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Steroidogenesis inhibitors alter but do not eliminate androgen synthesis mechanisms during progression to castration-resistance in LNCaP prostate xenografts

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ABSTRACT

In castration-resistant prostate cancer (CRPC) many androgen-regulated genes become re-expressed and tissue androgen levels increase despite low serum levels. We and others have recently reported that CRPC tumor cells can *de novo* synthesize androgens from adrenal steroid precursors or cholesterol and that high levels of progesterone exist in LNCaP tumors after castration serving perhaps as an intermediate in androgen synthesis.

Herein, we compare androgen synthesis from [^{3}H-progesterone] in the presence of specific steroidogenesis inhibitors and anti-androgens in steroid starved LNCaP cells and CRPC tumors. Similarly, we compare steroid profiles in LNCaP tumors at different stages of CRPC progression.

Steroidogenesis inhibitors targeting CYP17A1 and SRD5A2 significantly altered but did not eliminate androgen synthesis from progesterone in steroid starved LNCaP cells and CRPC tumors. Upon exposure to inhibitors of steroidogenesis prostate cancer cells adapt gradually during CRPC progression to synthesize DHT in a compensatory manner through alternative feed-forward mechanisms. Furthermore, tumors obtained immediately after castration are significantly less efficient at metabolizing progesterone (~36%) and produce a different steroid profile to CRPC tumors. Optimal targeting of the androgen axis may be most effective when tumors are least efficient at synthesizing androgens. Confirmatory studies in humans are required to validate these findings.

1. Introduction

Progression of prostate cancer (CaP) emerging after therapeutic approaches to block testicular androgen synthesis leads to "androgen-independent" or "castration resistant" prostate cancer (CRPC) which is the lethal component of this disease [1]. Several treatments targeting hormone synthesis and androgen receptor activation have been used both individually and in combinations in patients displaying CRPC disease [2–6]. These agents have been shown to alleviate symptoms of the disease and evoke prostate specific antigen (PSA) responses but have not yet proven to prolong survival [2–6]. In order to effectively treat CRPC patients and improve survival, a better understanding of the underlying mechanisms of CaP progression to CRPC (and how drugs affect these mechanisms) is necessary in order to strategically identify key targets, develop drugs inhibiting these targets and administer effective therapeutic interventions.

Analysis of CaP tumors from patients as well as human derived-xenograft tumors from CRPC progression models such as LNCaP has shown that many androgen-regulated genes, including the steroid metabolizing enzymes HSD3B2, SRD5A1, CYP17A1, AKR1C1, AKR1C2, AKR1C3 and SREBPs become re-expressed in CRPC tumors [7–9]. Recently, Titus et al. used liquid chromatography–mass spectrometry (LC–MS) to show that tumors obtained from recurrent CaP patients contain testosterone and dihydrotestosterone (DHT) in high enough levels to activate the androgen receptor (AR) in CRPC cells, despite observed low levels of circulating androgen in the serum [10,11]. Labrie and others have shown evidence suggesting that after castration steroid precursors obtained through circulation from the adrenals can be captured and utilized by CaP tumors to make these androgens [10,12,8]. We and others have demonstrated using radiotracing techniques that CRPC tumor cells can in fact *de novo* synthesize their own androgens from cholesterol and upstream precursors of cholesterol [13–15]. Combined,

