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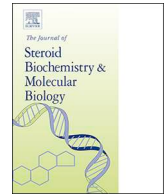
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The A-ring reduction of 11-ketotestosterone is efficiently catalysed by AKR1D1 and SRD5A2 but not SRD5A1



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ABSTRACT

Testosterone and its 5 α -reduced form, 5 α -dihydrotestosterone, were previously thought to represent the only active androgens in humans. However, recent studies have shown that the potent androgen, 11-ketotestosterone, derived from the adrenal androgen precursor, 11 β -hydroxyandrostenedione, may in fact serve as the primary androgen in healthy women. Yet, despite recent renewed interest in these steroids, their downstream metabolism has remained undetermined. We therefore set out to investigate the metabolism of 11-ketotestosterone by characterising the 5 α - or 5 β -reduction commitment step. We show that inactivation of 11-ketotestosterone is predominantly driven by AKR1D1, which efficiently catalyses the 5 β -reduction of 11-ketotestosterone, committing it to a metabolic pathway that terminates in 11-ketoetiocholanolone. We demonstrate that 5 α -reduction of 11-ketotestosterone is catalysed by SRD5A2, but not SRD5A1, and terminates in 11-ketoandrosterone, but is only responsible for a minority of 11-ketotestosterone inactivation. However, as 11-ketoetiocholanolone is also generated by the metabolism of the glucocorticoid cortisone, 11-ketoandrosterone should be considered a more specific urinary marker of 11-ketotestosterone production.

1. Introduction

Although once forgotten, several recent studies have shown that 11-oxygenated androgens play an important role in human androgen biology [1–3]. Their precursor, 11 β -hydroxyandrostenedione (11OHA4), is an abundant product of adrenal steroidogenesis [4] produced by the 11 β -hydroxylation of androstenedione (A4), catalysed by the cytochrome P450 11 β -hydroxylase (CYP11B1) [5]. 11OHA4 can

subsequently be converted to 11-ketoandrostenedione (11KA4) in peripheral tissues expressing 11 β -hydroxysteroid dehydrogenase type 2 (HSD11B2) [5,6]. 11KA4 in turn serves as a preferred substrate for the androgen-activating enzyme 17 β -hydroxysteroid dehydrogenase type 5 (HSD17B5, also known as AKR1C3) [6,7], yielding 11-ketotestosterone (11KT), which binds to and activates the human androgen receptor (AR) with an affinity and potency comparable to that of testosterone (T) [6,8]. Several studies have confirmed that the aforementioned 11-

Abbreviations: 11KA4, 11-ketoandrostenedione; 11KAN, 11-ketoandrosterone; 11K-5 α -dione, 11K-5 α -androstenedione; 11K-5 α -DHT, 11-keto-5 α -dihydrotestosterone; 11K-5 β -dione, 11-keto-5 β -androstenedione; 11K-5 β -DHT, 11-keto-5 β -dihydrotestosterone; 11KEdiol, 11-ketoetiocholanediol; 11KET, 11-ketoetiocholanolone; 11KT, 11-ketotestosterone; 11OHA4, 11 β -hydroxyandrostenedione; 11OHAN, 11 β -hydroxyandrosterone; 11OHEt, 11 β -hydroxyetiocholanolone; 11OHT, 11 β -hydroxytestosterone; HSD17B2, 17 β -hydroxysteroid dehydrogenase type 2; 5 α -DHT, 5 α -dihydrotestosterone; 5 α -dione, 5 α -androstenedione; 5 β -DHT, 5 β -dihydrotestosterone; 5 β -dione, 5 β -androstenedione; A4, androstenedione; Adiol, androstenediol; AKR1C3, 17 β -hydroxysteroid dehydrogenase type 5; AKR1C4, 3 α -hydroxysteroid dehydrogenase type 1; AKR1D1, aldo-keto reductase family member 1 type D1; An, androsterone; AR, androgen receptor; CYP11B1, cytochrome P450 11 β -hydroxylase; Ediol, etiocholanediol; Et, etiocholanolone; SRD5A1, steroid 5 α -reductase type 1; SRD5A2, steroid 5 α -reductase type 2; T, testosterone; UHPSFC-MS/MS, ultra-high performance supercritical fluid chromatography tandem mass spectrometry

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oxygenated androgens are present in circulation for both men and women [4,9–14]. Significantly, the circulating concentrations of 11KT have been shown to be equal to or higher than that of T in healthy women [11,13,14], and unlike the levels of T, appear not to decrease following menopause [13] or fluctuate during the menstrual cycle [14]. Moreover, significantly increased serum concentrations of 11KT and its precursors have been measured in patients with polycystic ovary syndrome and congenital adrenal hyperplasia, with 11KT being suggested to be the primary active androgen in these conditions [10,11,15].

Given the abundance and clear physiological importance of 11KT, it is unusual that this steroid went undetected in humans for so long. Failure to detect 11KT in circulation is likely explained by the predominant use of immunoassays, which focus on measuring single metabolites such as T, and often use antibodies that do not distinguish between closely related steroid metabolites [16]. While 11 β -hydroxyandrosterone (11OHA_n) has been described as a urinary steroid metabolite reflecting the 11-oxygenated androgen pathway using gas chromatography-mass spectrometry (GC-MS) analysis [17,18], this metabolite is mostly derived from 11OHA₄, with direct downstream metabolites of 11KT not yet described [19,20]. This led us to hypothesise that the urinary metabolite(s) of 11KT may be masked by other predominant urinary metabolites. In order to investigate this, we considered the standard metabolic pathways for the metabolism of T, which contains the same 3-keto- Δ^4 steroid moiety as 11KT [20].

The first step in T metabolism is either the 5 α - or the 5 β -reduction of carbon 5, both physiologically irreversible reactions that commit T to metabolism and excretion (Fig. 1) [20–23]. 5 α -Reduction is catalysed by the 5 α -reductase isozymes, SRD5A1 and SRD5A2 [22], which are expressed in a tissue specific manner [24,25]. While the resulting product, 5 α -dihydrotestosterone (5 α -DHT) is a more potent androgen than T, further metabolism yields inactive metabolites [26–28].

5 β -Reduction is catalysed by a single enzyme, aldo-keto reductase family 1 member D1 (AKR1D1; also known as 5 β -steroid reductase) [21,29]. AKR1D1 is essential to both bile acid biosynthesis and steroid hormone inactivation and clearance [30,31]. The AKR1D1-catalysed

5 β -reduction of T yields 5 β -dihydrotestosterone (5 β -DHT), a metabolite that has no androgenic activity due to the induction of a conformational change in the A/B-ring that hinders binding to the AR [32,33]. AKR1D1 is primarily expressed in the liver [34–37] where it competes with SRD5A isoforms for 3-keto- Δ^4 steroid substrates [21,24]. The relative expression levels and activity of these enzymes will therefore determine the ratio of 5 α - and 5 β -reduced products.

