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11-Oxygenated androgen precursors are the preferred substrates for aldo-keto reductase 1C3 (AKR1C3): implications for castration resistant prostate cancer.

Monique Barnard¹, Jonathan L. Quanson¹, Elahe Mostaghel², Elzette Pretorius¹, Jacky L. Snoep^{1,3,4}, and Karl-Heinz Storbeck^{1,#}

¹Department of Biochemistry, Stellenbosch University, Stellenbosch, 7600, South Africa ²Fred Hutchinson Cancer Research Centre, Seattle, WA, USA ³Department of Molecular Cell Physiology, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands ⁴MIB, University of Manchester, Manchester, UK

Abstract

The progression of castration resistant prostate cancer (CRPC) is driven by the intratumoral conversion of adrenal androgen precursors to potent androgens. The expression of aldo- keto reductase 1C3 (AKR1C3), which catalyses the reduction of weak androgens to more potent androgens, is significantly increased in CRPC tumours. The oxidation of androgens to their inactive form is catalysed by 17 β -hydroxysteroid dehydrogenase type 2 (17 β HSD2), but little attention is given to the expression levels of this enzyme. In this study, we show that the 11-oxygenated androgen precursors of adrenal origin are the preferred substrate for AKR1C3. In particular we show that the enzymatic efficiency of AKR1C3 is 8- and 24-fold greater for 11-ketoandrostenedione than for the classic substrates androstenedione and 5 α - androstenedione, respectively. Using three independent experimental systems and a computational model we subsequently show that increased ratios of AKR1C3:17 β HSD2 significantly favours the flux through the 11-oxygenated androgen pathway as compared to the classical or 5 α -androstenedione pathways. Our findings reveal that the flux through the classical and 5 α -androstenedione pathways are limited by the low catalytic efficiency of AKR1C3 towards classical androgens combined with the high catalytic efficiency of 17 β HSD2, and that the expression of the oxidative enzyme therefore plays a vital role in determining the steady state concentration of active androgens. Using microarray data from prostate tissue we confirm that the AKR1C3:17 β HSD2 ratio is significantly increased in patients undergoing androgen deprivation therapy as compared to benign tissue, and further increased in patients with CRPC. Taken together this study therefore demonstrates that the ratio of AKR1C3:17 β HSD2 is more important than AKR1C3 expression alone in determining intratumoral androgen levels and that 11-oxygenated androgens may play a bigger role in CRPC than previously anticipated.

Address all correspondence to: Karl-Heinz Storbeck, storbeck@sun.ac.za.

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Graphical abstract

Keywords

11-oxygenated androgens; aldo-keto reductase 1C3; 17β-hydroxysteroid dehydrogenase 2; 11-ketotestosterone; castration resistant prostate cancer

1. Introduction

Prostate cancer is a prevalent disease amongst men. The primary treatment of advanced cases of this androgen dependent disease is androgen deprivation therapy (ADT), which is accomplished by physical or chemical castration [1]. While initially effective the cancer invariably re-emerges, and is then termed castration resistant prostate cancer (CRPC).

Numerous studies have shown that CRPC remains androgen dependent despite the castrate levels of circulating testosterone and that CRPC is characterised by the reactivation of signalling via the androgen receptor (AR) [2]. Indeed, clinical trials with drugs such as abiraterone and enzalutamide, which target the reactivation of the AR-axis, have yielded compelling evidence for the continued role of androgens in CRPC [3-8]. Furthermore, numerous studies have shown that CRPC tumours upregulate the expression of key steroid metabolising enzymes and therefore acquire the ability to convert circulating adrenal androgen precursors to active androgens [9-11]. It should, however, be noted that other mechanisms, such as overexpression of AR, AR mutations and constitutively active AR splice variants, have also been implicated in the development of CRPC [12]. Nonetheless, the inhibition of adrenal derived androgens remains a promising treatment strategy [13].

Currently the most recognised pathway pertaining to the intratumoral activation of adrenal androgens is the so-called alternate 5α-dione pathway (Figure 1A). In this pathway, the adrenal androgen precursors DHEA and androstenedione (A4) are converted to the intermediate, 5α-androstenedione (5α-dione), while bypassing the production of testosterone (T) (Figure 1A). 5α-Dione is subsequently converted to the most potent androgen, 5α-dihydrotestosterone (DHT), by the enzyme aldo-keto reductase 1C3 (AKR1C3), otherwise known as 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) [14,15]. Studies by our group have recently shown that the 5α-dione pathway may not be the only pathway contributing to the intratumoral androgen pool in CRPC. We have shown that the abundant adrenal steroid, 11β-hydroxyandrostenedione (11OHA4), is not a dead-end product of adrenal steroidogenesis as previously thought, but rather a precursor to the potent androgens 11-ketotestosterone (11KT) and 11-keto-5α-dihydrotestosterone (11KDHT) (Figure 1B) [16-18]. 11OHA4 is converted to 11-ketoandrostenedione (11KA4), by 11β-

hydroxysteroid dehydrogenase type 2 (11 β HSD2). 11KA4 is subsequently converted to 11KT by AKR1C3. 11KT can then be reduced by steroid 5 α -reductase type 1 (SRD5A1), yielding 11KDHT, which is equipotent to DHT [16]. Both 11KT and 11KDHT have been shown to induce androgen dependent gene expression, protein expression and cell growth in androgen dependent cell lines and both steroids have been detected in prostate cancer tumours [16,19].

