Canonical Androstenedione Reduction is the Predominant Source of Signalling

Androgens in Hormone Refractory Prostate Cancer

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It has been known for some time that castration-resistant prostate cancer tissue contains levels of signalling androgens sufficient to activate the androgen receptor, but the source of these steroids is highly controversial. For the first time we have performed a comprehensive screen of the steroidogenic potential of human benign, hormone naïve and progressive hormonally treated prostate cancer tissue, and identified that reduction of androstenedione via canonical enzyme pathways is the predominant source of signalling androgens. Importantly, we found no evidence of significant contribution from the previously described ‘backdoor’ or ‘5α-dione’ pathway to androgen synthesis, in contrast to a body of work based on cell lines and xenograft models. These results have important implications for our understanding of continued androgen synthesis in human disease, as well as the rational design of future therapies.
**Abstract**

**Purpose**

It has been recognised for almost a decade that concentrations of signalling androgens sufficient to activate the androgen receptor are present in castration resistant prostate cancer tissue. The source of these androgens is highly controversial, with 3 competing models proposed. We therefore wished to determine the androgenic potential of human benign and malignant (hormone-naïve and treated) when incubated with various precursors, and examine concomitant changes in enzyme expression.

**Experimental Design**

Freshly harvested prostate tissue (benign, hormone-naïve & hormone refractory prostate cancer) was incubated in excess concentrations of cholesterol, progesterone, DHEA, androstenedione, or testosterone for 96 hours, and steroid concentrations in the conditioned media measured by GC-MS. Changes in the expression of androgen synthetic and/or degradative enzymes was determined by expression microarray and qPCR. Significant changes were confirmed in an independent dataset.

**Results**

Of the precursor molecules tested, only incubation with androstenedione gave rise to significant concentrations of signalling androgens. Although this was observed in all tissue types, it occurred to a significantly greater degree in hormone refractory compared to hormone naïve cancer. Consistent with this, GSEA of the expression microarray data revealed significant upregulation of 17HSD17B activity, with overexpression of the canonical enzyme AKR1C3 confirmed by qPCR in the same samples and in a publically available expression dataset. Importantly we found no evidence to support a significant contribution form either the ‘backdoor’ or ‘5-α dione’ pathway.

**Conclusions**
Reduction of androstenedione to testosterone by the canonical HSD17B AKR1C3 is the predominant source of signalling androgens in hormone refractory prostate cancer.
Introduction

Evidence accumulated over the last decade clearly indicates that continued activation of Androgen Receptor (AR) mediated signalling is critical to the development and progression of castration-resistant prostate cancer. This is supported by evidence of receptor upregulation and nuclear localisation both in experimental systems and clinical samples, as well as the increased expression of AR regulated transcripts (notably KLK3/PSA) with disease progression, and confirmed by the recent clinical success of second line hormonal agents that continue to target AR signalling(1-6). It is also clear that despite the presence of castrate levels of testosterone in serum, sufficient concentrations of the two most potent AR ligands, testosterone (T) and/or dihydro-testosterone (DHT) are present in human castration-resistant prostate cancer tissue at levels sufficient to activate AR, with potent inhibitors of androgen synthesis producing dramatic clinical responses in this phase of the disease(4, 7). The origin of these androgens in human disease is unknown and highly controversial.

Under normal physiological conditions, T, the most important circulating androgen is synthesised de novo from cholesterol by Leydig cells in the testes by two parallel pathways (recently reviewed by Chang and Sharifi(8)). In a common first step, C27 cholesterol is converted to C21 pregnenolone by CYP11A1 mediated cleavage of a C6 side-chain on the inner membrane of mitochondria. In a series of reactions, pregnenolone is then converted to C17 androstenedione by 17α-hydroxylase/17,20-lyase (CYP17A1) and 3β-hydroxysteroid dehydrogenase/Δ5→Δ4 isomerase (HSD3B), via the alternative intermediates DHEA or progesterone depending upon the sequence of enzyme action. Androstenedione is then reduced to T by 17β-hydroxysteroid dehydrogenase (HSD17B), which may be further reduced to DHT in specific target organs that express 5α-reductase (SRD5A1/2). The weak androgen DHEA, and its sulphated form DHEA-S, are also synthesised and released by the
adrenal cells in the zona reticularis, but must be converted to T and/or DHT in peripheral tissues.

