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# Inefficient UGT-conjugation of adrenal 11β-hydroxyandrostenedione metabolites highlights C11-oxy $C_{19}$ steroids as the predominant androgens in prostate cancer

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ABSTRACT

Although the adrenal $C_{19}$ steroids, androstenedione and testosterone, contribute to prostate cancer (PCa) progression the full complement of adrenal androgens, including the C11-oxy $C_{19}$ steroids, 11β-hydroxyandrostenedione (11OHA4) and 11β-hydroxytestosterone (11OHT) and their androgenic metabolites, 11keto-testosterone (11KT) and 11keto-dihydrotestosterone (11KDHT) have, to date, not been considered. This study investigated the contribution of 11OHA4 and 11OHT to the pool of active androgens in the prostate. Steroid profiles were determined in LNCaP, C4-2B and VCaP cell models, in PCa tissue, and in plasma focussing on the inactivation, reactivation and glucuronidation of 11OHA4, 11OHT and their downstream products using ultra-performance convergence chromatography tandem mass spectrometry ($UPC^2$-MS/MS). The C11-oxy $C_{19}$ steroids were the predominant steroids with the production of 11KT and 11KDHT in prostate cell models identifying 11β-hydroxysteroid dehydrogenase type 2 activity. Active:inactive steroid ratios indicated efficient inactivation of dihydrotestosterone (DHT) and 11KDHT by 3α-hydroxysteroid dehydrogenases, while the reactivation of DHT by retinol-like dehydrogenases was greater than the reactivation of 11KDHT. In PCa tissue, inactive C11-oxy $C_{19}$ steroids ranged from 27 to 30 ng/g, whereas inactive $C_{19}$ steroids were below 1 ng/g. Steroid glucuronidation was impeded: in VCaP cells, the C11-oxy $C_{19}$ steroids were unconjugated and the $C_{19}$ steroids fully conjugated; in C4-2B cells, all steroids were unconjugated, except for DHT of which 50% was conjugated; in LNCaP cells only androsterone, 11KT and 11β-hydroxyandrosterone were unconjugated. In PCa patients' plasma 11KDHT was present only in the unconjugated form, with 11KT also predominantly unconjugated (90–95%). Even though plasma and tissue sample numbers were limited, this study serves to demonstrate the abundance of C11-oxy $C_{19}$ steroids, with notable differences in their metabolism, dictated by steroidogenic enzymes and hampered conjugation, affecting active androgen levels. Larger cohorts are required to analyse profiles in modulated metabolic pathways, in order to shed light on treatment outcomes. The C11-oxy $C_{19}$ steroids are involved in PCa, with impeded glucuronidation in PCa ascribing a dominant role to these steroids in disease progression.



*Abbreviations:* UDP, uridine diphosphate; UGT, UDP-glucuronosyltransferases; 17βHSD, 17β-hydroxysteroid dehydrogenase; AKR1C3, 17β-hydroxysteroid dehydrogenase type 5; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; SRD5A, 5α-reductase; SRD5A1, 5α-reductase type 1; SRD5A2, 5α-reductase type 2; 3αHSD, 3α-hydroxysteroid dehydrogenase; UGT2B, UDP-glycosyltransferase 2 family polypeptide B; RL-HSD, retinol-like hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; A4, androstenedione; T, testosterone; 5αDIONE, 5α-androstanedione; DHT, dihydrotestosterone; AST, androsterone; 3αDIOL, 5α-androstane-3α 17β-diol; 11OHA4, 11β-hydroxyandrostenedione; 11OHT, 11β-hydroxytestosterone; 11KA4, 11keto-androstenedione; 11KT, 11keto-testosterone; 11KDHT, 5α-dihydro-11-keto-testosterone; 11OH-5αDIONE, 11β-hydroxy-5α-androstanedione; 11OHDHT, 5α-dihydro-11β-hydroxytestosterone; 11K-5αDIONE, 11-keto-5α-androstanedione; 11K-3αDIOL, 11-keto-5α-androstane-3α,17β-diol; 11OHAST, 11β-hydroxyandrosterone; 11KAST, 11-ketoandrosterone; -G, glucuronide.