Both 5 α -DHT and 5 β -DHT are subsequently 3 α -reduced by 3 α -hydroxysteroid dehydrogenase type 3/1 (AKR1C2/4) yielding androstenediol (Adiol) and etiocholanediol (Ediol), respectively [38,39]. These metabolites are subject to metabolism by oxidative 17 β -hydroxysteroid dehydrogenases (HSD17B) yielding androsterone (An) and etiocholanolone (Et), respectively, the primary T metabolites measured in urine [20]. Applying the same principles to the metabolism of 11KT would yield the end-products, 11-ketoandrosterone (11KAN) and 11-ketoetiocholanolone (11KEt) (Fig. 1). Although we have previously demonstrated the 5 α -reduction of 11KT by SRD5A1 and SRD5A2 [6], these reactions could not be quantified due to a lack of reference material at the time. Lower circulating concentrations of 11-keto-5 α -dihydrotestosterone (11K-5 α -DHT) than 5 α -DHT suggest that 11KT is not as efficiently 5 α -reduced as T [40]. Conversely, the AKR1D1 catalysed 5 β -reduction of 11KT has not been investigated to date. The proposed downstream metabolite, 11KEt, is routinely measured in urine as a prominent urinary steroid metabolite derived from cortisone [41–48].

In this study, we characterised AKR1D1, SRD5A1 and SRD5A2 activity towards T and 11KT using progress curve analysis and computational modelling. We demonstrate for the first time that the sequential metabolism of 11KT by AKR1D1, AKR1C2 and 17 β -hydroxysteroid dehydrogenase type 2 (HSD17B2) yields the steroid 11KEt, which is likely the primary urinary metabolite of 11KT.

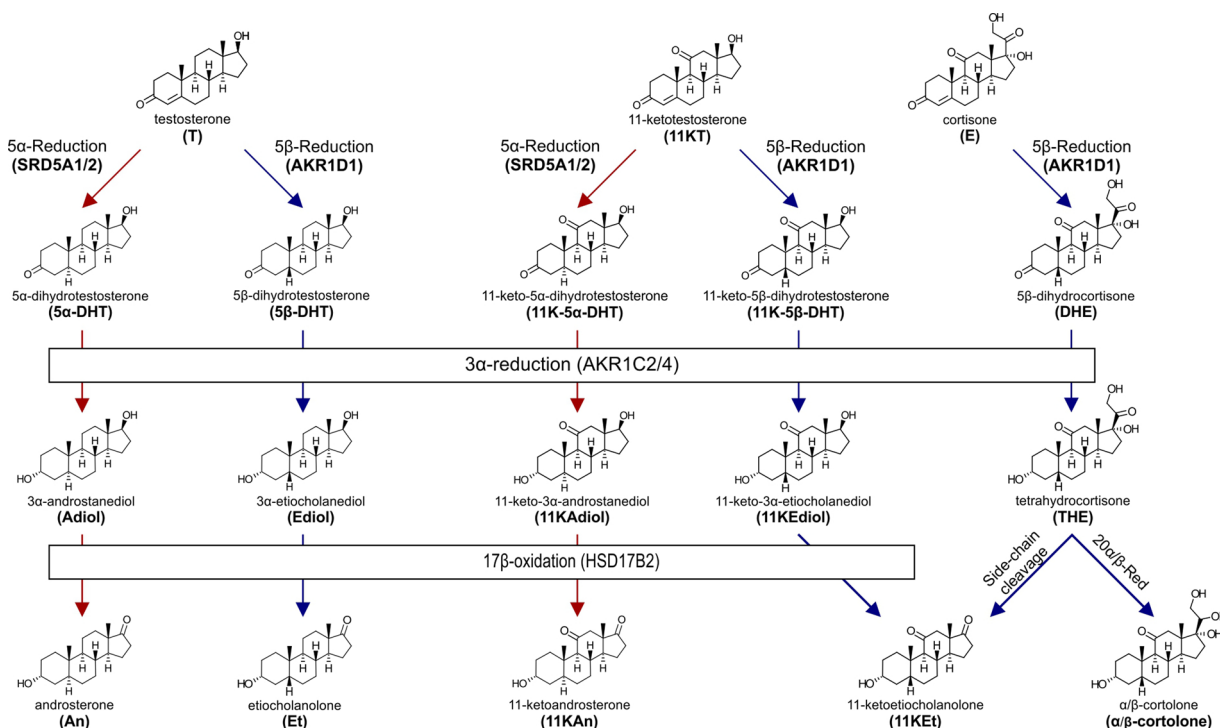


Fig. 1. Metabolism of T, 11KT and cortisone. Testosterone is either 5 α -reduced or 5 β -reduced, followed by sequential 3 α -reduction and 17 β -oxidation reactions yielding the 5 α -product androsterone (An) and the 5 β -product etiocholanolone (Et). The 5 β -reduction of 11KT leads to the production of 11-ketoetiocholanolone (11KEt), a metabolite also produced from the metabolism of the glucocorticoid, cortisone.

2. Materials and methods

2.1. Cell lines

HEK293 and HepG2 cells were purchased from America Type Culture Collection and were validated by STR profile analysis. Huh7 were purchased from the Japanese Cancer Research Resources Bank, where the cells were validated by isozyme analysis. All experiments were conducted within 20-passage numbers from purchase or re-validation. HEK293 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL streptomycin. HepG2 and Huh7 cells were cultured in DMEM supplemented with 10 % FBS, 1 % penicillin-streptomycin, 4.5 g/L glucose and 1 % non-essential amino acids (Thermo Fisher Scientific). Culturing conditions were kept stable at 37 °C, 90 % relative humidity and 5 % CO₂. All cells were regularly tested for mycoplasma contamination using MycoAlert™ mycoplasma detection kit (LT07-318) or MycoAlert™ assay control set (LT07-518) from Lonza.

2.2. Plasmid constructs

The pCMV6-XL4/AKR1D1 plasmid construct (CAT#: SC116410) was purchased from Origene Technologies. The pCMV7/SRD5A1 and pCMV7/SRD5A2 plasmid constructs were gifts from Prof. D.W. Russell (UT Southwestern Medical Centre, Dallas, USA). The pcDNA3/AKR1C2 and pcDNA4/17βHSD2 plasmid constructs were gifts from Prof. T.M. Penning (University of Pennsylvania, School of Medicine, Philadelphia, USA) and Prof. J. Adamski (Institute of Experimental Genetics, Helmholtz Zentrum München, Germany), respectively. Plasmid inserts were confirmed by sequence analysis.

2.3. Steroids

11β-hydroxyandrostenedione (11OHA4), 11β-hydroxytestosterone (11OHT), 11-keto-5α-dihydrotestosterone (11K-5α-DHT), 11-ketoandrosterone (11KAn), 11-ketoetiocholanolone (11KEt), 11-ketotestosterone (11KT), androsterone (An) and testosterone (T) were purchased from Steraloids. Deuterated 11β-hydroxyandrostenedione 2,2,4,6,6,16,16-D7 (D7-11OHA4) 98 %, androstenedione 2,2,4,6,6,16,16-D7 (D7-A4) 98 %, cortisol 9,11,12,12-D4 (D4-F) 98 % and testosterone 1,2-D2 (D2-T) 98 % were obtained from Cambridge Isotopes. Testosterone 16,16,17-D3 (D3-T) and 5α-dihydrotestosterone 16,16,17-D3 (D3-DHT) solutions from Cerilliant® were purchased from Sigma-Aldrich, while 11-ketotestosterone 16,16,17-D3 (D3-11KT) and androsterone 2,2,4,4-D4 (D4-An) solutions were obtained from IsoSciences.