As is evident from above, AKR1C3, plays an important role in both the 5 α -dione and 11OHA4 pathways of intratumoral androgen activation. It is therefore not surprising that numerous studies have shown that this is one of the key enzymes that is upregulated during the development of CRPC, presumably to maintain intratumoral androgen levels [10,20-22]. The upregulation of AKR1C3 has also been linked to resistance to both abiraterone and enzalutamide, with combined treatment with the AKR1C3 inhibitor, indomethacin reversing resistance towards both drugs *in vivo* and *in vitro* [23,24]. Furthermore, AKR1C3 expression has been correlated with Gleason score and recurrence status [23]. While AKR1C3 catalyses the 17 β -reduction of androgen precursors to their active forms, 17 β -hydroxysteroid dehydrogenase (17 β HSD2) catalyses the reverse oxidative reactions. The expression levels of 17 β HSD2 in CRPC are not often considered with only a few studies showing that the expression of the enzyme remains relatively constant or is downregulated [25]. As AKR1C3 and 17 β HSD2 catalyse opposing reactions it is important to consider the expression levels of both enzymes together with their kinetic parameters when considering the flux through intratumoral androgen pathways. We therefore set out to characterise both enzymes towards classical and 11-oxygenated substrates. We subsequently investigated the effect of different AKR1C3:17 β HSD2 ratios on the flux through the 5 α -dione and 11OHA4 pathways. Our results show that the 11-oxygenated androgen precursors, 11KA4 and 11keto-5 α -androstanedione (11 K-5 α -dione) are significantly better substrate for AKR1C3 than A4 or 5 α -dione, and that increased AKR1C3:17 β HSD2 ratios have a significantly greater effect on the flux through the 11OHA4 pathway when compared to the 5 α -dione pathway. This study therefore adds to the growing body of evidence that 11-oxygenated androgens should no longer be ignored when considering the development and progression of CRPC.

2. Materials and Methods

2.1 Cell lines

LNCaP and PC3 cells were purchased from the European Collection of Cell Cultures (ECACC). LNCaP cells were cultured in RPMI-1640 media supplemented with 10% FBS, 1.5 g/L NaHCO₃, 2.5 g/L D-(+)-Glucose, 1% HEPES, 1% sodium pyruvate and 1% penicillin-streptomycin, while PC3 cells were cultured in HAMs F12K media supplemented with 10% FCS, 1.5 g/L NaHCO₃ and 1% penicillin-streptomycin. HEK293 and VCaP cells were purchased from the American Type Culture Collections (ATCC) and cultured in DMEM media supplemented with 10% FCS, 1.5 g/L NaHCO₃, 1% sodium pyruvate and 1% penicillin-streptomycin. C4-2B cells, a generous gift from Professor D Neal (University of Oxford, UK) were cultured in RPMI-1640 media supplemented with 10% FBS, 1.5g/L NaHCO₃, 2.5 g/L D-(+)-Glucose and 1% penicillin-streptomycin. All cell lines were grown at 37 °C in 90% humidity and 5% CO₂.

2.2. Steroids

17 β -Dihydroandrosterone (5 α -androstane-3 α ,17 β -diol; 3 α -adiol), Androstanedione (5 α -androstane-3,17-dione; 5 α -dione), Androstenedione (androstene-3,17-dione; A4), Androsterone (5 α -androstan-3 α -ol-17-one; AST), 5 α -dihydrotestosterone (5 α -androstan-17 β -ol-3-one; DHT), Drospirenone (17-Hydroxy-6 β ,7 β :15 β ,16 β -dimethylene-3-oxo-17 α -pregn-4-ene-21 carboxylic acid, DRSP), dutasteride ((5 α ,17 β)-*N*-[2,5-Bis(trifluoromethyl)phenyl]-3-oxo-4-azaandrost-1-ene-17-carboxamide), gestodene (4,15-estradien-17 β -ethynyl-18-homo-17 α -ol-3-one, GES), indomethacin (1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid) and testosterone (4-androsten-17 β -ol-3-one; T) were purchased from Sigma Aldrich (St. Louis, USA). 11-Ketoandrostenedione (4-androstene-3,11,17-trione; 11KA4), 11-ketotestosterone (4-androsten-17 β -ol-3,11-dione; 11KT), 11-keto-5 α -dihydrotestosterone (5 α -androstan-17 β -ol-3,11-dione; 11KDHT) and 11-ketoandrosterone (5 α -androstan-3 α -ol-11,17-dione; 11KAST) were purchased from Steraloids (Wilton, USA). Testosterone-1, 2-D2 (T-D2) was purchased from Cambridge Isotope Laboratories (Andover, USA).

2.3 Plasmids

A pcDNA3 vector containing AKR1C3, as well as a pcDNA4 vector containing 17 β HSD2 were both generous gifts from Prof. J. Adamski (Institute of Experimental Genetics, Helmholtz Zentrum München, Germany).