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## 1. Introduction

It is widely accepted that although the adrenal $C_{19}$ steroids, commonly referred to as androgens, do not exhibit androgenic activity, they serve as precursors to more potent androgens biosynthesized in peripheral tissue. This holds true when considering dehydroepiandrostenedione (DHEA), androstenedione (A4) and 11β-hydroxyandrostenedione (11OHA4) (Bloem et al., 2015; Chang et al., 2011; Du Toit et al., 2017; Mizokami et al., 2009; Mohler et al., 2011; Mostaghel, 2014; Storbeck et al., 2013; Swart et al., 2013). In addition, the adrenal also produces 11β-hydroxytestosterone (11OHT) and 11keto-testosterone (11KT) (Rege et al., 2013) which, at 10 nM, have been shown to be capable of activating the androgen receptor (AR), comparable to dihydrotestosterone (DHT) (Bloem et al., 2015). Testosterone (T) is targeted in androgen deprivation therapy (ADT) aimed at reducing DHT, and has been the primary approach in the treatment of metastatic prostate cancer (PCa) for decades. The disease, which is dependent on active androgens, nevertheless progresses to castration-resistant prostate cancer (CRPC) with intratumoral DHT levels still capable of activating the AR (Chang et al., 2011; Mohler et al., 2011; Mostaghel, 2014). Adrenal A4 has been implicated in driving PCa in the absence of testicular T as it is converted to DHT in the prostate via 5α-androstanedione (5αDIONE) in the alternative pathway. The conversion of A4 to 5αDIONE, catalysed by 5α-reductase (SRD5A) is more efficient than that of T, with the upregulation of SRD5A type 1 (SRD5A1) and the concomitant down-regulation of SRD5A type 2 (SRD5A2) confirmed in the progression to CRPC (Chang et al., 2011; Montgomery et al., 2008; Stanbrough et al., 2006; Thomas et al., 2008; Titus et al., 2005).

A4 is however not the only adrenal androgen precursor contributing to CRPC with 11OHA4 also in circulation with levels having been reported to be ~2 fold higher than A4 (Rege et al., 2013). We have shown that 11OHA4 is converted to potent androgens, 11KT and 11-ketodihydrotestosterone (11KDHT) by the same steroidogenic enzymes catalysing the production of DHT with 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) and SRD5A playing pivotal roles (Bloem et al., 2015; Storbeck et al., 2013; Swart et al., 2013). The adrenal also produces 11-ketoandrostenedione (11KA4), 11OHT and 11KT, albeit at levels considerably lower than 11OHA4 (~0.99, 0.48, 0.39 and 157 nM, respectively) measured in female adrenal vein samples (Rege et al., 2013). These C11-oxy steroids are readily reduced by SRD5A, with 11KT and 11KDHT capable of activating the AR to the same degree as DHT, and of inducing androgen dependent gene expression (Bloem et al., 2015; Pretorius et al., 2016; Storbeck et al., 2013). Due to the improved capabilities of analytical technologies in the field of liquid chromatography tandem mass spectrometry (LC-MS/MS), we have also shown that these steroids are present in serum and tissue of PCa patients (Du Toit et al., 2017), which therefore begs the question as to the exclusivity of DHT being the sole potent androgen in terms of contributing to the progression of PCa. Furthermore, it is evident that the steroid profile in PCa remains incomplete and skewed since neither the C11-oxy androgens nor their conjugation are taken into account.

The biosynthesis of active androgens from their adrenal precursor $C_{19}$ steroids and their subsequent inactivation are dependent on the expression levels of the relevant steroidogenic enzymes in peripheral tissue. Furthermore, the conjugation by the uridine diphosphate (UDP)-glucuronosyltransferase (UGT) isoforms adds another level of complexity with the expression of these enzymes characterized by copy number variation, the use of alternative promoters, polymorphisms, alternative splicing and exon skipping (Guillemette et al., 2010; Du Toit and Swart, 2016). We have shown 11KT is not conjugated as readily as T, DHT or 11KDHT by the UGT enzymes (Du Toit et al., 2017). The irreversible conjugation by the UGT enzymes contributing to the inactivation and reactivation equilibrium directly impact active androgen levels, not only in the prostate affecting PCa treatment strategies, but also in other clinical conditions which are characterized by adrenal-derived androgen excess. Increased C11-oxy $C_{19}$ steroid levels have been detected in classic 21-hydroxylase deficiency patients and it has been suggested that these steroids could act as specific biomarkers of adrenal androgen excess (Turcu et al., 2016).