2.4. Enzyme assays in transiently transfected HEK293 cells

HEK293 cells were seeded into 100 mm dishes at a cell density of 2×10^5 cells/mL in a total volume of 10 mL. Following a 24 h incubation, each dish was transfected with 10 µg of either pCMV6-XL4/AKR1D1, pCMV6/SRD5A1, pCMV6/SRD5A2 or pCneo (as negative control) using XtremeGene HP® DNA transfection reagent (Roche) according to the supplier's protocol. After a further 24 h, cells were replated into 48-well Corning® CELLBIND® plates at a cell concentration of 1×10^5 cells/mL in 250 µL. For co-expression investigations, AKR1D1 and either SRD5A1 or SRD5A2 transfected cells were mixed in a 1:1 ratio and 500 µL (1×10^5 cell/mL) plated into 24-well Corning® CELLBIND® plates. Cells were incubated for a further 24 h after which the media was replaced with media containing the appropriate steroid substrates. These included 0.1, 1 and 10 µM A4, T, 11KA4 or 11KT. Aliquots of 250 µL were collected from 48-well plates at 1, 2, 3, 4, 6, 8, 10 and 12 h. A4 and 11KA4, and T and 11KT samples were combined allowing for multiplexing during ultra-high performance supercritical

fluid chromatography tandem mass spectrometry (UHPSFC-MS/MS) analysis. Aliquots of 500 µL were collected after 24 h from 24-well plates containing SRD5A1/2 and AKR1D1 co-expressing cells.

2.5. Promoter reporter assays

To determine AR activation, HEK293 cells were transiently transfected with either pCMV6-XL4/AKR1D1 or empty plasmid vector for 48 h as previously described [49], followed by treatments with un-supplemented phenol-red free DMEM (Thermo Fisher Scientific) containing 200 nM of either T or 11KT for 24 h. Cell media aliquots (500 µL) were collected and stored at -20 °C. In another set of experiments, HEK293 cells were transiently co-transfected with a pcDNA3.1 + AR construct and an androgen responsive element (ARE) reporter - a mixture of an inducible ARE-responsive firefly luciferase construct and a constitutively expressing renilla luciferase construct (#CCS-1019 L, QIAGEN). After 48 h, cell media was replaced with the stored steroid containing media aliquots described above, and cells were incubated for 24 h. Cell lysates were then harvested in passive lysis buffer, and reporter activity was measured using the Luciferase Assay System (Promega) and an EnSpire Multimode plate reader (PerkinElmer). The data were presented as the ratio of firefly to renilla luciferase activity (Fluc/Rluc) in percentage.

2.6. Sequential metabolism of 11KT using conditioned media

AKR1D1, AKR1C2 and HSD17B2 transfections, performed as described above, were staggered sequentially by 24 h. First AKR1D1 transfected cells (2×10^5 cells/mL, 2 mL, 6well plates) were incubated for 24 h after which the media was removed and replaced with 2 mL media containing 4 µM T or 11KT. Following 24 h, 1 mL conditioned media was carried over to cells transfected with AKR1C2 (2×10^5 cells/mL, 2 mL, 6-well plate). The conditioned media was diluted 1:1 with 1 mL fresh media. After a further 24 h, 500 µL conditioned media was transferred to cells transfected with HSD17B2 (2×10^5 cells/mL, 500 µL, 24-well plate) and incubated a further 24 h. A final steroid concentration of 2 µM was anticipated following the dilution of the conditioned media. Aliquots (500 µL) were collected at each step for UPHSFC-MS/MS analysis.

2.7. Steroid extraction

All steroid samples were brought to room temperature before the addition of internal standards (1.5 ng D2-T and 15 ng of D7-11OHA4, D7-A4 and D4-F for the HEK293 experiments or 10 ng D3-T, D3-11KT, D3-DHT, D4-An and D4-F for the HepG2 and Huh7 experiments). Steroids were extracted using three volumes of tert-Butyl methyl ether (MTBE). Samples were mixed by vortexing and incubated at -80 °C for 1 h. The organic layer was subsequently transferred to a clean test tube and dried under a stream of nitrogen at 45 °C. The dried steroid residue was suspended in 50 % MeOH in water (150 µL) and stored at -20 °C until analysis by UHPSFC-MS/MS.

2.8. UHPSFC-MS/MS analyses of steroid metabolites

Steroid metabolites were separated and quantified using an ACQUITY UPC² (Waters Corporation) coupled to a Xevo-TQS (Waters Corporation) triple quadrupole mass spectrometer. Chromatographic separation was achieved using an ACQUITY UPC² Torus 1-AA column (3.0 mm × 50 mm; 1.7 µm) (Waters Corporation) at 60 °C with liquid CO₂ (Solvent A) modified with absolute MeOH (solvent B) as the mobile phases. A linear gradient increased solvent B from 20 % to 30 % over 2.5 min at a flow rate of 1.2 mL/minute. The automated backpressure regulator was set to 2000 psi. The make-up solvent consisted of 1% formic acid in MeOH and fed into the system at a flow rate of 0.2 mL/minute. The injection volume was 2 µL. Steroids were quantified by

multiple reaction monitoring (MRM) in positive electrospray ionisation mode. The instrument settings were as follows: source temperature: 150 °C; capillary voltage: 3.8 kV; cone voltage: 25–125 V; cone gas flow: 150 L/hour; desolvation temperature: 500 °C; desolvation gas flow: 750 L/hour; collision gas flow: 0.2 mL/minute. All data were collected and analysed using the MassLynx Software Package (Version 4.1) (Waters Corporation).

2.9. Determination of kinetic parameters for AKR1D1, SRD5A1 and SRD5A2 and computational model construction

The time course data collected from HEK293 cells transiently transfected with SRD5A1, SRD5A2 or AKR1D1 were used to determine kinetic parameters (apparent V_{\max} and K_m) in Wolfram Mathematica (version 12). Irreversible Michaelis-Menten enzyme kinetics were assumed for the determination of all parameter values. An ordinary differential equation (ODE) based model was constructed and fitted to the data. Non-linear regression was used to minimise the objective function, which is the sum squared residual values (SSR) between the model fit and the data weighted by the variance. The kinetic parameters were validated by their ability to predict the experimental data from independent time course experiments. Differences in transfection efficiencies between experiments were accounted for by measuring the initial rates of selected substrates for each experiment.

The validated kinetic constants were subsequently used to construct a model of SRD5A1, SRD5A2 and AKR1D1 co-expression. The model was validated by the ability to predict the metabolism of T and 11KT by a 1:1 mixture of cells expressing SRD5A1 and AKR1D1 or SRD5A2 and AKR1D1. Following validation, the model was used to predict the metabolism of T or 11KT by various combinations of SRD5A1, SRD5A2 and AKR1D1 activity. The computational model is available on JWS online (<https://jij.bio.vu.nl/>), and is also available in SBML format upon request.