2.4 Determination of kinetic parameters for AKR1C3 and 17 β HSD2 and computational model construction

HEK293 were plated in in a 75 cm² flasks until 80% confluent and subsequently transfected with 1 μ g of 17 β HSD2 using X-tremeGENE transfection DNA reagent (Roche) according to the manufactures protocol. Twenty-four hours later cells were re-plated at a density of 5×10^4 cells/well (300 μ L) in Corning® CELLBIND® 48 well plates. Cells were incubated for a further 24 hours to ensure reattachment. The media was subsequently replaced with media containing the appropriate steroid substrates (1 - 20 μ M for AKR1C3 and 0.25 - 20 μ M for 17 β HSD2) and aliquots (250 μ L) were collected at suitable time intervals. Steroid metabolism was quantified using ultra-high performance supercritical fluid chromatography tandem mass spectrometry (UHPSFC-MS/MS) as described below. Kinetic parameters were subsequently determined in Wolfram Mathematica (version 11) using triplicate data obtained from the same transfection as to ensure equal enzyme expression. We assumed a rapid equilibration of the substrate and product over the cell membrane. To estimate the kinetic parameters for the individual enzymes we assumed Michaelis Menten kinetics and fitted the $V_{\max, \text{app}}$ and $K_{\text{m, app}}$ values by minimising the sum of the squared differences between the data and model, weighted by the variance using the NMinimize function. The kinetic parameters were subsequently validated by their ability to predict the experimental data from subsequent independent experiments. Differences in transfection efficiencies between experiments were accounted for by measuring the initial rates of selected substrates for each experiment. The validated kinetic parameters were subsequently used to model subsequent experimental data. The model consists of ordinary differential equations (ODEs) for the metabolites, e.g. $d(11KA4)/d(T) = v_{17\beta HSD2}(11KT) - v_{AKR1C3}(11KA4)$, and was

numerically integrated using the NDSolve routine in Mathematica to obtain metabolite profiles that can be compared to the experimental data. In addition, the model was used to calculate the steady state concentrations of intermediates for different AKR1C3:17 β HSD2 ratios (Section 2.5), by setting the ODEs to zero and then solving for the experimentally determined metabolite concentrations.”

2.5. Determination of the effect of different AKR1C3:17 β HSD2 ratios

HEK293 cells were transfected with 1 μ g of 17 β HSD2, AKR1C3 or pCINeo respectively as described above. Twenty-four hours later cells were counted and combined in different ratios of 17 β HSD2:AKR1C3 before re-plating into Corning® CELLBIND® surface 24 well plates at a density of 2.1×10^5 cells per well (500 μ L). After 24 hours, the media was replaced with media containing either 0.1 or 1 μ M A4, 11KA4, 5 α -dione or a combination of A4 and 11KA4. Aliquots were collected 24 hours after the addition of substrate.

PC3 cells were plated in 12 well plates at a density of 2×10^5 cells/well (1 mL). After 24 hours, the cells were transfected with a total of 1 μ g plasmid DNA per well using XtremeGENE transfection DNA reagent. Plasmid DNA consisted of 0 μ g, 0.25 μ g, 0.5 μ g or 1 μ g of the AKR1C3 plasmid. The plasmid vector, pCINeo, was used to ensure all ratios contained an equal amount of plasmid DNA. After 72 hours, the media was replaced with media containing either 0.1 or 1 μ M A4, 11KA4, 5 α -dione or a combination of A4 and 11KA4. Dutasteride (10 μ M) was included in all PC3 cell experiments in order to inhibit downstream metabolism. Aliquots (500 μ L) were collected 24 hours after the addition of substrate and subsequently analysed by UHPSFC-MS/MS, as described below.

2.6. Steroid metabolism in prostate cancer cell lines

VCaP, LNCaP and C4-2B cells were plated in 24 well plates at cell density of 1×10^5 cells/well (500 μ L) for VCaP cells and 2.5×10^4 cells/well (500 μ L) for LNCaP and C4-2B cells. After 24 hours, the media was replaced with media containing 0.1 μ M A4, 11KA4, T or 11KT as well as 10 μ M dutasteride. Aliquots (500 μ L) were collected every 24 hours for a total of 4 days. All samples were deconjugated with β -glucuronidase from bovine liver prior to extraction. Samples were acidified to a pH of 6.5 using 1% acetic acid. Eight-hundred units of β -glucuronidase was added to each sample after which the samples were incubated for 24 hours at 37 °C. Steroid extraction and quantification by UHPSFC-MS/MS was subsequently performed, as described below.

2.7. RNA isolation

Total RNA isolation was isolated using Tri-Reagent® (Sigma) and cDNA subsequently synthesized using the The GoScript™ Reverse Transcription System kit (Promega). Quantitative PCR was performed using the KAPA SYBR® FAST qPCR Master Mix for LightCycler® and a LightCycler® 96, rapid thermal cycler instrument (Roche Life Science). Primer sequences were as follows, 17 β HSD2 (forward) 5' - TCTTTGGAAGTGTGGAGGTCACAAAGACGT -3' [25], 17 β HSD2 (reverse) 5' - AGAGAAGTCCCTTGCTGGCTAACGAGTTGAT -3' [25], AKR1C3 (forward) 5' - AGCCAGGTGAGGAAGTTTC -3' [26], AKR1C3 (reverse) 5' - ATCACTGTTAAATAGTGGAG -3' [26], the reference gene, delta-aminolevulinic

synthase (ALAS) (forward) 5'- TTCCACAGGAGCCAGCATAC -3' [16] and ALAS (reverse) 5'- GGACCTTGGCCTTAGCAGTT -3' [16]. PCR efficiency exceeded 90% for all primer sets.