Three competing, although not mutually exclusive mechanisms have been proposed to explain the presence of significant concentrations of T/DHT in castration-resistant prostate cancer. The most widely accepted view is that these potent androgens are derived from the intra-tumoural conversion of weaker adrenal androgens based on normal physiological pathways. The critical feature of this mechanism is that T is an essential element, either as an AR ligand in its own right or as an immediate precursor for DHT. Two alternative pathways to DHT that bypass T have been recently described: de novo steroidogenesis via the ‘backdoor pathway’, as well direct conversion of androstenedione to DHT via the ‘5α-dione pathway’(9, 10). The more contentious is the de novo mechanism, which proposes that castration-resistant prostate cancer cells develop the ability to generate steroids directly from cholesterol, with synthesis of DHT in a 7-8 step reaction that bypasses the canonical androstanedione-T axis. This entails conversion of pregnenolone to androstenediol via androsterone, and then directly to DHT by a mix of both canonical (CYP17A, HSD17B) and backdoor (RDH5, SRD5A1) enzymes. In the more recently described ‘5α-dione’ pathway, it is proposed that adrenally derived androstenedione is reduced to 5α-androstanedione by SRD5A1, and then converted directly to DHT by HSD17B (see Figure 1).

Although there is significant overlap in the enzymes involved in each pathway, accurate knowledge of the exact mechanism involved is important for the rational design of more targeted inhibitors, with the potential for increased potency and fewer side-effects than currently available drugs. Unfortunately direct evidence to support any of the proposed mechanisms is lacking, with most of the data being derived from human cell lines or xenograft models, which may not accurately reflect the changes that occur in clinical disease. We were therefore interested in directly determining the ability of hormone-refractory
prostate cancer to generate the putative signalling ligands T and DHT from a range of androgen precursor molecules, and correlate this with changes in pertinent enzyme expression.

**Materials and Methods**

**Tissue incubation with androgen precursors**

Following institutional review board approval, fresh prostate tissue was collected from patients with benign prostatic hyperplasia, hormone-naïve or hormone-refractory prostate cancer undergoing transurethral resection (TURP) or radical prostatectomy for clinical indications. Patients with hormone refractory disease had evidence of clinical progression with or without a rising PSA despite treatment with androgen deprivation therapy (Table 1). **No patient was being treated with a 5α-reductase inhibitor (finasteride, dutasteride) at the time of surgery.** Collected tissue was placed in PBS in a specimen jar on ice, weighed, and then gently teased apart with scalpel and forceps under sterile conditions. As transurethral resection results in significant surface charring, tissue samples for steroid assays were preferentially prepared from the centre of larger prostate chips. The resulting minced tissue aggregates were then divided into 7 approximately equal portions, reweighed, and 6 of the 7 samples cultured in 2 ml of phenol red free RPMI (Gibco) containing 5% charcoal-stripped serum and 1% penicillin/streptomycin in a 24-well plate (Falcon). The remaining sample of fresh tissue was snap frozen for RNA analysis. To five of the six tissue-containing wells 1μl of a 20μg/μl stock of testosterone, cholesterol, progesterone, androstenedione or DHEA was added, while the final well contained no additive. To account for continued androgen production in the absence of exogenous precursors, steroid measurements in individual patients were standardized to this negative control. The addition of exogenous steroids was repeated in corresponding wells containing no tissue as controls. Due to limited
tissue availability, only one technical replicate per patient was possible, and the mean
(standard deviation) weight of fresh tissue used in each steroid assay was 135 (97) mg. The
plate was then incubated at 37°C in 5% CO₂ for 4 days, and the contents of the wells then
spun at 5000 rpm for 5 minutes and the supernatant collected for hormone extraction. **As**
androgen metabolism is a dynamic process, a time-course experiment may give a more
comprehensive picture of the intermediate steroids generated from each precursors,
particularly if short-lived. However given the limited amount of tissue available as well
as the number or precursors we wanted to test, we were limited to a single time-point.
Although many of the synthetic reactions occur within minutes or hours in enzyme
kinetic studies and cell culture experiments (10, 11), similar experiments using
xenografts and/or human tissue have shown continued accumulation of measurable
androgens in tissue supernatants using various precursors out to 72-96 hours(9, 10). As
tissue culture beyond 5 days in our hands resulted in a significant drop off in cell
viability (data not shown), we chose 96 hours as a ‘cumulative’ time-point to best assess
the potential contribution of the ‘de novo’ and 5α-dione pathways to DHT synthesis.
Remainin tumour tissue was fixed in formalin, paraffin-embedded and sections stained with
H&E for pathological assessment