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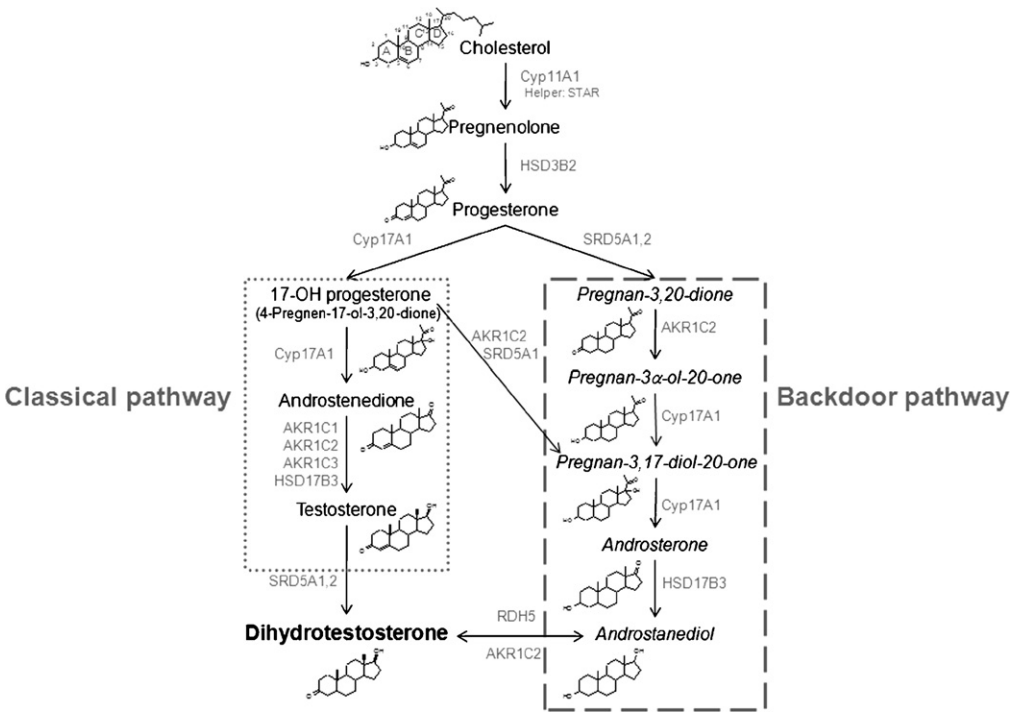

Fig. 1. DHT synthesis pathway from cholesterol with characteristic enzymes and intermediates. Progesterone can be metabolized via the classical pathway or the backdoor pathway to DHT. This figure has been adapted from [13].

these studies suggest that tumor cells can develop an ability to evade castration induced steroid starvation by utilizing upregulated androgen synthesis enzymes to produce their own androgens for AR activation and progression to CRPC.

In concordance with these discoveries several drug candidates targeting the androgen axis were being developed and evaluated in their ability to treat CRPC patients. Ketoconazole, an azole antifungal agent which exerts its clinical effects through inhibiting CYP17A1 (and other cytochrome P450s) can induce reasonable PSA response rates in CRPC patients but no studies have demonstrated improvement in overall survival [16,17]. A new more specific CYP17A1 hydroxlase and lyase inhibitor, abiraterone acetate, can trigger declines in PSA of greater than 30%, 50% and 90% in 14, 12 and 6 CRPC patients (out of 21 patients total), respectively, in Phase I and II clinical trials [6]. SRD5A1/2 inhibitors are used in the treatment of hair loss, benign prostatic hyperplasia and prevention of CaP [18–21]. Clinical trials evaluating the use of these drugs, finasteride and dutasteride, in treating CRPC disease are currently underway [22–24]. These steroidogenesis inhibitor drugs and other developing candidates targeting androgen synthesis pathways show significant promise in treating CaP advancing to CRPC through androgen synthesis mechanisms.

In this study we evaluated the effects of various steroidogenesis inhibitors and anti-androgens on androgen synthesis pathways in steroid-starved LNCaP cells and CRPC xenografts. Better understanding of how these agents alter androgen synthesis in CaP tumors will optimize their use therapeutically. Using LC–MS, we previously observed that LNCaP tumors excised shortly after castration, as compared to tumors from intact (pre-castration) mice, contain elevated levels of progesterone relative to testosterone and DHT, despite low serum levels [13]. Furthermore, mRNA levels of enzymes responsible for progesterone synthesis (CYP11A1, StAR and HSD3B2) and metabolism (CYP17A1, AKR1C1 and SRD5A2) increased during progression to CRPC [8,13,15], suggesting that high progesterone levels may be involved in androgen synthesis under steroid-deprived conditions. We therefore chose to study progesterone as a key steroidal precursor and investigate its downstream androgen synthesis mechanisms. Classically progesterone is converted to 17-OH progesterone and androstenedione by CYP17A1 [25], subsequently converted to testosterone by AKR1C3 [26,27]/HSD17B3 [28] and finally DHT by SRD5A1/2 (Fig. 1). Auchus et al. also described a second "backdoor" pathway to DHT synthesis that bypasses testosterone as an intermediate (Fig. 1) [29–31]. In this pathway progesterone is initially converted to pregnan-3,20-dione by SRD5A1/2 before undergoing conversion to pregnan-3α-ol-20-one by AKR1C2. This intermediate is then converted by CYP17A1 to androsterone and further bioconversion by HSD17B3 to androstanediol. Androstanediol can then be reversibly converted to DHT by RDH5.