2.8. Steroid quantification by ultra-high performance supercritical fluid chromatography tandem mass spectrometry (UHPSFC-MS/MS).

Steroids were extracted from media using tert-Butyl methyl ether (MTBE) at a ratio of 3:1 (MTBE:media) as previously described [27]. Briefly, samples were shaken for 10 min, allowed to stand for 5 min and then incubated at -80 °C for an hour to allow the aqueous layer to freeze. The MTBE layer containing the steroids was subsequently transferred to a clean test tube and evaporated under a stream of nitrogen gas. Samples were then resuspended in 50% methanol (150 uL) and stored at -20 °C prior to analysis.

Steroids were separated using an ACQUITY UPC² system (Waters Corporation, Milford, USA) with an ACQUITY UPC² BEH 2-EP column (3 mm X 100 mm, 1.7 µm particle size) as previously reported [27]. Supercritical CO₂ was used as the primary mobile phase and was combined with methanol, which was used as an organic modifier. The gradient profile for the 5 min run is described by Quanson et al. [27]. The flow rate was kept constant at 2.0 mL/min and the column temperature and automated back pressure regulator (ABPR) were set to 60 °C and 2000 psi, respectively. The injection volume was 2 µL for all standards and samples. A make-up pump was set to feed 1% formic acid in methanol at a constant flow rate of 0.2 mL/min to the mixer preceding the MS line. A Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. Multiple reaction monitoring (MRM) was used to analyse all steroids with a positive ionization mode electrospray probe (ESI +). The capillary voltage was set to 3.8 kV and the source and the desolvation temperature was set to 120 °C and 500 °C respectively, while the desolvation and cone gas were set to a flow rate of 1000 L/h and 150 L/h, respectively. This method was modified for the analysis of samples containing only A4, T, 11KA4, 11KT, 5α- dione and DHT. Solvent B was increased from 4% to 8% in 1.5 minutes and then to 25% in 0.1 minutes. All data was collected and analyzed using MassLynx 4.1 (Waters Corporation).

2.9 Computational modelling of experimental data

To test whether the conversion profiles in the prostate cancer cell lines could be described by activity of AKR1C3 and 17βHSD2, we fitted the ODE model that was based on the experimentally determined parameter values for the enzymes (see section 2.4), on the metabolite profiles, where the only fitting parameter was the expression level of the enzymes. Thus, the binding constants and the relative activities of the enzymes for the different substrates were fixed to the experimentally measured values (Tables 1 and 2), and the expression levels were fitted, and used for all the conversions in that cell line. The same model was fitted to all experimental data simultaneously, with only the initial conditions (substrate concentrations) differing. After showing that the model could approximate the experimental data, we subsequently simulated the effect of different AKR1C3:17βHSD2 ratios on the steady state concentrations of activated androgens.

2.10. Clinical samples

Primary tumor samples were from men enrolled in a previously published study of neoadjuvant androgen deprivation prior to prostatectomy in men with localized prostate cancer (T1c-T3, N0/NX) and Gleason score ≤ 7 [28]. Patients were randomly assigned to 3 months of neoadjuvant therapy with (1) goserelin (Zoladex; AstraZeneca, London, United Kingdom) 10.8 mg with high-dose dutasteride (Avodart, GlaxoSmithKline, London, United Kingdom) 3.5 mg per day (ZD; $n = 12$); (2) goserelin, bicalutamide (Casodex; AstraZeneca) 50 mg per day, and dutasteride (ZBD; $n = 12$); or (3) goserelin, bicalutamide, dutasteride, and ketoconazole 200 mg three times per day (with prednisone 5 mg per day; ZBDK; $n = 13$). Experimental cohorts were combined to form a single ADT-treated cohort for the analyses below. CRPC tumor samples comprised 149 CRPC metastases from 63 patients collected at rapid autopsy as previously published [29].

2.10. Transcript profiling in clinical samples

Benign and tumor epithelium from the ADT-treated patients in the neoadjuvant study, from a contemporaneous cohort of similarly high risk patients ($n=7$) who did not receive ADT, and from the CRPC tumor samples were subject to laser capture microdissection (LCM), microarray hybridization to Agilent 44K whole human genome expression oligonucleotide microarrays (Agilent Technologies Inc), and data normalization as previously described (Gene Expression Omnibus accession number GSE74685) [29]. Systematic batch effects were normalized by application of the ComBat function within the sva Bioconductor package to the log₂ Cy3 signal intensities. The unlogged combat normalized Cy3 signal intensities for AKR1C3, 17 β HSD2 or the ratio of AKR1C3 to 17 β HSD2 in each group were compared using unpaired t tests. P values <0.05 and <0.10 were considered as significant and trending towards significance, respectively.

3. Results

3.1. 11-oxygenated androgen precursors are the preferred substrate for AKR1C3.

The role of AKR1C3 in the production of potent androgens from precursor C19 steroids is well documented [14,30-34]. Given the recent interest in 11-oxygenated androgens [16-18,35-38] we set out to characterise the activity of AKR1C3 towards this group of steroids. First, we showed that while AKR1C3 catalysed the conversion of both classical and 11-keto C19 steroids, the enzyme demonstrated limited to no activity towards the 11 β -hydroxyl C19 steroids, 11OHA4, 11OH-5 α -dione and 11OHAST (Figure 2).