**Hormone extraction**

The hormone extraction was carried out on 2ml of the supernatant from the tissue incubation.
The samples were transferred into a 12 x 75mm glass disposable culture tube (Kimble Chase)
and 1μl of a 20μg/μl stock of 16,16,17,d3-testosterone was added to each sample as an
internal standard. Ethyl acetate (Sigma-Aldrich) was added to the sample at a ratio of 1:1, the
tube was then capped and inverted twice, the lid removed and the sample allowed to settle for
10 min before transferring the top ethyl acetate layer to a new labelled glass tube. A second
ethyl acetate extraction was performed on the original sample and added to the first
extraction. The tubes were then placed in a heating block at 60°C overnight and the sample dried, capped and stored at 4°C. The dried samples were then sent to the National Measurement Institute (Sydney, Australia) where 50µl of 4ppm Methyl-testosterone was added before being dried using a TurboVap (Zymark) with nitrogen gas and a 40°C water bath. 50µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide was added before 2µl was injected onto the GC/MS (Shimadzu GCMS-QP2010 Plus with the AOC-20S auto-sampler) and individual steroid species identified using the method of Schanzer and Donike(12). At least 3 technical replicates of known standards containing 0, 200 and 400ng of each of the 15 different hormones detected; androsterone, androstenedione, androstenediol, 5α,3α-Androstanediol, 5β,3α-Androstanediol, 5α,3β-Androstanediol, cholesterol, DHEA, DHT, Progesterone, 17-OH-Progesterone, Pregnan-3α-ol-20-one, Prenone-3,20-dione, 17α-OH-Pregnenolone and Testosterone were also analysed per run in order to construct a calibration curve for each of the hormones detected. This curve was then used to determine the concentration of the unknown hormone.

**RNA tissue extraction**

Total RNA was extracted from snap frozen TURP tissue using the miRNeasy mini kit (Qiagen). The tissue was kept at -20°C and handled with RNA free instruments. No more than 50mg of frozen tissue was added to 700µl of QIAzol lysis reagent in a 14ml round bottom Falcon tube. The sample was then homogenised for 40sec using the TissueRuptor homogeniser (Qiagen) ensuring the sample was uniformly homogeneous with no small particles of tissue remaining. The sample was then transferred to a 1.5ml Eppendorf tube and incubated at RT for 5min before adding 140µl of chloroform, capping the lid and shaking vigorously for 15sec. The tube was allowed to settle at RT for 5min before centrifuging at 8,500rpm at 4°C for 15min, after which the sample was separated into three phases, the upper colourless aqueous layer containing the RNA, a white interphase containing the DNA and a
lower red organic layer containing the proteins. The upper layer containing the RNA was transferred to a new 1.5ml Eppendorf tube and 525µl of 100% ethanol was mixed in thoroughly by pipette. The sample was then transferred to an RNeasy mini spin column and centrifuged at 13,000rpm for 15sec at RT before discarding the flowthrough and adding 700µl of buffer RWT and centrifuging for a further 15sec at 13,000rpm at RT, discarding the flowthrough. 500µl of buffer RPE was added to the spin column and centrifuged at 13,000rpm for 15sec at RT before discarding the flowthrough and washing the column a second time with 500µl of buffer RPE and centrifuging for 2min at 13,000rpm at RT to dry the membrane. The spin column was placed in a fresh collection tube and centrifuged at 13,000rpm for 1min to remove any residual buffer RPE. The spin column was then transferred into a labelled Eppendorf tube and 37µl of RNase free water added to elute the RNA before centrifuging at 13,000rpm for 1min at RT. The concentration and purity of RNA was then determined using the NanoDrop 3300, before running a sample on an RNA gel to check RNA quality.

**Gene Expression Microarray**

The HumanHT-12v4 BeadChip (BD-103-0204, Illumina) was utilized for gene expression microarray profiling. Briefly, 100ng of total RNA was used as input for cDNA library construction with the Illumina®TotalPrep™RNA Amplification kit (AMIL1791, Applied Biosystems) as per manufacturers instructions. Poly-A RNA was selected and reverse transcribed to single stranded cDNA, converted to double stranded DNA and purified. The DNA was then transcribed in vitro overnight in the presence of biotinylated UTP and CTP to produce biotin-labelled cRNA. This cRNA was hybridised to the BeadChip over 16 hours. Following addition of streptavidin-Cy3, the BeadChip was read using an iScan System (Illumina) and raw intensities generated from laser-excited fluorescence.