We aim herein to explore the effects of steroidogenesis inhibitors on androgen production in this dynamic steroid synthesis system in *ex vivo* CRPC tumors using progesterone as a steroidal precursor. Further to these studies we aim to evaluate and compare the ability of *ex vivo* LNCaP xenograft tumors from different stages of the disease to synthesize androgens from progesterone.

2. Materials and methods

2.1. Materials

[1,2,4,5,6,7-^{3}H (N)]-DHT (110.0 Ci/mmol, PerkinElmer Life Sciences, Inc., Wellesley, MA) and [1,2,6,7-^{3}H (N)]-Progesterone (90.0 Ci/mmol, PerkinElmer Life and Analytical Sciences, Wellesley, MA) were used for *in vitro* incubations and radiometric standards. Stock solutions of testosterone-16,16,17-d3 (deuterated testosterone) (CDN Isotopes, Pointe-Claire, Quebec, Canada); 4-pregnen-17-ol-3,20-dione, 5β-pregnan-3α-27-diol-20-one and 5β-pregnan-3,20-dione (Steraloids, Inc., Newport, RI); androsterone, 4-androstene-3,17-dione, 5α-androstan-17β-ol-3-one, pregnenolone, progesterone, testosterone and dihydrotestosterone (Sigma–Aldrich, Oakville, Ontario, Canada); and R1881 (Dupont, Boston, MA) were prepared in 100% methanol for use as standards for mass spectrometry and 100% ethanol for *in vitro* incubations. Inhibitors: ketoconazole, finasteride, cinnamic acid, RU-486 and

casodex (Sigma–Aldrich, Oakville, Ontario, Canada) were prepared in ethanol.

2.2. In vitro models: LNCaP cells

LNCaP cells (passage 40–48; American Type Culture Collection, Rockville, MD) were cultured in RPMI-1640 (without phenol red) with L-glutamine, penicillin–streptomycin (PS) and 5% fetal bovine serum (FBS, Hyclone, Logan, UT) or 5% charcoal stripped serum (CSS, Hyclone, Logan, UT). Cells were maintained and grown in FBS; however, prior to all treatments cells were cultured in 5% CSS for 48 h.

2.3. In vivo model: LNCaP tumor progression to castration-resistance

All animal experimentation was conducted in accord with accepted standards of the UBC Committee on Animal Care. LNCaP xenograft tumors were grown in athymic nude mice at four sites as modified from a previously reported method [9]. Also as done before [13], PSA levels were measured by tail vein sera samples weekly using an immunoassay kit (ClinPro, Union City, CA). At 6 weeks post-inoculation mice were castrated. Tumors were harvested from the same mouse (16 mice total) pre-castration (PSA androgen-dependent-AD), 8 days post-castration (PSA nadir-N) and 35 days post-castration (PSA castration-resistant-CRPC) (see supplementary data section for PSA and tumor volume profiles). Tumors were excised and immediately placed in phenol red-free RPMI-1640 media supplemented with 5% CSS.

2.4. Treatments of LNCaP cells and castration-resistant xenograft tumors

In every *ex vivo* assay cells were teased apart from a fixed weight of xenograft tumor section and debris was removed prior to plating and treatment in CSS supplemented media. Steroid starved (CSS) cells and AD, N and CRPC xenograft tumor cells were treated with 1 μCi/mL [^{3}H]-progesterone for 48 h. Steroid starved cells and CRPC tumor cells were treated with an additional 1 nM R1881 and inhibitors of CYP17A1, SRD5A2, AKR1C3, steroid receptors and AR: 20 μM ketoconazole, 25 μM finasteride [32,33], 50 μM cinnamic acid [34], 10 μM RU-486 [35] and 25 μM casodex [36], respectively for the same 48-h period to determine the effect of inhibitors on steroidogenesis downstream of progesterone. Dose titrations of steroid starved LNCaP cells with 10 μM progesterone and 0, 0.1, 1, 10, 20/25, 50, 100 and 1000 μM ketoconazole/finasteride, were conducted in order to verify optimal dosing for metabolism studies.