2.10. Steroid metabolism in Huh7 and HepG2 liver cell lines

HepG2 and Huh7 cells were plated into 24-well Corning® CELLB-IND® plates at a cell density of 1.6×10^5 cells/mL (500 μ L/well). After 24 h, cells were treated with either 20 nmol of AKR1D1 siRNA or scrambled control (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) prepared in OPTIMEM serum free media (Invitrogen) as previously reported [49]. The cells were incubated for 48 h after which the media was removed and replaced with unsupplemented phenol-red free DMEM (Thermo Fisher Scientific) containing 1 μ M T or 11KT (500 μ L/well). Cell media samples were collected after 24 h for Huh7 cells and 48 h for HepG2 cells and stored at -20 °C prior to steroid extraction.

2.11. GC–MS analysis

Measurement of steroid metabolites was carried out by GC–MS as previously described [19,50]. In brief, free and conjugated steroids were extracted from 1 mL cell culture media by solid-phase extraction. Steroid conjugates were enzymatically hydrolysed, re-extracted, and chemically derivatised to form methyloxime trimethyl silyl ethers. GC–MS was performed on an Agilent 5975 instrument operating in selected-ion-monitoring (SIM) mode to achieve sensitive and specific detection and quantification of 38 selected steroid metabolites.

2.12. Human samples

Human liver biopsies were collected from healthy female individuals subject to their written informed consent (University of Oxford, Translational gastroenterology biobank ethics NRES ref: 16/YH/0247). Intraoperatively, a wedge biopsy was taken from the anatomical left lobe of the liver after pneumoperitoneum was established.

Liver tissue was washed in 0.9 % NaCl and then stored in RNAlater stabilization solution at -80 °C until RNA extraction.

2.13. RNA extraction and gene expression analysis

Total RNA was extracted from cells using Tri-Reagent (Sigma-Aldrich) and RNA concentrations were determined spectrophotometrically (OD_{260}) using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed in a total volume of 20 μ L: 1 μ g of total RNA, incubated with 10x RT Buffer, 100 mM dNTP mix, 10x RT Random Primers, 50 U/ μ L MultiScribe Reverse Transcriptase and 20 U/ μ L RNase Inhibitor (Thermo Fisher Scientific). Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min and then terminated by heating to 85 °C for 5 min.

All quantitative PCR (qPCR) experiments were conducted using a QuantStudio 7 Realtime PCR System (Applied Biosystems). Reactions (6 μ L) were performed in 384-well plates in reaction buffer containing 3 μ L of 2x Kapa Probe Fast qPCR Master Mix (Sigma-Aldrich). All probes were supplied by Thermo Fisher Scientific as predesigned TaqMan Gene Expression Assays (FAM dye-labelled). The reaction conditions were: 95 °C for 3 min, then 40 cycles of 95 °C for 3 s and 60 °C for 20 s. Relative expression of AKR1D1 in each sample was determined by a pre-existing equation and expression values normalised to *18S rRNA*, unless stated otherwise [37,51].

2.14. Cell lysis and immunoblotting

Cells lysis was performed using RIPA buffer (150 mM NaCl, 1.0 % IGEPAL® CA630, 0.5 % sodium deoxycholate, 0.1 % SDS, and 50 mM Tris, pH 8.0) (Sigma-Aldrich), and protease inhibitor cocktail (Thermo Fisher Scientific). Protein concentrations were measured using a commercially available assay (Bio-Rad Laboratories Inc.) according to the manufacturer's protocol. Primary human AKR1D1 (dilution 1:500 - HPA057002, Atlas Antibodies AB), β -tubulin (#15115, monoclonal) (Cell Signalling), and secondary antibodies (P044801-2, polyclonal) from Dako (Agilent) were used at a dilution of 1:1000 (primary) and 1:2000 (secondary) respectively. Bands were visualised with Bio-Rad Clarity Western ECL and ChemiDocXS imager (Bio-Rad). Western blots were quantified by densitometry analysis using ImageJ (NIH: <http://rsb.info.nih.gov/ij/>), normalised to β -tubulin to correct for variability in loading.

3. Results

3.1. 11KT is efficiently metabolised by AKR1D1 and SRD5A2, but not SRD5A1

In order to evaluate the potential metabolism of 11KT, we characterised the activity of SRD5A1, SRD5A2 and AKR1D1 towards T and 11KT using progress curve analyses (Fig. 2 and supplementary data: Fig. 1). Both SRD5A2 and AKR1D1 catalysed the conversion of T and 11KT to their respective 5 α - and 5 β -products. T was also effectively 5 α -reduced by SRD5A1, however, this isozyme did not efficiently catalyse the 5 α -reduction of 11KT. While the determined apparent K_m values for T and 11KT were similar (0.55 μ M vs 0.64 μ M) for SRD5A1, the apparent V_{\max} was 10-fold higher for T compared to 11KT. The resulting ratio of V_{\max}/K_m , which serves as an estimate of catalytic efficiency, therefore demonstrated the clear preference of SRD5A1 for T over 11KT (0.12 h $^{-1}$ vs 0.01 h $^{-1}$) (Table 1). Unlike SRD5A1, SRD5A2 efficiently catalysed the 5 α -reduction of both T and 11KT, although a preference for T was observed in both the apparent K_m and V_{\max}/K_m values (Table 1). No steroid conversion was observed in cells transfected with empty vector.

Notably, we show for the first time that 11KT is a substrate for AKR1D1. In fact, the V_{\max} measured for 11KT was 16-fold greater than