**qRT-PCR**
For each sample analysed, 200 ng of RNA, extracted from prostate tissue was reverse transcribed into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. qRT-PCR was performed using 1 μl of cDNA, 0.5 μl Taqman primers (pre-designed and commercially available from Applied Biosystems), 5 μl of Taqman Fast Advanced Master Mix (Applied Biosystems) and made up to 10 μl volume per well with UltraPure distilled water (Gibco). Samples were run on a 384-well plate using a Viia7 PCR machine (Applied Biosystems) under the following conditions: UNG incubation at 50°C for 2 mins; polymerase activation at 95°C for 20 secs; denature at 95°C for 1 sec; anneal/extend at 60°C for 20 secs. Taqman primers (Applied Biosystems) to steroidogenesis associated genes were as follows: CYP11A1 (cat no Hs00897320_m1); HSD17B4 (Hs01069900_m1); AKR1C3 (Hs00366267_m1); RDH5 (Hs00161263_m1); SRD5A1 (Hs00602694_m1); SRD5A2 (Hs00936406_m1).

Bioinformatics processing
BeadChip array analysis was performed using the limma package as part of Bioconductor in R(13). Raw intensities were background corrected using negative control probes and quantile normalisation was performed using negative and positive control probes. If there were multiple probes mapping to a gene, only the probe showing the maximum average intensity was retained. Batch effects were corrected using ComBat(14). Heatmaps were generated using the heatmap.2 package and all intensities were normalised by row (gene). Gene set enrichment analysis (GSEA) was performed using the mean-rank gene set test in limma. P-values were corrected for multiple testing using the Benjamini-Hochberg method(15) and q-values derived. The q-value for each hypothesis is the minimum False Discovery Rate at which the test may be called significant. For example, if a hypothesis has a q value of 0.1, then the null hypothesis can be rejected if it is accepted a priori that 1 in 10 rejected null hypotheses are incorrect. To convert p-values to q-values using the
Benjamini-Hochberg method, the p-values are first arranged from smallest to largest, and the largest p-value is then multiplied by the number of tests. Following this, the next largest p-value is multiplied by the total number of tests divided by the rank of that p-value in the list. This is repeated for all p-values in the list to derive a q-value for each test.

Results

Androstenedione is the Preferred Precursor for Intra-Prostatic Testosterone Production

To determine the efficiency whereby various precursors undergo intra-prostatic conversion to more potent androgens, we measured the concentrations of 15 different steroids in the conditioned media following incubation of fresh BPH, androgen-naïve or hormone refractory prostate cancer tissue for 96 hours with excess cholesterol, progesterone, androstenedione, DHEA and testosterone. We observed that only incubation with androstenedione led to the generation of significant concentrations of T productions across all tissue types, and appreciable quantities of DHT were identified only when T was the precursor (Figure 2). Notably we did not identify any significant DHT (or T) production when prostate tissue was incubated with two precursors of the ‘de novo’ pathway, cholesterol and progesterone, nor did we detect any significant production of DHT from DHEA or directly from androstenedione by the ‘5α-dione’ pathway (Figure S1), despite evidence of both SRD5A (T \(\rightarrow\) DHT) and 3αHSD (androstenedione \(\rightarrow\) androsterone) activity in hormone-refractory prostate cancer tissue. Small quantities of androsterone were identifiable in media from both benign and castration-resistant prostate tissue when incubated with cholesterol, suggesting that de novo synthesis may occur at a low background level, although there was no significant upregulation with hormone-resistance. When analysed by tissue type, hormone refractory prostate cancer (HRPC) was more efficient at converting androstenedione to
testosterone than both benign tissue and androgen-naïve prostate cancer (Figure 2 inset), although there was no significant difference in the ability to reduce T to DHT. Interestingly, T was more efficiently converted to androstenedione rather than DHT in both benign and castration-resistant prostate tissue, mirroring the results observed when androstenedione was used as a precursor, indicating preferential upregulation of HSD17B activity in these tissues. Consistent with previous reports, progesterone, DHEA, androstenedione and T lead to increases in cholesterol production, particularly in hormone resistant tumour tissue(16).