2.5. Cell viability determination

Cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega, Madison, WI). 20 μL of reagent was added to each well (96-well plate) and left to incubate at 37 °C in the dark for 1 h. The viability of the cells was determined based on measuring spectrometer absorbances at 490 nm wavelength and comparing these values to those measured for ethanol control treated cells.

2.6. PSA determination

Secreted PSA levels were determined from 10 μL of media or sera diluted in 40 μL of H_2O using an immunoassay kit (ClinPro, Union City, CA). Concentration was determined using a standard curve (0–120 ng/L). The intra-assay and inter-assay coefficients of variation for this assay were measured to be 3.4% and 4.8%, respectively.

2.7. Steroid extraction for liquid chromatography–mass spectrometry (LC–MS) and radiometric analysis

Pellets underwent a rigorous freeze–thaw protocol with liquid nitrogen and boiling water three times. Then supernatants and pellets were extracted twice with ethyl acetate (EtOAc) (v:v, 1:1), washed with H_2O (v:v, 1:1) once and dried down using a Centrivap™ centrifugal evaporation system (35 °C). Samples were then reconstituted in 100 μL of 50% methanol.

2.8. Steroid analysis by LC–MS

LC–MS protocols were carried out as developed previously [13]. A Waters 2695 Separations Module coupled to a Waters Quattro Micro was used for LC–MS analysis. All MS data was collected in electrospray ionization positive (ESI+) mode with capillary voltage at 3 kV, source and desolvation temperatures of 120 °C and 350 °C respectively and N_2 gas flow of 450 L/h. Chromatographic separations were carried out using a Waters Exterra 2.1 mm × 50 mm 3.5 μm C18 column equilibrated with 20:80 ACN:H_2O, ramped to 80:20 ACN:H_2O from 0.5 to 8.0 min, further to 95:5 from 8.0 to 9.0 min and returned to 80:20 ACN:H_2O from 10.0 to 10.5 min with a total run time of 15 min. Flow rate was 0.3 mL/min, column temperature 35 °C and 0.05% formic acid was present throughout the run. Extracted ion chromatograms from extracted samples of radiolabeled alone (Hot) versus radiolabeled plus non-radiolabeled (Cold) progesterone (H + C) spiked incubations were compared and LC retention time alignments were used to identify potential metabolites as conducted previously [13]. Precursor ions unique to the H + C sample fractions collected by high-performance liquid chromatography (HPLC) radiometric detection were selected for further collision induced dissociation (CID) at both 11 and 22 V CE resulting in positive identification of progesterone, 17-OH progesterone, pregnan-3,17-diol-20-one, androsterone and testosterone.

2.9. HPLC separation and radiometric detection of [^{3}H]-labeled steroids

HPLC-radiometric detection methods were developed previously [13]. A Waters 2695 Separations Module coupled with a Packard (PerkinElmer, Wellesley, MA) Radiomatic™ Model 150TR detector equipped with a 0.5 mL flow cell provided chromatographic separation and detection of radiolabeled analytes. Separations of [^{3}H]-labeled steroids were performed using a Waters Exterra 2.1 mm × 150 mm C18 column equilibrated with 10:90 acetonitrile (ACN):H_2O, ramped to 25:75 ACN:H_2O (0.75–1.5 min), further to 35:65 ACN:H_2O (1.5–20 min), then to 45:55 ACN:H_2O (25–30 min). Isopropanol (IPA) was introduced at this time from 45:0:55 ACN:IPA:H_2O to 45:55:0 ACN:IPA:H_2O (30–50 min), retained at 45:55:0 until 55 min and returned to starting conditions at 57 min for re-equilibration up to a 70 min run length. LC flow rate was 0.3 mL/min, column temperature was 30 °C and Radiomatic™ scintillation fluid (Ultima Flo M, PerkinElmer, Wellesley, MA) flow rate was 1 mL/min. DHT identification was evidenced based on RT match-up to available radiolabeled and non-labeled steroid standards on the same LC gradient (Fig. 2 Table 1). Radiometric retention times (RT) were observed to lag MS RT by ~1 min when using this LC setup with the Quattro Micro and this normalization factor was applied for the additional non-labeled standards. Statistics on intra-run variation in the retention time (RT) of both [^{3}H-DHT] and [^{3}H-Progesterone] standards were conducted by LC-radiometric detection to ensure consistency in peak identification and RT match up to steroidal standards by LC–MS. RT shift was