Kinetic characterisation of AKR1C3 subsequently revealed that the 11-keto C19 substrates 11KA4 and 11K-5 α -dione are the preferred substrate for AKR1C3. The kinetic parameters were determined using progress curve analysis and subsequent validation due to the low rate of catalysis towards selected substrates (supplementary figure 1). While the apparent K_m ($K_{m, app}$) values of the 11-keto androgen precursors were all similar to that of their classical counterparts, the apparent V_{max} ($V_{max, app}$) values were 12- to 55-fold greater (Table 1). As a result, the V_{max}/K_m values, which serve as an approximation of enzyme efficiency, were 8 to 24-fold higher for the 11-keto androgen precursors when compared to the respective classical androgen precursors (Table 1). Our data therefore shows that the substrate

preference for AKR1C3 is 11KA4 > 11 K-5 α -dione > 11KAST > A4 > AST > 5 α -dione. We subsequently determined the kinetic parameters of 17 β HSD2 using initial rates and subsequent validation by progress curve analysis (supplementary figures 2 and 3). Interestingly, neither the $K_{m, app}$ or $V_{max, app}$ values differed substantially between the 11-keto and classical androgen precursors, resulting in V_{max}/K_m values within the same order of magnitude (Table 2).

3.2. An increased AKR1C3:17 β HSD2 ratio favours the conversion of 11KA4 to 11KT

Given that AKR1C3 is significantly upregulated in CRPC, while the expression of 17 β HSD2 remains constant or is downregulated, we next investigated the effect of altered AKR1C3:17 β HSD2 ratios on the metabolism of classical and 11-oxygenated androgens. First, HEK293 cells transiently transfected to express either AKR1C3 or 17 β HSD2 were mixed at different ratios. The number of cells expressing 17 β HSD2 were kept constant, while the number of cells expressing AKR1C3 was increased incrementally. Cells transfected with the pCI-Neo plasmid were used to keep the total cell number constant for all ratios.

Increasing the ratio of AKR1C3:17 β HSD2 from 1:1 to 6:1 had no effect on the conversion of 0.1 μ M A4 or 5 α -dione to T or DHT, respectively (Figure 3A). Conversely, increased AKR1C3:17 β HSD2 ratios resulted in significant increases in the conversion of 11KA4 to 11KT (Figure 3A). The addition of the AKR1C3 inhibitor, indomethacin, significantly reduced conversion of 11KA4 to 11KT at all ratios tested, thereby confirming that the observed effect was due to the increased expression of AKR1C3 (Figure 3B and C). Similar trends were observed when A4 and 11KA4 were added simultaneously as substrates or when 1 μ M substrate was used (data not shown).

A mathematical model for the conversion of A4, 5 α -dione and 11KA4 by different ratios of AKR1C3:17 β HSD2 was constructed based on the kinetic parameters determined for AKR1C3 and 17 β HSD2 (Table 1 and 2). The resulting model was then used to predict the metabolism of 0.1 μ M A4, 5 α -dione or 11KA4 by different ratios of AKR1C3:17 β HSD2. The predicted conversions correlated well with what was observed experimentally after 24 hours confirming that increased AKR1C3:17 β HSD2 ratios have a substantially greater effect on the conversion of 11-keto C19 steroids when compared to their classical C19 counterparts (Figure 3A).

We chose A4 as a proxy for 5 α -dione, which is the physiologically more relevant substrate, for subsequent experiments based on the following: (1) the kinetic parameters for both A4 and 5 α -dione, and their products were similar (Table 1 and 2); (2) the effect of the different AKR1C3:17 β HSD2 ratios were experimentally and mathematically similar (Figure 3); and (3) downstream metabolism of A4 and T could be inhibited in prostate cancer cell lines by the addition of the SRD5A inhibitor, dutasteride, while downstream metabolism of 5 α -dione and DHT could not be prevented and would have complicated the analysis.

First we investigated the effect of different AKR1C3:17 β HSD2 ratios in the androgen independent PCa cell line, PC3, which endogenously expresses 17 β HSD2, but does not express AKR1C [39]. PC3 cells were transiently transfected with increasing amounts of

AKR1C3 and qPCR analysis confirmed that the mRNA levels of AKR1C3 increased proportionally to the amount of AKR1C3 plasmid DNA included, while 17 β HSD2 levels remained constant (Figure 4 A and B). Unsurprisingly, the metabolism of A4 and 11KA4 in this model system mirrored the results of the HEK293 ratio experiments described above (Figure 3). Only very low levels of A4 were converted to T under all conditions (Figure 4C), while the amount of 11KT produced increased significantly when the cells were transfected with increased levels of AKR1C3 (Figure 4D). The specific SRD5A inhibitor, dutasteride, was included in all experiments to prevent the downstream metabolism of substrates and products. UHPSFC-MS/MS analysis confirmed that no other metabolites were formed (data not shown).

