.2	30	97.2	22	109.0	22	-	-
5α-dione	3.55	289.2	30	119.2	35	171.2	35	-	-
DHT	3.33	291.2	25	255	15	273	20	-	-
AST	3.57	273.2	15	105.3	30	147	25	-	-
3α-adiol	3.24	275.2	15	161	15	175	15	257	15
11OHA4 method									
11OHA4	3.15	303.2	30	121	30	267.2	15	-	-
11OHT	3.00	305.3	35	121	20	269	15	-	-
11KA4	3.31	301.2	35	241	30	257	25	-	-
11KT	2.97	303.2	30	121	20	267	20	-	-

Possible interference by rooibos in the analysis of steroids by UPLC-MS/MS was investigated, and no interference was detected when rooibos (1 mg/mL and 4.3 mg/mL) in culture media only, was analysed by UPLC-MS/MS. No change to the base line spectra was detected (fig. 7.2 a/b).
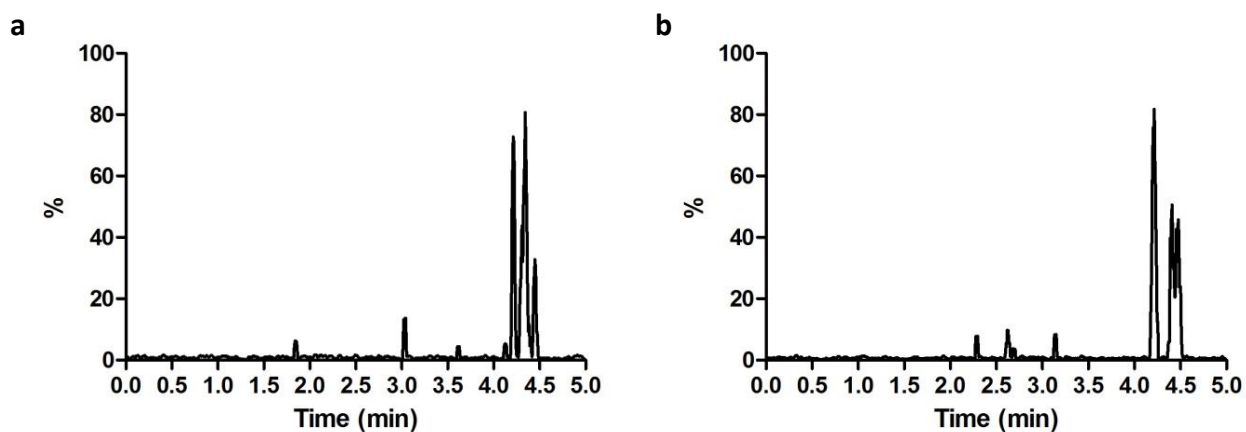
Figure 7.2: UPLC-MS/MS chromatogram of: (a) rooibos (1 mg/mL) in culture media; and (b) rooibos (4.3 mg/mL) in culture media. Cluster of peaks detected at ±4.1-4.5 min represents the background 'noise' generated by the UPLC-MS/MS.

7.2.11 Statistical analysis

All experiments were performed in triplicate and the subsequent results are given as means ± SEM. Statistics were calculated by an unpaired t-test or by a one-way ANOVA, followed by a Dunnett's multiple comparison test using GraphPad Prism (version 5) software (GraphPad Software, San Diego, California). Differences were considered statistically significant at $P<0.05$.

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