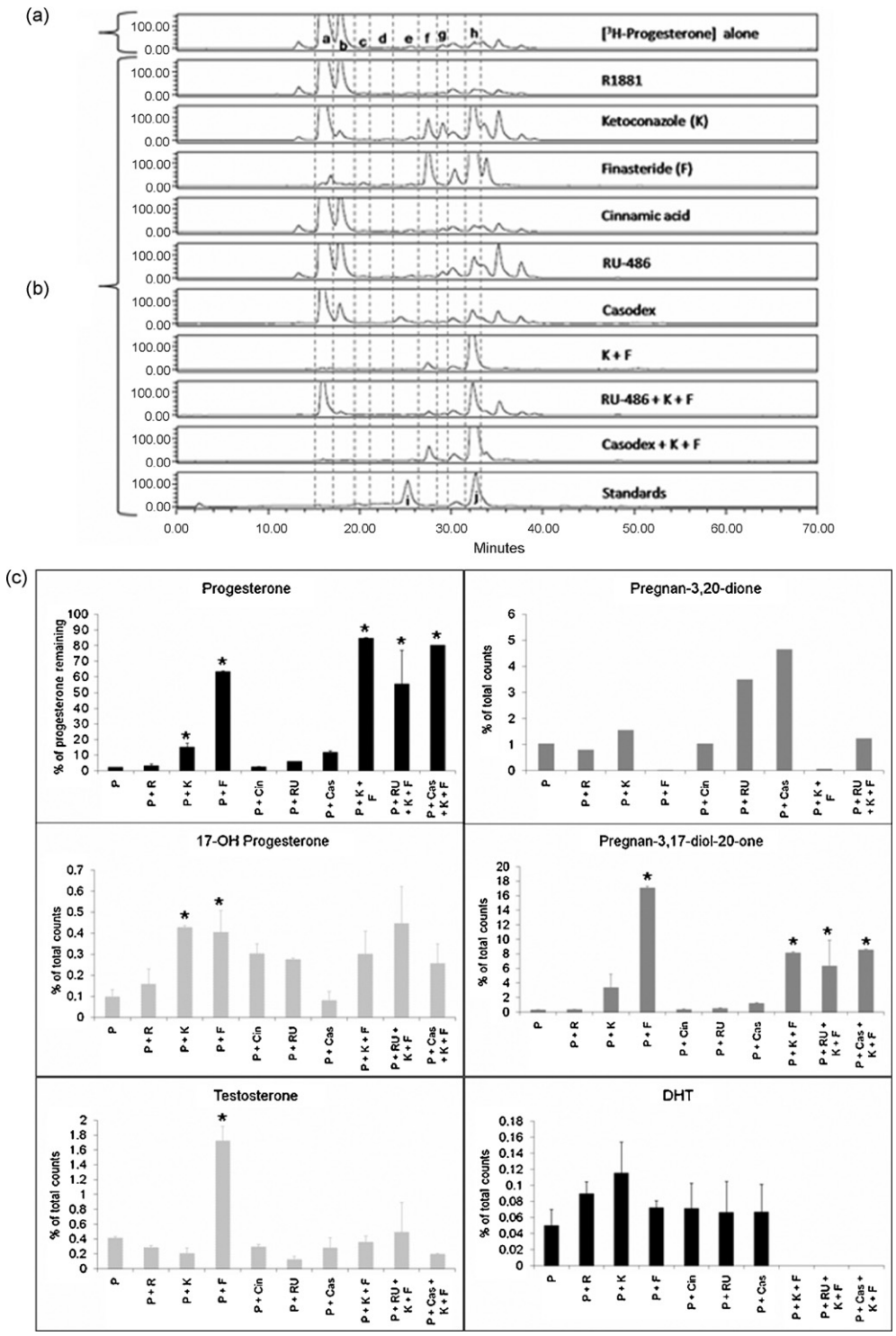

Fig. 2. (a) Example chromatographic profile of metabolites from steroid starved LNCaP + [^{3}H]-Progesterone by HPLC-radiometric detection. (b) Example chromatographic profile of metabolites from LNCaP + [^{3}H]-Progesterone and 1 nM R1881 or inhibitors 20 μM ketoconazole (K) (CYP17A1), 25 μM finasteride (F) (SRD5A2), 50 μM cinnamic acid (AKR1C3), 10 μM RU-486 (PR and AR), 25 μM casodex (AR), 20 μM ketoconazole + 25 μM finasteride in combination, 10 μM RU-486 + 20 μM ketoconazole (K) + 25 μM finasteride (F) in combination and 25 μM casodex + 20 μM ketoconazole (K) + 25 μM finasteride (F) in combination by HPLC-radiometric detection. (c) Effect of 1 nM R1881 and inhibitors: 20 μM ketoconazole (CYP17A1), 25 μM finasteride (SRD5A2), 20 μM ketoconazole and 25 μM finasteride in combination, 50 μM cinnamic acid (AKR1C3), 10 μM RU-486 (RU) (PR and AR) and 25 μM casodex (AR) on the conversion of progesterone to downstream steroids in the classical pathway and backdoor pathway. Graph displayed of each metabolite steroid as % of total counts in [^{3}H-Progesterone] (P), P + ketoconazole (P + K), P + finasteride (P + F), P + cinnamic acid (P + Cin), P + RU-486 (P + RU), P + casodex (P + Cas), P + ketoconazole + finasteride (P + K + F), P + RU-486 + ketoconazole + finasteride (P + RU + K + F), P + casodex + ketoconazole + finasteride (P + Cas + K + F) treated LNCaP cells (mean + SEM). *Statistically different from LNCaP cells with no treatment ($P < 0.01$). All experiments were conducted in triplicate.

Table 1
Radiometric standards [^{3}H-DHT] and [^{3}H-Progesterone] were analyzed for retention time (RT) match up to LC–MS standards. HPLC-radiometric detection identified peaks (a–j) matched up to RT of steroidal standard as determined by LC–MS. SIR/MRM precursor masses and fragment masses were used to identify and quantify steroids listed. All experiments were conducted in triplicate.

^{5}H peak	Steroid standard	Radiometric RT (min)	SIR/MRM
a	Metabolite 1	15.9	317
b	Metabolite 2	17.9	331
c	Testosterone	20.4	289 > 97
d	17-OH Progesterone	22.7	331 > 97
e	DHT	25.2	287 > 97
f	Pregran-3,17-diol-20-one	27.5	331 > 97
g	Androsterone	29.4	273 > 255
h	Progesterone	32.5	315 > 97
i	[^{3}H]-DHT standard	25.1 ± 0.1	–
j	[^{3}H]-Progesterone standard	32.6 ± 0.1	–

found to be ±0.1 min SEM from run to run confirming the reproducibility of this assay.

3. Results

3.1. Steroidogenesis enzyme inhibitors alter the de novo conversion of [^{3}H]-Progesterone to DHT in LNCaP cells and CRPC tumors