Next, three PCa cell lines, VCaP, C42B and LNCaP, were chosen to investigate the effect of endogenous AKR1C3 and 17 β HSD2 activity on the metabolism of A4, T, 11KA4 and 11KT. Endogenous mRNA expression levels were confirmed by qPCR and the ratio AKR1C3:17 β HSD2 ratio calculated. The AKR1C3:17 β HSD2 ratio was significantly higher in VCaP cells when compared to C42B and LNCaP cells (Figure 5). Each cell line was subsequently incubated with 0.1 μ M of each substrate (A4, 11KA4, T and 11KT) in the presence of dutasteride. Significantly, higher rates of conversion were observed for 11KA4 when compared to A4 in all three cell lines. Overall, the highest levels of conversion were observed in the VCaP cells, which is not surprising given that these cells expressed the highest levels of AKR1C3 and had the highest AKR1C3:17 β HSD2 ratio (Figure 5). After 96 hours only 12% A4 was converted to T, while 80% 11KA4 was converted to 11KT (Figure 5). Interestingly, although VCaP cells express only low levels of 17 β HSD2, T was efficiently converted to A4 when added as a substrate (88% metabolised after 96 hours). In contrast, only low levels of 11KA4 were produced when 11KT was added as a substrate (14% metabolised after 96 hours) (Figure 5).

Despite the lower AKR1C3:17 β HSD2 ratios observed in C4-2B and LNCaP cells, both cell lines showed a significant preference for the conversion of 11KA4 (Figure 5). Conversely, a significant preference was observed for the conversion of T when T and 11KT were added as substrates (Figure 5). Furthermore, the highest level of conversion from 11KT to 11KA4 was observed in LNCaP cells (Figure 5) which correlated to the observation that this cell line expressed the highest levels of 17 β HSD2.

Subsequent fitting of the previously determined kinetic parameters (Table 1 and 2) yielded good approximations of the experimental data. The only parameter which was fitted during these simulations was the $V_{\max}$ of each enzyme as to account for the different expression levels between cell lines. The ratio of the $V_{\max}$ values for AKR1C3:17 β HSD2 predicted by the model correlated well with the ratio of the expression levels of AKR1C3:17 β HSD2 as determined by qPCR (Figure 5 C), thereby confirming the validity of this approach. We subsequently used the model to simulate the steady state concentration for each cell line. These simulation showed that only the VCaP cells approached steady state after 96 hours (Figure 5). The model also confirmed that the steady state level for each cell line was independent of the initial substrate. For example, the same steady state was achieved irrespective of whether 11KA4/A4 or 11KT/T were used as the starting substrate (note that the steady state concentrations of 11KT/T and 11KA4/A4 are always additive to the starting

concentration of 0.1 μM). In all cases the model showed that the predicted steady state concentrations of 11KT were substantially higher than those predicted for T. This was further illustrated by simulating the effect of different AKR1C3:17 β HSD2 ratios on the steady state concentrations of 11KT and T (Figure 6). Moreover, this simulation revealed this effect is not influenced significantly by the substrate concentration. Simulation of the steady state concentrations of DHT following the conversion from 5 α -dione, confirmed that A4 and 5 α -dione and their resulting products are metabolised in a similar manner, as illustrated by the overlapping model outputs in figure 5. All data, from this study is available at <https://fairdomhub.org/studies/368>.

3.3. Expression of AKR1C3 and 17 β HSD2 in prostate cancer

mRNA expression of AKR1C3 and 17 β HSD2 were compared between the following groups (Figure 7): benign tissue with no treatment; prostate cancer with retreatment; benign tissue with ADT; prostate cancer with ADT; and CRPC. AKR1C3 expression was significantly increased in patients undergoing ADT (2.5 fold) and further increased in patients with CRPC (5.4 fold). Interestingly, while AKR1C3 was not increased in patients who received no treatment, a significant decrease in 17 β HSD2 expression (-2.4 fold) was observed in these patients. No further decreases were observed in patients undergoing treatment and levels remained decreased in those progressing to CRPC. Only a small increase in the AKR1C3:17 β HSD2 ratio was observed (1.5 fold) in patients with prostate cancer who received no treatment, though this was not significant ($p=0.085$). Significant increases in the AKR1C3:17 β HSD2 ratio were observed in patients undergoing ADT (3.9 fold) with further significant increases those progressing to CRPC (5.8 fold CRPC vs ADT, 22.7 fold CRPC vs NP).

4. Discussion

There is little doubt that intratumoral androgens derived from circulating adrenal precursors play a vital role in CRPC. To date, however, DHT is the only androgen which is considered in this regard, with the production being dependent on the expression of SRD5A1 and AKR1C3 [14]. Both enzymes have been shown to be upregulated in CRPC and the substrate preference of SRD5A1 for A4 over T is believed to be the determining factor in the 5 α -dione pathway which bypasses T (Figure 1) [14,15]. The results from this study show that while A4 has been considered one of the major substrates for AKR1C3, this reaction is not catalysed efficiently. The low enzyme efficiency of AKR1C3 towards A4 therefore no doubt contributes significantly towards the preferential conversion of A4 to 5 α -dione in the alternate 5 α -dione pathway. The subsequent conversion of 5 α -dione to the potent androgen, DHT, is also dependent on AKR1C3 activity. Interestingly, in our hands this reaction is even less efficient than the conversion of A4 to T (Table 1). Nonetheless, there is sufficient evidence that this reaction does take place within CRPC tumours and is aided by the significant up regulation of AKR1C3 [14]. However, to date studies have failed to account for the possibility that 11-oxygenated androgens may also play an important role on in the activation of the AR and that the upregulation of AKR1C3 may also affect the metabolism of these steroids.