**HRPC is Associated with a Significant Enrichment of HSD17B Expression**

The synthesis of T and DHT from precursor molecules in androgenic tissue under normal physiological conditions occurs via a well-established sequence of reversible reactions, although more recently a number of alternative pathways have been described that bypass this canonical pathway in castration-resistant prostate cancer. As androgen levels may be affected by both changes in synthetic and degradative enzyme expression, and given the number of possibly redundant pathways involved, to obtain an unbiased global estimate of the expression of potential genes of interest we analysed gene expression in the same benign, androgen-naïve and hormone refractory prostate cancer specimens using the Illumina HumanHT-12 microarray. As shown in Figure 3a, although the expression of a number of genes of interest was consistently higher in hormone refractory compared to androgen-naïve prostate cancer samples (HSD17B4, 7 & 12), the magnitude of measurement difference was small and did not meet significance using the stringent false discovery cut-offs. This is not surprising, given the small number of cases within each group, variance in expression, as well as the potential redundancy of activity across a number of different iso-enzymes. We therefore performed geneset enrichment analysis using a number of pre-specified gene lists as shown in TableS1, and identified significant enrichment for HSD17B
activity at a q-value <0.1 (Figure 3b). This is consistent with the results of our culture experiments, as HSD17B isoenzymes are responsible for the reversible 17-keto reduction of androstenedione to T.

**HRPC is Associated with Overexpression of the Canonical Isoenzyme AKR1C3**

To confirm some of the findings identified using the expression microarray, we performed quantitative PCR using RNA isolated from a subset of the same tissue samples, with at least 3 biological replicates in each group. Given the limited amount of starting RNA available from the sample set, we focussed on a small number of genes of interest, including the ‘backdoor’ genes CYP11A1, HSD17B9 (RDH5) and SRD5A1, as well as the canonical 17β-hydroxysteroid dehydrogenase HSD17B5 (AKR1C3) and the alternative isoform HSD17B4, which appeared to be consistently upregulated compared to naïve tissue, and overexpression of which has previously been associated with poor outcome in prostate cancer(17). We found that the expression of AKR1C3 was significantly elevated in HRPC samples compared to both benign and androgen-naïve samples, whereas RHD5, was actually significantly decreased in CRPC samples compared to benign tissue (Figure 4). Interestingly, consistent with previous reports, the expression of SRD5A1 was upregulated in hormone refractory tumours(18), whereas SRD5A2 levels were unchanged.

**AKR1C3 is Overexpressed in Hormone Refractory Metastases**

It is clear that like many solid tumours, prostate cancers are molecularly heterogeneous, and it is possible that different mechanisms are preferentially active in tissue from alternative clinical sites. Due to the limited tissue availability from biopsy of metastases, for our steroidogenesis and expression assays we used tumour derived from transurethral resection or obtained at prostatectomy, which clearly represents events occurring in localised disease. To determine if
similar changes in enzyme abundance occur in the metastatic setting, we downloaded and re-analysed expression data from the Taylor cohort (29 matched normal prostate samples, 131 primary prostate cancers and 19 metastases) which were interrogated using the Affymetrix Human Exon 1.0 ST array(19). Based on the clinical annotations provided we probed the differences in expression between non-castration resistant (lymph node metastases obtained at the time of planned prostatectomy ± neo-adjuvant androgen deprivation) and hormone refractory metastases (distant metastases + prior hormonal treatment ± prior chemotherapy), in comparison to both localised prostate cancer and benign tissue. Consistent with our qPCR results, we saw no increase in the expression of the backdoor enzymes CYP11A1 and RDH5 in castration-resistant metastasis (Figure 5). In contrast, although the expression of the AKR1C3 in non-castration resistance metastases was similar to both normal prostate and localised prostate cancer, expression in castration metastases was increased by 153%, 112% and 148% respectively. The expression of SRD5A1 was similarly highest in castration-resistance metastases, although a more incremental increase in expression was observed across the tissue types. **In contrast, SRD5A2 expression was significantly decreased in metastatic lesions compared to localised prostate tumours regardless of hormonal status.** No significant differences in CYP17A1, HSD17B1, HSD17B2, HSD17B3, HSD17B4, HSD17B6, HSD17B7, HSD17B9 (RDH5), HSD17B10, HSD17B11, HSD17B12, HSD17B13, HSD3B1, HSD3B2, or SRD5A3 expression between castration-resistant and non-castration resistant metastases was observed (data not shown).

**Discussion**

Ever since concentrations of potent signalling androgens sufficient to activate the AR under experimental conditions were measured in castration-resistant prostate cancer tissue, there has been much speculation as to their source. The orthodox view is that they are generated by
the intra-tumoural conversion of adrenal precursors utilising physiological androgenic enzymes and reactions, culminating in the reduction of T to DHT. Recently two alternative reaction pathways that bypass T as the immediate precursor of DHT (‘de novo’ and 5α-dione pathway) have been proposed to be important in human disease. In this study, which is the first to comprehensively address androgen production by human prostate tissue at distinct stages of disease progression, we have identified that the only significant source of androgen precursor in any tissue type is the intermediate steroid androstenedione, and that the predominant potent androgen generated is T. Conversion of androstenedione to T was observed in both benign and malignant prostate tissue, suggesting that this is a normal physiological pathway, although the generation of T was significantly more efficient in hormone-resistant compared to hormone-naïve tumour. In particular, we did not identify any evidence of DHT production from the ‘de novo’ precursors cholesterol or progesterone, nor did we observe direct conversion of androstenedione to DHT via the 5α-dione pathway, despite evidence within the assay of necessary enzyme activity.