We initially investigated the ability of steroidogenesis inhibitors and anti-androgens to alter androgen synthesis pathways from radioactively labeled progesterone in both steroid starved LNCaP cells and CRPC tumor cells. Both ketoconazole and finasteride but not R1881 or cinnamic acid appeared to alter progesterone metabolism to DHT in serum starved LNCaP cells and CRPC xenograft tumors (Figs. 2a,b and 3). Ketoconazole significantly inhibited progesterone conversion to downstream metabolites ($P = 0.003$) and also altered the relative amounts of progesterone metabolites that were still able to form (Fig. 2c). Metabolite 1 was formed in a similar manner to that observed when cells undergo progesterone treatment alone; however much less of metabolite 2 formed (Fig. 2a and b). Furthermore, 17-OH progesterone formation was not inhibited by ketoconazole as predicted by CYP17A1 inhibition (Fig. 2c), in fact it was increased ($P = 0.001$), as was pregnan-3,17-diol-20-one (not statistically significant). The rate-limiting step of dual enzyme CYP17A1 is believed to be its lyase action [30]. Formation of 17-OH progesterone and pregnan-3,17-diol-20-one via hydroxylation of progesterone and other progesterone-derived steroids upstream of CYP17A1 lyase action or perhaps the existence of another enzyme that is capable of hydroxylation at the 17-C site may therefore account for the increased levels of these steroids (Fig. 2c). Nonetheless, this data suggests that ketoconazole affects conversion of progesterone to DHT via both the classical and backdoor pathways.

Finasteride inhibition of progesterone conversion ($P = 0.001$) appeared to affect formation of DHT by both the classical and backdoor pathways (Fig. 2c). Formations of Metabolites 1 and 2 were both dramatically inhibited (Fig. 2a and b). As expected, testosterone levels were significantly increased by finasteride treatment ($P = 0.001$) [22] (Figs. 1 and 2c). Both 17-OH progesterone and pregnan-3,17-diol-20-one were also significantly increased by finasteride treatment and this inhibition profile was similar to that observed with ketoconazole ($P = 0.049$, $P < 0.001$ respectively). These results suggest that finasteride inhibits the conversion of progesterone via both the classical and backdoor pathways and upon inhibition of SRD5A2 activity, CYP17A1 conversion of progesterone to 17-OH progesterone in the classical pathway and downstream conversion pregnan-3,17-diol-20-one (via SRD5A1) in the backdoor pathway are increased in a compensatory manner.

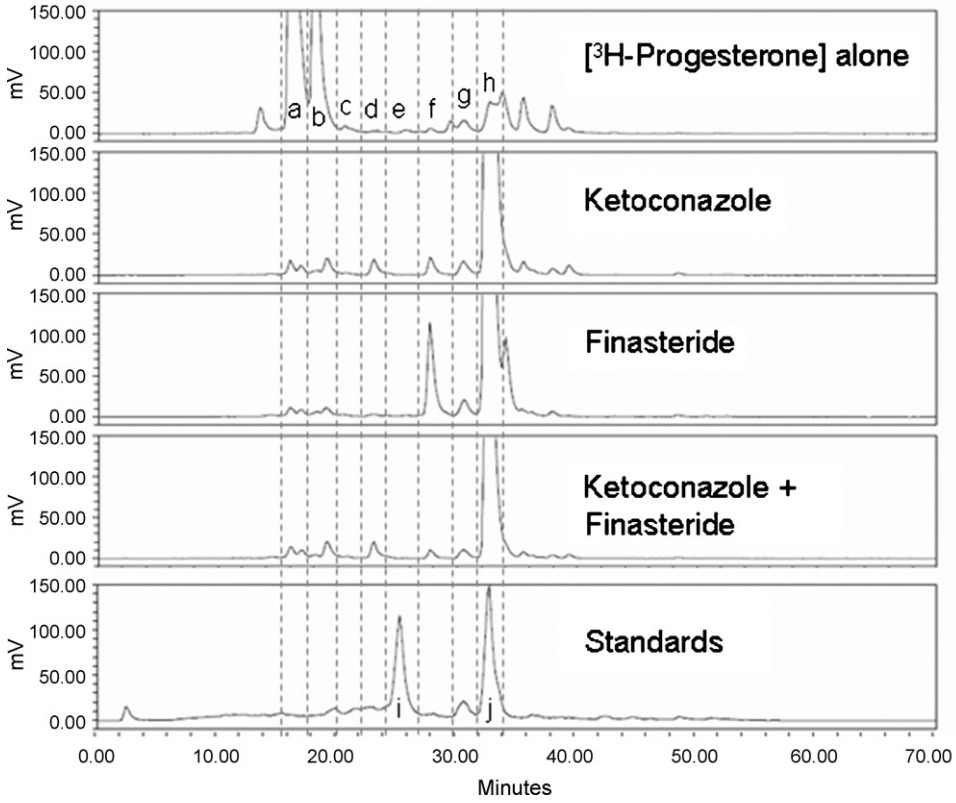

Fig. 3. Example chromatographic profile of metabolites from CRPC xenograft tumor *ex vivo* cells + [^{3}H]-Progesterone and inhibitors 20 μM ketoconazole, 25 μM finasteride, and 20 μM ketoconazole + 25 μM finasteride in combination by HPLC-radiometric detection.