In the $C_{19}$ steroid metabolic pathway, T, DHT, androsterone (AST) and 5α-androstane-3α,17β-diol (3αDIOL) are substrates for the conjugation by the UDP-glycosyltransferase 2 family, polypeptide B (UGT2B) enzymes (Fig. 1). DHT is inactivated by the 3α-hydroxysteroid dehydrogenase (3αHSD) enzymes to 3αDIOL, and, if not conjugated and secreted, 3αDIOL may be reactivated to DHT by the retinol-like hydroxysteroid dehydrogenase (RL-HSD) enzymes. AST can undergo the same conversion by the RL-HSD enzymes to 5αDIONE, followed by the 17β-hydroxysteroid dehydrogenase (17βHSD) reduction producing DHT (Supplemental Fig. 1).

In the C11-oxy $C_{19}$ steroid metabolic pathway, 11OHT, 11KT, 11β-hydroxydihydrotestosterone (11OHDHT), 11KDHT, 11β-hydroxyandrosterone (11OHAST), 11-ketoandrosterone (11KAST) and 11-keto-5α-androstane-3α,17β-diol (11K-3αDIOL) are potential substrates for glucuronidation enzymes (Fig. 1). 11KDHT may be inactivated by the 3αHSD enzymes to 11K-3αDIOL, which may be reactivated to 11KDHT by the RL-HSD enzymes if unconjugated. 11OHAST and 11KAST may undergo the same conversion by RL-HSD enzymes to 11β-hydroxy-5α-androstenedione (11OH-5αDIONE) and 11-keto-5α-androstenedione (11K-5αDIONE), respectively. The subsequent conversion of 11OH-5αDIONE by 11βHSD2 to 11K-5αDIONE, followed by the 17βHSD reduction of the latter leads to the production of 11KDHT. 11OHDHT is not converted by the 3αHSD enzymes (Supplemental Fig. 1).

The aim of this study was to investigate the inactivation, reactivation and UGT-conjugation in the metabolism of the $C_{19}$ and the C11-oxy $C_{19}$ steroids in PCa cell models, plasma and tissue. The metabolism of 11OHA4 and 11OHT was firstly assayed in C4-2B and VCaP cell models together with that of T and 11OHDHT. Steroid profiles were established which allowed the analyses of active and inactive androgen levels. The reversible inactivation and reactivation of DHT and 11KDHT by 3αHSD and RL-HSD enzymes were subsequently assayed in LNCaP, VCaP and C4-2B prostate models expressing these enzymes endogenously. Analyses of the unconjugated steroids in prostate tissue of a CRPC patient produced profiles for comparative analyses of the full spectrum of active and inactive androgens. In addition, PCa cell models were used to assess glucuronidation in terms of unconjugated and conjugated metabolites. The $C_{19}$ and the C11-oxy $C_{19}$ glucuronide metabolites were subsequently identified in normal and in PCa plasma.

## 2. Material and methods

### 2.1. Tissue and plasma

Human PCa tissue and plasma samples were collected in a study investigating clinical markers of PCa (reference no. N09/11/330; Faculty of Medicine and Health Sciences, Stellenbosch University and Tygerberg Hospital, South Africa). The PCa tissue sample was obtained from a patient aged 83, classified as having CRPC, who underwent a bilateral orchiectomy (BO). Plasma was obtained from a normal subject, aged 59 and PCa patients, aged 74 who underwent BO and aged 66 who received luteinising hormone-releasing hormone analogue treatment (LHRHa). The tissue sample was snap-frozen in liquid nitrogen upon resection and stored with plasma samples at −80 °C.

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