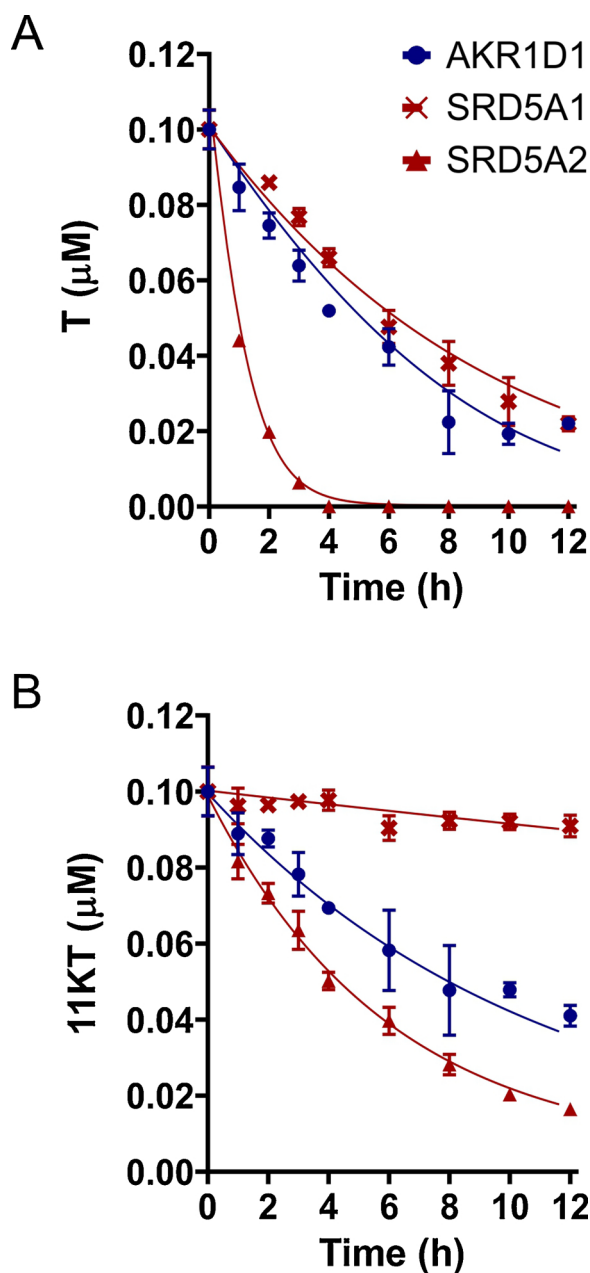


Fig. 2. Metabolism of 100 nM T (A) and 11KT (B) by AKR1D1, SRD5A1 and SRD5A2 expressed in non-steroidogenic HEK293 cells. Experimental results are shown as the mean \pm SD from a representative experiment used to determine the kinetic parameters. Model fits are shown by the solid lines. Kinetic parameters were validated by their ability to predict the experimental data from subsequent independent time course experiments. All fits and validations are available in Supplementary data: Fig. 1.

Table 1

Kinetic parameters (K_m , app ; V_{max} , app ; V_{max}/K_m) determined for AKR1D1, SRD5A1 and SRD5A2. N.A., not available. Values in brackets refer to the upper and lower limits of the model prediction.

	Substrate	Product	$V_{max, app}$ ($\mu\text{M}/\text{h}$)	K_m, app (μM)	V_{max}/K_m
AKR1D1	T	5 β -DHT	0.02 (0.02–0.03)	0.10 (0.05–0.17)	0.24
	11KT	11K-5 β -DHT	0.37 (0.28–0.57)	4.09 (2.98–6.34)	0.09
SRD5A1	T	5 α -DHT	0.07 (0.05–0.10)	0.55 (0.33–0.99)	0.12
	11KT	11K-5 α -DHT	0.01 (0.00–N.A.)	0.64 (0.23–N.A.)	0.01
SRD5A2	T	5 α -DHT	0.22 (0.20–0.25)	0.21 (0.17–0.25)	1.06
	11KT	11K-5 α -DHT	0.71 (0.46–1.66)	4.28 (2.59–10.56)	0.17

the V_{max} for T (0.37 $\mu\text{M}/\text{h}$ vs 0.02 $\mu\text{M}/\text{h}$). However, this was offset by a lower apparent K_m for T when compared to 11KT (Table 1), resulting in a moderately more efficient reaction for T as estimated from the V_{max}/K_m values (0.24 h^{-1} vs 0.09 h^{-1}). Comparing the V_{max}/K_m values for all three enzymes indicates that the order of estimated enzyme efficiency is SRD5A2 > AKR1D1 > SRD5A1 for both T and 11KT, but with significant differences observed between the two substrates. A similar trend was observed for the androgen precursors A4 and 11KA4 (Supplementary data: Fig. 1 and Table 1). Neither 11OHA4 nor 11 β -hydroxytestosterone (11OHT) were metabolised by AKR1D1 (data not shown).

3.2. AKR1D1 catalyses the inactivation of 11KT

The 5 α -reduction of T and 11KT yields 5 α -DHT and 11K-5 α -DHT, respectively, which are both more potent agonists of the AR than their direct precursors [6,8]. Conversely, AKR1D1 catalysed 5 β -reduction of T yields 5 β -DHT, which unlike its precursor, is not an agonist of the AR [52]. Here we show that similar to the case with T, AKR1D1 inactivates 11KT (Fig. 3), demonstrating that 11K-5 β -DHT is an inactive metabolite.

3.3. Co-expression of SRD5A and AKR1D1 favours the 5 β -reduction of 11KT

Given that SRD5A1, SRD5A2 and AKR1D1 are co-expressed in the liver (Supplementary data: Fig. 3), the primary site of steroid metabolism, we next determined the effect of co-expressing either SRD5A1 or SRD5A2 with AKR1D1. This was accomplished by transiently transfecting HEK293 cells with either SRD5A1, SRD5A2 or AKR1D1 and then mixing the SRD5A and AKR1D1 transfected cells at a ratio of 1:1. The metabolism of T yielded 5 α -DHT and 5 β -DHT in both cases, but with the 5 α -reduced product dominating in the presence of SRD5A2 (Fig. 4A and C). Similarly, co-expression of SRD5A2 and AKR1D1 yielded more 11K-5 α -DHT than 11K-5 β -DHT from incubations with 11KT, however the ratio of 5 β :5 α -products was 3.7-fold higher than that observed for T (0.37 vs 0.10). Furthermore, co-expression of SRD5A1 and AKR1D1 yielded minimal 5 α -reduced product as expected due to the inability of SRD5A1 to efficiently catalyse the 5 α -reduction of 11KT (Figs. 3 and 4B, Table 1).

Metabolism of A4 and 11KA4 yielded similar trends to that of T and 11KT, with A4 metabolism leading to predominantly 5 α -reduced products irrespective of which SRD5A isozyme was co-expressed with AKR1D1 (Supplementary data: Fig. 2A and C). Conversely, the co-expression of SRD5A1 or SRD5A2 with AKR1D1 favoured the production of 11-keto-5 β -androstenedione (11K-5 β -dione) or 11-keto-5 α -androstenedione (11K-5 α -dione), respectively (Supplementary data: Fig. 2B and D). Neither 11OHA4 nor 11OHT showed significant conversion to 5 β -products in either co-expression system (data not shown).