Consistent with the results of our steroid assay, we observed upregulation of HSD17B genes which are responsible for the reduction of androstenedione to T in castration-resistant prostate cancer samples on GSEA, and confirmed over-expression of the canonical substrate-specific 17β-hydroxysteroid dehydrogenase isoenzyme AKR1C3 by PCR. A similar finding was observed specifically in castration-resistant metastases in the Taylor dataset, strongly suggesting that this mechanism is not limited to localised tissue. AKR1C3 expression has been previously shown to be hormonally regulated, in that treatment of cell lines with the synthetic androgen R1881 suppressed transcript levels, whereas castration leads to its upregulation in xenograft models(20). In addition, AKR1C3 expression is consistently upregulated as measured in both global and gene level analysis of human castration resistant compared to hormone naïve prostate tissue, as well as by immunohistochemistry of
corresponding tissue sections(21, 22). Taken together these results strongly suggest that
upregulation of AKR1C3 mediated conversion of androstenedione to T is the predominant
source of potent androgens in castration-resistant prostate cancer. In contrast to previously
reported castration-resistant LNCaP xenografts and consistent with previous reports of human
tissue analysis, we did not find significant enrichment for enzymes implicated in the
backdoor pathway(20).

Chang et al have proposed based on their analysis of a variety of prostate cancer cell
lines and two metastatic castrate resistant prostate cancer fresh tumour tissue samples, the
existence of a 5α-dione intermediate pathway whereby androstenedione is converted
eventually to DHT via the intermediate 5α-androstanedione(10). They demonstrated the
accumulation of DHT from the androstenedione precursor in a time course experiment for
both LNCaP and LAPC4 cell lines, however this was not demonstrated for the two castrate
resistant patient tissue samples they examined with only the conversion of androstenedione to
the intermediate 5α- androstanedione and conversion of T to DHT data presented.
Interestingly, conversion of androstenedione to T was clearly demonstrated for their patient 1
sample and no loss in cumulative levels of T was observed for the patient 2 sample,
suggesting that very little of the T added to the patient samples was actually converted to
DHT. These results are actually in accordance with our findings of conversion of
androstenedione to T and no measurable accumulation of DHT from androstenedione. We
did not measure the 5α-androstanedione intermediate produced from our samples upon
addition of the steroid precursor androstenedione, however we did measure detectable
levels of the downstream intermediates 3α-androsterone and 5α-androstane-3α,17β-
diol, suggesting that reasonable levels of 5α-androstanedione were produced upon
addition of androstenedione which were then further catalyzed by 3αHSD
(AKR1C3/AKR1C1) and AKR1C3/17βHSD3 to the respective downstream metabolites.
In fact, we observed that the concentrations of androsterone measured in culture supernatants mirrored that of T when androstenedione was used as a precursor, suggesting that reduction to 5α-androstanedione is at least as efficient as conversion to T in any given tissue type; however we have no evidence that any intermediates were further processed into detectable levels of DHT. Given that AKR1C3 catalyses both 5α-androstanedione $\rightarrow$ DHT and 5α-androstanedione $\rightarrow$ androsterone reactions, and that its expression is clearly upregulated in hormone refractory prostate cancer, it is not immediately clear as to why the more convoluted enzymatic generation of androsterone should be favoured compared to the more straightforward conversion to DHT. There are however at least 3 splice variants of the AKR1C3 gene and to date over 67 SNPs in coding regions of the AKR1C3 gene have been identified, with 47 of these being nonsynonomous and potentially able to change the functional activity of the enzyme(23). Subtle changes in substrate specificity and/or catalytic activity of AKR1C3 variants may favour conversion of substrates down one particular pathway over other potential alternatives which may account for the unexpected accumulation of androsterone in the primary prostate tissues we measured. It was also surprising that despite elevated expression of the T to DHT converting enzyme SRD5A1 in our hormone resistant samples compared to the hormone naïve and BPH samples, we did not observe any increase in DHT. Raised levels of SRD5A1 have been observed in numerous studies of CRPC tissue samples though our results suggest that the raised levels of messenger RNA detected in these samples do not equate with raised functional activity(21, 24, 25). Interestingly, the paradoxical observation of elevated T levels despite increased expression of the T converting enzyme SRD5A1 has long been recognized in CRPC tissue samples, although there is usually a concomitant decline in SRD5A2 expression similar to that demonstrated in the Taylor dataset(21, 25). Physiologically, SRD5A2 is the only significant form of 5a-reductase in the
intact male prostate with little detectable levels of SRD5A1 observed(26). Genetic studies of mutant individuals have also confirmed the physiological role of SRD5A2 with individuals with 5a-reductase deficiency where pseudohermaphroditism and hypogonadism are exclusively mutants in the SRD5A2 gene but not in SRD5A1(27). This suggests that the key regulator of T to DHT conversion in prostate tumour tissues is in fact SRD5A2, which consistently declines in castrate resistant tissue due in part to lack of the well-known DHT feed forward regulatory effect(28, 29). This would explain the relative accumulation of T over DHT in castrate resistant tissues, with SRD5A1 despite its relative increase in expression being unable to efficiently catalyze conversion of T to DHT in prostatic tissues despite evidence of its ability to do so in cell lines(10). Supporting this is the observation that DHT was generated from precursor T across tissue types at a relative efficiency commensurate with SRD5A2, but not SRD5A1 expression. It is important to note however that we did not include metastatic tissue in our functional assays, and that much of the pre-clinical evidence supporting both the ‘backdoor’ and 5α-dione pathways has been generated using cell lines derived from clinical metastases. Therefore although the expression of genes involved in androgen synthesis in castration-resistant metastases is consistent with our observations in primary tumours, it remains possible that alternative pathways play a more prominent role in secondary sites of disease.