The abundant 11-oxygenated steroid 11OHA4 is the product of the CYP11B1 catalysed 11 β -hydroxylation of A4 in the adrenal cortex and circulates at higher levels than A4 [36,40]. Interestingly, we have shown that neither 11OHA4, nor any of its 11 β -hydroxy C19 counterparts, are substrates for AKR1C3 (Figure 2). Within the context of CRPC, 11OHA4 would therefore first have to be converted to 11KA4 by 11 β HSD2 prior to the conversion by AKR1C3. Indeed, 11 β HSD2 is expressed in prostate tissue and we have previously reported the conversion of 11OHA4 to 11KA4 within PCa cell lines [18,41,42]. In this study, we have used three independent experimental systems to show that 11KA4 is in fact a preferred substrate for AKR1C3, and that the upregulation of AKR1C3 relative to the oxidative enzyme 17 β HSD2 significantly favours the conversion of 11KA4 to 11KT over that of either A4 or 5 α -dione to T or DHT, respectively. A validated mathematical model subsequently revealed that a steady state is established between the reductive and oxidative activities of AKR1C3 and 17 β HSD2, respectively. The model showed that increases in the AKR1C3:17 β HSD2 ratio results in significant changes in the steady state ratio of 11KT:11KA4 as the catalytic efficiency of AKR1C3 for the conversion of 11KA4 to 11KT is similar to that of the catalytic efficiency of 17 β HSD2 for the conversion of 11KT to 11KA4 (Figure 6). Conversely, the catalytic efficiency of 17 β HSD2 for T and DHT is substantially higher than that of AKR1C3 for A4 or 5 α -dione and as a result, elevated levels of AKR1C3 had a minimal effect on the conversion of A4 or 5 α -dione to T or DHT due to the rapid conversion of T back to A4 by 17 β HSD2. Significantly, higher levels of AKR1C3 relative to 17 β HSD2 are therefore required to overcome this barrier. While this can be achieved by increased expression of AKR1C3, a combination of increased AKR1C3 expression and decreased 17 β HSD2 expression would have a greater effect. Indeed, expression data revealed that 17 β HSD2 expression is decreased 2.4-fold in prostate cancer and that expression remains at this decreased level during treatment and the development of CRPC. Our data therefore suggests that this decrease in 17 β HSD2 expression is equally important as the increase in AKR1C3 expression in shifting the steady state. The importance of oxidative 17 β HSD was recently confirmed in a study investigating the role of 17 β HSD4 in CRPC [43]. The study showed that 17 β HSD4 splice variant 2, like 17 β HSD2, catalyses the inactivation of DHT and that loss of this splice variant favours the development of CRPC. This finding therefore corroborates the idea that the expression and activity of oxidative 17 β HSD enzymes are equally as important as the overexpression of the reductive enzyme AKR1C3 when considering intratumoral androgen activation.

Moreover, while the data presented in this study clearly reveals that the production of active 11-oxygenated androgens by AKR1C3 occurs at a higher rate when compared to classical androgens, we have previously shown that the inactivation of 11KT and 11KDHT by androgen dependent prostate cancer cell lines occurs at a significantly lower rate than that of T and DHT [16]. Taken together, this strongly suggests that potent 11-oxygenated androgens could accumulate at higher levels than DHT in CRPC tissue. Indeed, a recent study has shown that 11-oxygenated metabolites are more abundant in PCa tissue compared to metabolites from the classical pathway, thus highlighting that the 11-oxygenated steroids may accumulate [19]. Given that 11KT and 11KDHT are potent and efficacious androgens [16], their contribution to the development and progression of CRPC should therefore no longer be overlooked and more studies are required to delineate the role of these steroids in

CRPC. Although the relative contributions and importance of the classical and 11-oxygenated pathways remain to be elucidated, the involvement of both pathways strongly suggests that AKR1C3 is an ideal candidate for the treatment of CRPC through the inhibition of intratumoral androgen biosynthesis, especially considering that this enzyme has been associated with resistance to current treatment strategies [23,24].

Finally, this study has also demonstrated the utility of a combined experimental and computational approach. A computational model was constructed using a system expressing only the desired enzyme; model validation was performed in systems with both enzymes expressed at different ratios; and the model was subsequently applied to more complex prostate cancer cell lines to estimate expression levels of the two enzymes, and substrate conversion. Given the success of this approach, it is feasible to consider that the next step will be to predict intratumoral steroid metabolism in cancer patients based on qPCR measurements of steroid enzymes and mass spectrometry based measurements of circulating steroid levels, thereby opening the door to novel approaches in personalised medicine.

5. Conclusion

This study has conclusively shown that 11-keto androgen precursors are the preferred substrate for AKR1C3 and that both the reductive and oxidative 17 β HSDs need to be taken into account when making deductions pertaining to androgen activation. Significantly, increased AKR1C3:17 β HSD2 ratios were shown to favour the activation of 11-keto androgen precursors. Future studies should therefore consider the contribution of 11-oxygenated androgens when measuring intratumoral androgens. Further computational modelling of all steroidogenic enzymes may also shed more light on the relative contributions of the different androgen pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 11-oxygenated androgen precursors are the preferred substrates for AKR1C3
- Oxidative 17 β HSD enzymes regulate androgen activation
- Increased AKR1C3:17 β HSD2 ratios favour the activation of 11-oxygenated androgens
- Computational modelling provides a valuable tool to study androgen metabolism

Figure 1:
Schematic of intratumoral androgen activation. The preferred reactions in the alternate 5 α -dione (A) and 11OHA4 (B) pathways are shown in bold.