Using the kinetic parameters established in Table 1, we constructed a computational model for SRD5A and AKR1D1 co-expression. By adjusting the model for the activity of SRD5A and AKR1D1 measured in the co-expression experiments, the model was able to accurately predict the metabolism of T and 11KT in our experimental co-expression

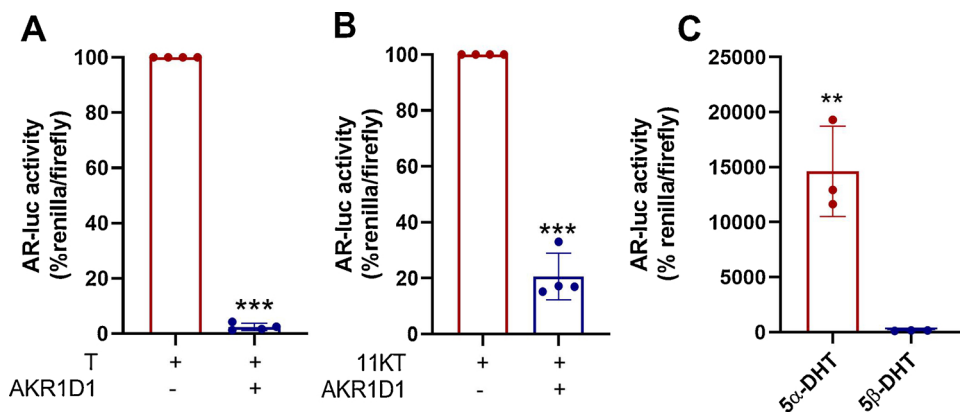
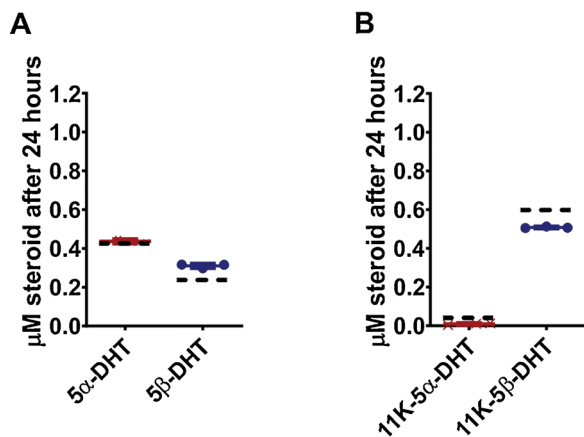


Fig. 3. AKR1D1 catalyses the inactivation of 11KT. AR-activation by 5 β -DHT (A) and 11K-5 β -DHT (B). AR activity was measured in HEK293 cells transfected with ARE and a luciferase reporter construct and treated with conditioned media from incubations of HEK293 cells transiently transfected with AKR1D1 or empty vector. The activity of the promoter-reporter system was confirmed by direct treatments with 5 α -DHT and 5 β -DHT (C). Results represent four independent experiments performed in triplicate shown as the mean \pm SEM. Statistical significance was determined using a paired *t*-test with ***, *p* < 0.001.

AKR1D1:SRD5A1



AKR1D1:SRD5A2

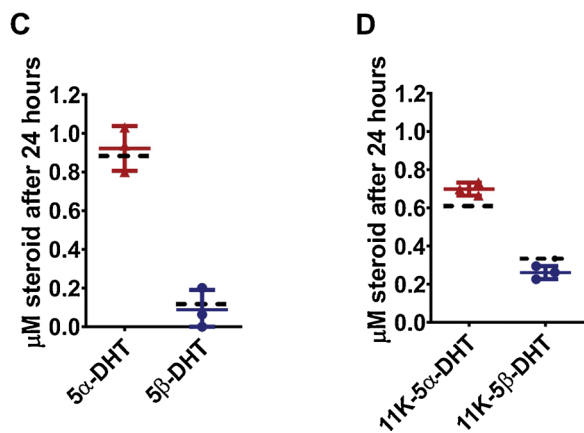


Fig. 4. The co-expression of AKR1D1 and SRD5A isozymes determine the metabolic fate of T (A and C) and 11KT (B and D). HEK293 cells expressing AKR1D1 or either SRD5A1 (A and B) or SRD5A2 (C and D) were mixed at a ratio of 1:1. The resulting 5 α - and 5 β -reduction of 1 μ M T (A and C) and 11KT (B and D) were measured after 24 h using UHPSFC-MS/MS. Experimental results (mean \pm SD) are shown in red (5 α -product) and blue (5 β -product), while the computational model predictions are shown with black dashed lines.

systems (Fig. 4). Given the accuracy of the model, we next used it as a tool to investigate the simultaneous expression of all three enzymes. During these simulations we fixed the activity of SRD5A1 while varying the activity of AKR1D1 and SRD5A2 10-fold in either direction. Using this approach we were able to show that the proportion of 5 α -reduced

products remains lower for 11KT than for T under all simulated conditions (Fig. 5).

Model predictions for A4 and 11KA4 metabolism with the different levels of enzyme activity were also generated (Supplementary data: Figure: 4). Similar results were observed with the proportion of 5 α -reduced products remaining lower for 11KA4 than for A4 under all simulated conditions.

3.4. The sequential metabolism of 11KT by AKR1D1, AKR1C2 and HSD17B2 yields 11-ketoetiocholanolone

After showing that 11KT is efficiently 5 β -reduced by AKR1D1, and that this 5 β -reduction makes a considerable contribution to 11KT metabolism under physiological conditions, we next investigated the sequential metabolism of 11KT by AKR1D1, AKR1C2 and HSD17B2 in an effort to replicate the established 5 β -metabolic pathway followed by T (Fig. 1). T was used as a control in these experiments to ensure that the experimental system was able to produce Et (1 μ M) from T, thereby confirming the predicted enzymatic activities (data not shown). Using this system, we demonstrated that 11KT is sequentially converted to 11KEt (1.34 μ M) as anticipated (Fig. 6). Moreover, using UHPSFC-MS/MS we confirmed the formation of the predicted intermediate products, 11K-5 β -DHT and 11-keto-etiocholanediol (11KEdiol), though we were unable to quantify these due to a lack of commercially available standards.

3.5. 11KEt is the principal metabolite from 11KT metabolism in HepG2 cells

We next investigated the metabolism of 11KT and T in HepG2 human liver cells, which endogenously express SRD5A1, AKR1D1, AKR1C2 and HSD17B2, but not SRD5A2 [28,37,53]. Using this model system, we were able to demonstrate the conversion of T to An and Et and 11KT to 11KAn and 11KEt as predicted (Fig. 7). The amount of 5 β -reduced products were > 10-fold higher than that of the 5 α -reduced products for both T and 11KT. The ratio of 5 β :5 α -products were, however, higher for 11KT than T (22.7 vs 13.6) as predicted by our computational model (Fig. 5).

To ensure that this observation was due to AKR1D1 we knocked down the expression of AKR1D1 using siRNA (Fig. 7A and B). This resulted in a significant reduction in the levels of both Et and 11KEt. Furthermore, the ratios of 5 β :5 α -products were significantly reduced for both T (13.6 to 2) and 11KT (22.7 to 4.7) (Fig. 7E and H). Similar results were observed in Huh7 cells treated with 11KT (Supplementary data: Fig. 5).