We note that appreciable concentrations of DHT were identified only when T was used a direct precursor, however in hormone resistant tumour tissue oxidation back to androstenedione was more efficient than reduction to DHT, suggesting that production of DHT in castrate resistant tissues is an inefficient process. It has been recognised for some time that the normal T to DHT ratio of approximately 1:10 is reversed in castration resistance, with T being the predominant androgen identified. It is interesting to speculate as to whether this switch in relative abundance of AR ligands has functional consequences for
disease progression or whether it is merely a consequence of it. Two large prostate cancer prevention trials have which have sought to manipulate the T to DHT ratios in the prostates of subjects potentially suggest that there may be functional consequences of switching these ratios within the prostate. The Prostate Cancer Prevention Trial showed that finasteride, a selective SRD5A2 inhibitor reduced the risk of prostate cancer by 25%, but among the tumours that were detected, there was a 27% increase in the number of those that had higher Gleason scores of 7 to 10(30). Similarly, the REDUCE trial examined the incidence of prostate cancer detected on biopsy among men at increased risk for the disease treated with dutasteride which unlike finasteride, inhibits both isoforms of 5α-reductase(31). During years 3 and 4 of this trial there was a significantly increased number (P=0.003) of Gleason 8 to 10 tumours detected in the dutasteride group versus placebo(32). There are many competing explanations for the increased risk of higher grade tumours detected in both these 5α-reductase inhibitor trials, however it remains plausible that the observed increases in high grade disease are at least in part due to the relative accumulation of T over DHT, a state commensurate with the prostatic milieu associated with advanced castrate resistant disease.

Collectively our results add further credence to calls for a comprehensive orchestrated targeting of the AR axis at the initiation of endocrine therapy rather than the sequential phasing in of such treatments as is commonly the practice today. Particularly pertinent to our results is the development of specific AKR1C3 inhibitors which would be interesting adjunct treatments that might specifically interfere with the switch in T to DHT ratios in the prostate(33-37).

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36. Byrns MC, Steckelbroeck S, Penning TM. An indomethacin analogue, N-(4-chlorobenzoyl)-melatonin, is a selective inhibitor of aldo-keto reductase 1C3 (type 2 3alpha-HSD, type 5 17beta-HSD, and prostaglandin F synthase), a potential target for the treatment

Table 1. Clinical and Pathological Characteristics of the prostate cancer patients