Figure 2:
AKR1C3 catalyses the reduction of 11-keto androgens, but not that of 11 β -hydroxy androgens. The bars indicate the percentage conversion of classical, 11-keto and 11 β -hydroxy androgen precursors (1 μ M) after 24 h incubation.

Figure 3:

Increased AKR1C3:17βHSD2 ratios favour the activation of 11KA4 over that of A4 and 5α-dione. The experimental data from the ratio experiments described in section 2.5 are shown as points with error bars (A), while the computational models' predictions are shown with the solid lines. The effect of AKR1C3 inhibition by the selective AKR1C3 inhibitor, Indomethacin, is shown for metabolism of (0.1 μM) A4 and 11KA4 respectively in (B) and (C). All results are shown as means ± SEM of three independent experiments performed in triplicate.

Figure 4:

Increased expression of AKR1C3 in PC3 cells endogenously expressing 17βHSD2 favours the activation of 11K4 over A4. qPCR analysis of expression levels are shown for 17βHSD2 (A) and AKR1C3 (B). The metabolism of 0.1 μM A4 and 11KA4 are shown in (C) and (D), respectively. Results are shown as means ± SEM of three independent experiments each performed in triplicate. qPCR data from the individual experiments was normalized using log transformation and mean centering prior to analysis. Letters a, b, c and d represents significant differences between samples ($p > 0.05$), while values which do not significantly differ are assigned the same letter.

Figure 5:

(A) The activation of 11KA4 is preferred over A4 in prostate cancer cell lines endogenously expressing AKR1C3 and 17 β HSD2. Experimental data for the metabolism of 0.1 μM A4, 11KA4, T and 11KT by VCaP, C42B and LNCaP cell lines is shown as points with error bars, while the computational models predictions are shown with the solid lines (A). The dashed lines indicate estimated steady state concentrations for the different metabolites. qPCR analysis of the endogenous expression of AKR1C3:17 β HSD2 is shown in (B). The correlation between the fitted AKR1C3:17 β HSD2 V_{max} values and the AKR1C3:17 β HSD2

expression ratios is shown in (C). Results are shown as means $\pm$ SD of two independent experiments each performed in triplicate.

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Figure 6:

A computational model shows that increased AKR1C3:17 β HSD2 ratios favour higher steady state concentrations of 11KT than T or DHT. Simulation of the effect of different AKR1C3:17 β HSD2 ratios on the steady state ratios of 11KT:11KA4, T:A4 and DHT:5 α -dione were performed using 0.01 – 1 μ M substrate. The points indicate, from left to right, the values determined in LNCaP, C42B and VCaP cells respectively (Figure 5). The shaded area indicates the effect of different initial substrate concentrations.

Figure 7:

The AKR1C3:17βHSD2 expression ratio is increased significantly in men with CRPC. Expression levels of AKR1C3 (A), 17βHSD2 (B) and corresponding AKR1C3/17βHSD2 ratios (C) are shown for normal and prostate cancer tissue. NP, normal prostate; PCa, prostate cancer; No Rx, no treatment; ADT, androgen deprivation therapy; CRPC, castration resistant prostate cancer. Data are shown as median with the boxes indicating the 25th and 75th percentiles and the whiskers the minimum and maximum values. P values were calculated using unpaired t-tests between the indicated groups (P values <0.05 and <0.10 were considered as significant and trending towards significance, respectively).

Table 1:

Kinetic constants ($K_{m, app}$; $V_{max, app}$; V_{max}/K_m) determined for AKR1C3.

Substrate	Product	$K_{m, app}$ (μM)	$V_{max, app}$ ($\mu M/h$)	V_{max}/K_m
A4	T	0.06 (0.03-0.09)	0.02 (0.01-0.02)	0.3
11KA4	11KT	0.11 (0.07-0.13)	0.27 (0.25-0.30)	2.4
5 α -dione	DHT	0.12 (0.08-0.20)	0.01 (0.01-0.02)	0.1
11K-5 α -dione	11KDHT	0.23 (0.16-0.33)	0.55 (0.44-0.72)	2.4
AST	3 α -adiol	0.27 (0.14-0.55)	0.05 (0.04-0.08)	0.2
11KAST	11K-3 α -adiol	0.34 (0.24-0.50)	0.59 (0.48-0.78)	1.8

Table 2:Kinetic constants (K_m , app; V_{max} , app; V_{max}/K_m) determined for 17 β HSD2.

Substrate	Product	K_m , app (μ M)	V_{max} , app (μ M/h)	V_{max}/K_m
T	A4	5.08 (4.56-5.67)	3.71 (3.41-4.04)	0.73
11KT	11KA4	9.11 (8.24-10.12)	4.35 (4.01-4.73)	0.47
DHT	5 α -dione	3.77 (3.40-4.17)	2.25 (2.07-2.44)	0.59
11KDHT	11K-5 α -dione	7.72 (6.67-9.02)	6.40 (5.70-7.23)	0.83
3 α -adiol	AST	5.86 (4.94-7.03)	3.99 (3.49-4.57)	0.68