4. Discussion

Although underappreciated for decades, it is now clear that 11-oxygenated androgens are important components of the androgen pool

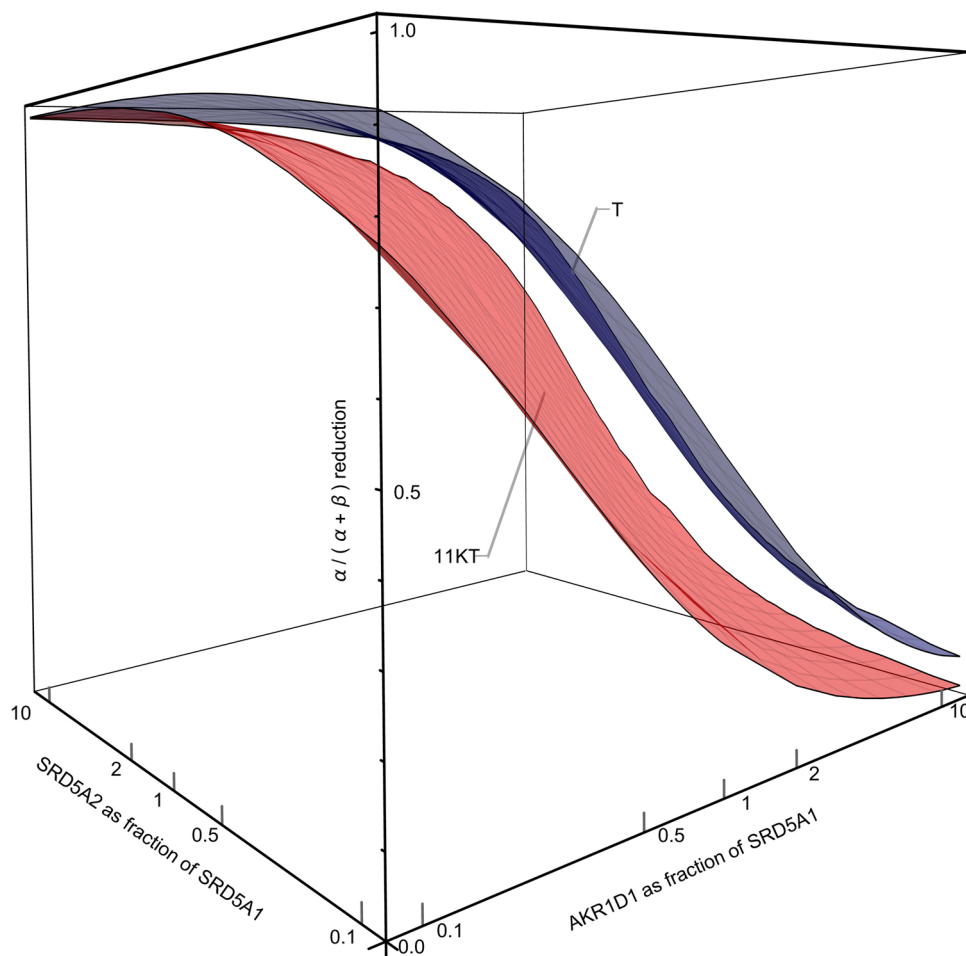


Fig. 5. Computational model of the effect of varying AKR1D1 and SRD5A2 activities. SRD5A1 activity was fixed while the activity of AKR1D1 and SRD5A2 were varied 10-fold in both directions. The resulting effects on the 5 α - and 5 β -reduction of T (blue) and 11KT (red) are shown as the proportion of 5 α -reduced products.

in humans. Yet, despite recent renewed interest in these steroids, the downstream metabolism of the potent androgen, 11KT, has remained undetermined. Therefore, we set out to characterise the activity of AKR1D1 and SRD5A isozymes towards T and 11KT, which accounts for the first commitment step of 3-keto Δ^4 steroid metabolism [20]. Our results show for the first time that 11KT is a substrate for 5 β -reduction by AKR1D1 (Figs. 3B, 4 B and D and 5; Table 1), and that the resulting product 11K-5 β -DHT cannot activate the human AR (Fig. 3). Moreover, we show that the 5 α -reduction of 11KT is isozyme specific with

SRD5A2, but not SRD5A1, efficiently catalysing this reaction (Fig. 3B, 4B and D; Table 1).

Considering that the liver is the primary site of steroid metabolism, and that both 5 β and 5 α -reductases are expressed in this tissue (Supplementary data: Fig. 3), we considered the effect of the coexpression of SRD5A1, SRD5A2 and AKR1D1 using both experimental and computational models. Our data demonstrates that the relative levels of AKR1D1 and SRD5A2 activity will regulate the metabolism of 11KT due to the limited SRD5A1 activity towards 11KT. Higher ratios of

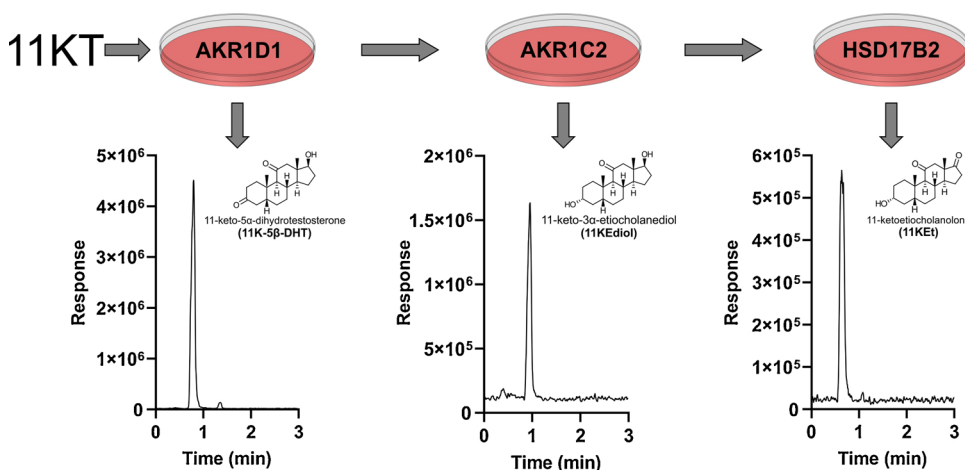


Fig. 6. The sequential production of 11KET from 11KT. 11KT was 5 β -reduced by AKR1D1 expressed in HEK293 cells. Incubation of HEK293 cells expressing AKR1C2 with the resulting 11K-5 β -DHT containing conditioned media subsequently yielded the product 11KEdiol. Further incubation of HEK293 cells expressing HSD17B2 with the 11KEdiol containing media resulted in the production of 11KET. The identity of each product was confirmed by UHPSFC-MS/MS at each step.