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age</th>
<th>Disease</th>
<th>Gleason Score</th>
<th>Mets</th>
<th>Progression</th>
<th>Prior Hormonal Therapy</th>
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</thead>
<tbody>
<tr>
<td>TURP_1</td>
<td>57</td>
<td>HRPC</td>
<td>5+5</td>
<td>Yes</td>
<td>Yes</td>
<td>LHRH agonist</td>
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<tr>
<td>TURP_8</td>
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<td>Yes</td>
<td>LHRH agonist, Bicalutamide, Abiraterone</td>
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<td>TURP_9</td>
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<td>TURP_13</td>
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<td>LHRH agonist</td>
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<td>TURP_14</td>
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<td>PP100</td>
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<td>Nil</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** Schematic outlining recognised pathways of androgen synthesis. The canonical pathway is unmarked, whereas the ‘backdoor’ and ‘5α-dione’ pathways are marked in grey and pink respectively. Adapted from Locke et al.(9) and Chang and Sarifi(8).

**Figure 2.** (i) Mean + SEM concentration of indicated steroids measured by GC/MS in the conditioned media derived from prostate tissue (benign prostatic hyperplasia (BPH) n= 9, hormone naïve prostate cancer (CaP) n=4 or hormone-refractory CaP (HRPC) n=3 or 4) when cultured in the presence of various precursors at a concentration of 10 μg/ml for 96 hr. Inset shows mean + SEM concentration of testosterone measured in the conditioned media of prostate tissue categorized by pathology. *p<0.05 vs hormone naïve using a 2-sided t-test. (ii) Mean + SEM concentration of indicated steroids measured in the conditioned media derived from BPH, hormone naïve CaP or HRPC when cultured in the presence of androstenedione 10 μg/ml for 96 hr.

**Figure 3.** (i) Heatmap depicting the relative gene expression of enzymes implicated in androgen synthesis or degradation in prostate samples measured using the Illumina HT12 BeadChip and grouped by disease. (ii) Geneset enrichment analysis of the gene expression data using custom gene lists as detailed in the supplementary tables, with the associated p and q values.

**Figure 4.** Mean + SEM logfold concentration of selected gene transcripts in BPH (n=3 or 4), hormone naïve CaP (n=3 or 4) or HRPC (n=3 or 4) compared to a standard curve and normalized to various housekeeping genes. P values refer to the significance of 2-sided t-tests between indicated groups.
**Figure 5.** Boxplot of median expression + 95% CIs of selected gene expression in the Taylor dataset categorized by tissue type and hormone status of metastases. LNCaP xenografts represent n=1 and are represented by a horizontal bar for interest only. Outliers are represented by single dot points. Differences between groups were determined using independent 2-sided t-tests.
Figure 1

Cholesterol $\xrightarrow{CYP11A1}$ Pregnenolone $\xrightarrow{HSD3B}$ Progesterone $\xrightarrow{SRD5A}$ Pregnan-3,20-dione

17α-Hydroxypregnenolone $\xrightarrow{CYP17A1}$ 17α-Hydroxyprogesterone $\xrightarrow{HSD3B}$ 17α-Hydroxyprogesterone

DHEA $\xrightarrow{HSD3B}$ Androstenedione $\xrightarrow{SRD5A}$ 5α-androstane-3α,17β-diol-3-one $\xrightarrow{3αHSD (AKR1C1/3)}$ Androsterone $\xrightarrow{CYP17A1}$ Androsterone

Androstenediol $\xrightarrow{HSD3B}$ Testosterone $\xrightarrow{SRD5A}$ Dihydrotestosterone $\xrightarrow{HSD17B3 \ AKR1C3}$ 5α-androstane-3α,17β-diol-3-one $\xrightarrow{RDH5}$ 5α-androstane-3α,17β-diol-3-one

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Figure 2

(i)

(ii)

Androstanedione

- Benign Prostate
- Hormone naïve CaP
- mHRPC
Hormone-naïve CaP CRPC

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Hormone-naïve CaP</th>
<th>CRPC</th>
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<tr>
<td></td>
<td>p value</td>
<td>q value</td>
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<td>HSD17 activity</td>
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<td>Backdoor pathway</td>
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<tr>
<td>Degradation</td>
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<td>1.0000</td>
</tr>
</tbody>
</table>

Figure 3
Figure 4

CYP11A1

RDHS

p = 0.039

SRD5A1

HSD17B4

p = 0.035

AKR1C3

p = 0.012

p = 0.028

SRD5A2
Figure 5
Clinical Cancer Research

Canonical Androstenedione Reduction is the Predominant Source of Signalling Androgens in Hormone Refractory Prostate Cancer

Matthew Fankhauser, Yuen Tan, Geoff Macintyre, et al.

Clin Cancer Res Published OnlineFirst April 25, 2014.