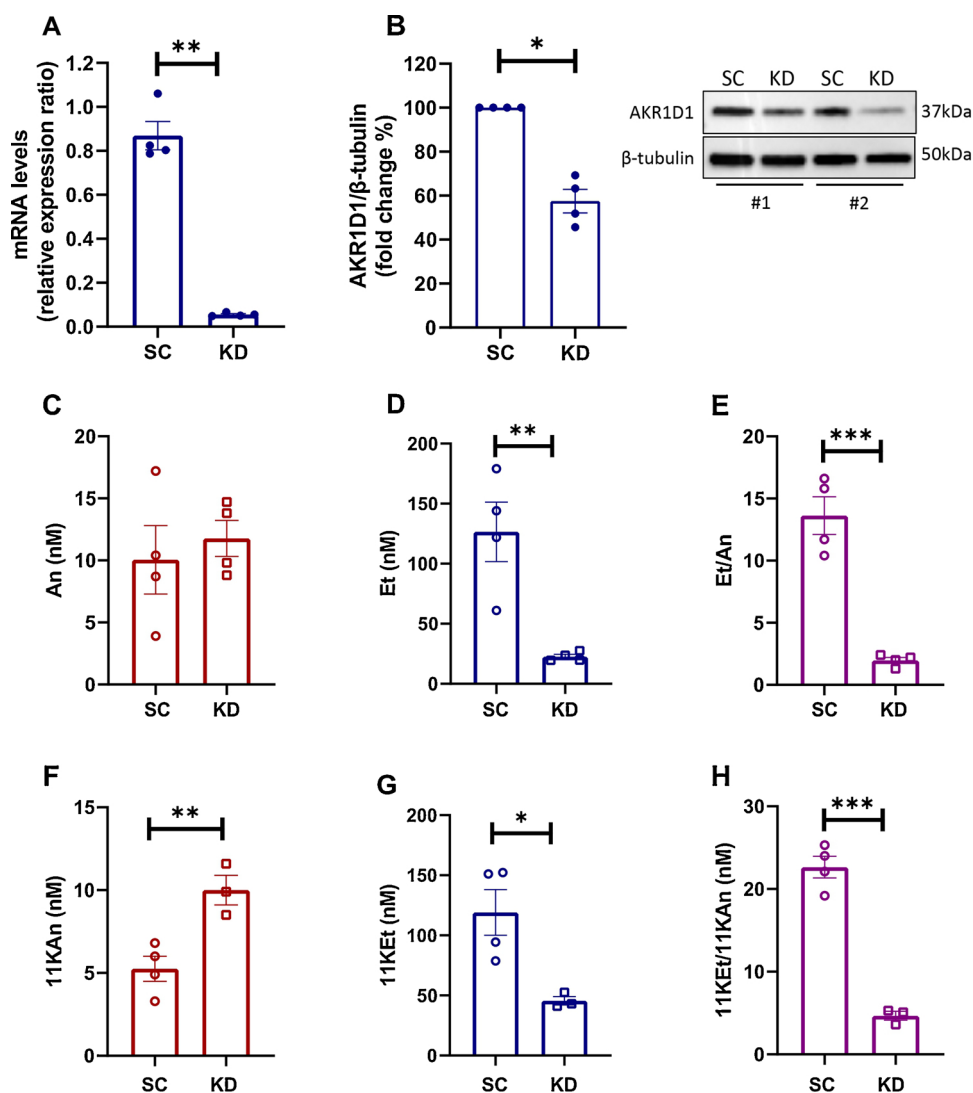


Fig. 7. Formation of Et and 11KEt by HepG2 cells after knockdown of AKR1D1. Relative mRNA levels of AKR1D1 after knockdown (A). Relative protein levels of AKR1D1 to β -tubulin after knockdown (B). An (C) and Et (D) and Et:An (E) production from T metabolism in HepG2 cells. 11KAN (F) and 11KEt (G) and 11KEt:11KAN (H) production from 11KT metabolism in HepG2 cells. Results represent four biological repeats each performed in duplicate shown as the mean \pm SEM. Statistical significance was determined using unpaired t-tests with *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$ and ****, $p < 0.0001$. SC, scrambled control; KD, knockdown.

AKR1D1:SRD5A2, as we report here for female liver (Supplementary data: Fig. 3), will favour the flux towards the 5β -reduced metabolite, 11KEt (Fig. 5).

Up until now, 11KEt was believed to be an exclusive urinary metabolite of the glucocorticoid, cortisone. Our data show that 11KT also contributes to 11KEt production, although its contribution is likely lower due to the higher circulating levels of cortisone relative to 11KT [20]. Nonetheless, this observation confirms that a major metabolite of 11KT is masked in urine metabolome assays and explains why urinary metabolites have not been assigned to 11KT. This overlap also shows that urinary 11KEt cannot be used as a surrogate to serum 11KT. Since we have illustrated that 11KA4 is also readily 5β -reduced by AKR1D1, with a 4-fold higher catalytic efficiency than 11KT (Table 1 and Supplementary data: Table 1), 11KA4 metabolism will also contribute to the production of 11KEt. Our data also shows that a proportion of 11KT metabolism would yield 11KAN, which is currently not routinely included in GC-MS urine analyses [19,54]. Furthermore, the metabolism of 11KA4, the direct precursor to 11KT, will also contribute to the production of 11KAN as 11KA4 is efficiently 5α -reduced by both SRD5A1 and SRD5A2 (Table 1 and Supplementary data: Table 1). Moreover, the glucocorticoid, cortisone, cannot be 5α -reduced and as a

result cannot contribute to the production of 11KAN. We therefore recommend that 11KAN is included as a urinary analyte in future studies, since this serves as a specific marker for 11KT and its precursor, 11KA4, and is the only marker of 11-oxygenated androgens not affected by glucocorticoid metabolism. Our data also demonstrate that neither 11OHA4 nor 11OHT are substrates for AKR1D1 and as a result, their further downstream metabolism would only contribute to urinary 11OHAn, and not to 11 β hydroxyetiocholanolone (11OHEt). 11OHAn is already generally accepted as a urinary marker for 11OHA4, with minor (< 10 %) contribution from the glucocorticoid pathway [17,18].

Another significant finding from this study is that 11KT is not efficiently 5α -reduced by SRD5A1, which is the predominant SRD5A isozyme expressed in the majority of peripheral tissues [22,24,25]. This observation likely explains why the circulating levels of 11K-5 α -DHT are substantially lower than those of 5 α -DHT [40] and suggests that 11KT is the primary active 11-oxygenated androgen. Moreover, 11KT may be resistant to peripheral inactivation by glucuronidation [8,55]. Thus, it appears that the peripheral metabolism and inactivation of 11KT is substantially different to that of T. We therefore propose that AKR1D1-catalysed 5β -reduction of 11KT plays a vital role in the inactivation and clearance of this potent androgen. When considering the

11-oxygenated androgens tested in this study, it is interesting to note that AKR1D1 demonstrated a significant substrate preference towards the 11-keto substrates, 11KT and 11KA4, and was unable to catalyse the 5 β -reduction of the 11hydroxysteroids, 11OHT or 11OHA4. We have previously reported a similar finding with the androgen activating enzyme AKR1C3, which efficiently catalyses the conversion of 11KA4 to 11KT, but does not accept 11OHA4 as a substrate [7]. Similarly, cortisone, which contains an 11keto moiety, is preferentially 5 β -reduced [34,56,57].

5. Conclusion

This study has shown for the first time that the AKR1D1-catalysed inactivation of 11KT leads to the production of 11KET, a urinary metabolite previously only associated with the metabolism of the glucocorticoid cortisone. Furthermore, we have shown that 11KT is not a substrate for SRD5A1 and that it can only be efficiently 5 α -reduced by SRD5A2. The relative expression ratios of SRD5A2:AKR1D1 therefore determines the metabolic fate of 11KT, with higher hepatic levels of AKR1D1 favouring the 5 β -reduction of 11KT. Some 11KT is however 5 α -reduced leading to the production of 11KAN, which we propose should be used as a specific urinary marker for 11KT and 11KA4, as unlike 11KET, it does not overlap with glucocorticoid metabolism.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jsmb.2020.105724>.

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