Combined finasteride + ketoconazole treatment inhibited progesterone conversion to a greater extent than finasteride or ketoconazole monotherapy ($P = 0.002$) (Fig. 2a–c). In fact, the formation of metabolites 1 and 2 was significantly blocked upon combination treatment with ketoconazole + finasteride ($P < 0.001$) (Fig. 2a and b). Levels of pregnan-3,17-diol-20-one, albeit much lower than in finasteride only treated cells, were still significantly higher than those produced in the progesterone alone treated cells ($P < 0.001$) (Fig. 2c). Furthermore, upon combination treatment of cells with finasteride and ketoconazle DHT levels became undetectable (Fig. 2c).

Neither R1881 nor cinnamic acid significantly affected the *in vitro* conversion of [^{3}H-progesterone] by steroid starved LNCaP cells in the presence of exogenous progesterone treatment (Fig. 2b). The lack of effect observed by cinnamic acid treatment suggests that either an alternative enzyme is capable of metabolizing steroids similarly to AKR1C3 (such as HSD17B3) or that this compound is not effective in inhibiting AKR1C3 in steroid starved LNCaP cells at the dose previously reported by Brozic et al. [34].

In CRPC xenograft tumors [^{3}H]-Progesterone also appeared to be metabolized to DHT (Fig. 3) and inhibitors ketoconazole, finasteride and ketoconazole + finasteride combination treatment appeared to significantly inhibit this metabolism. This result demonstrates that these inhibitors effect androgen synthesis intratumorally at CRPC by altering steroid production via both the classical and backdoor pathways.

In conclusion, conversion of progesterone to downstream steroids is significantly and differentially altered by both ketoconazole and finasteride treatments in both steroid starved LNCaP and CRPC xenograft tumors. When finasteride + ketoconazole were used in combination, progesterone metabolism was inhibited to a much larger extent. These results suggest that inhibition of enzymes in either the classical or backdoor pathway may lead to a compensatory increase in the steroid levels of other respective pathways which in turn can provide the cells with alternative androgen synthesis mechanisms to AR activation.

3.2. Receptor antagonists in combinational treatments with steroidogenesis inhibitors alter the de novo conversion of [^{3}H]-Progesterone to DHT in steroid starved LNCaP cells

Steroid receptor antagonists RU-486 (inhibits PR and AR) and casodex (inhibits AR) [35,36] dosed individually did not appear to alter progesterone metabolism to DHT synthesis via either pathway, however they did appear to enhance the production of more hydrophobic metabolites (longer RT) (Fig. 2a and, b). Furthermore, while we saw large variation in results, combination treatment with RU-486 + finasteride + ketoconazole appeared to significantly inhibit progesterone metabolism ($P < 0.001$) but not to the same extent as finasteride + ketoconazole alone (Fig. 2c). Casodex + finasteride + ketoconazole also inhibited progesterone metabolism ($P < 0.001$) more so than finasteride + ketoconazole alone (Fig. 2c). All combinational treatments increased the amount of pregnan-3,17-diol-20-one produced in the backdoor pathway ($P_{\text{finasteride+ketoconazole}} < 0.001$, $P_{\text{RU-486+finasteride+ketoconazole}} < 0.001$, $P_{\text{casodex+finasteride+ketoconazole}} < 0.001$) (Fig. 3c).

3.3. Steroidogenesis inhibitors and receptor antagonists significantly decrease but do not eliminate progesterone-induced secretion of PSA in steroid starved LNCaP cells

We and others have previously hypothesized that cancer cells synthesize DHT at levels high enough to activate AR leading to a cascade of events linked to tumor growth and proliferation [8,10,12–15]. Thus we deemed that the effect of steroidogenesis inhibitors and receptor antagonists on PSA secretion is appropriate

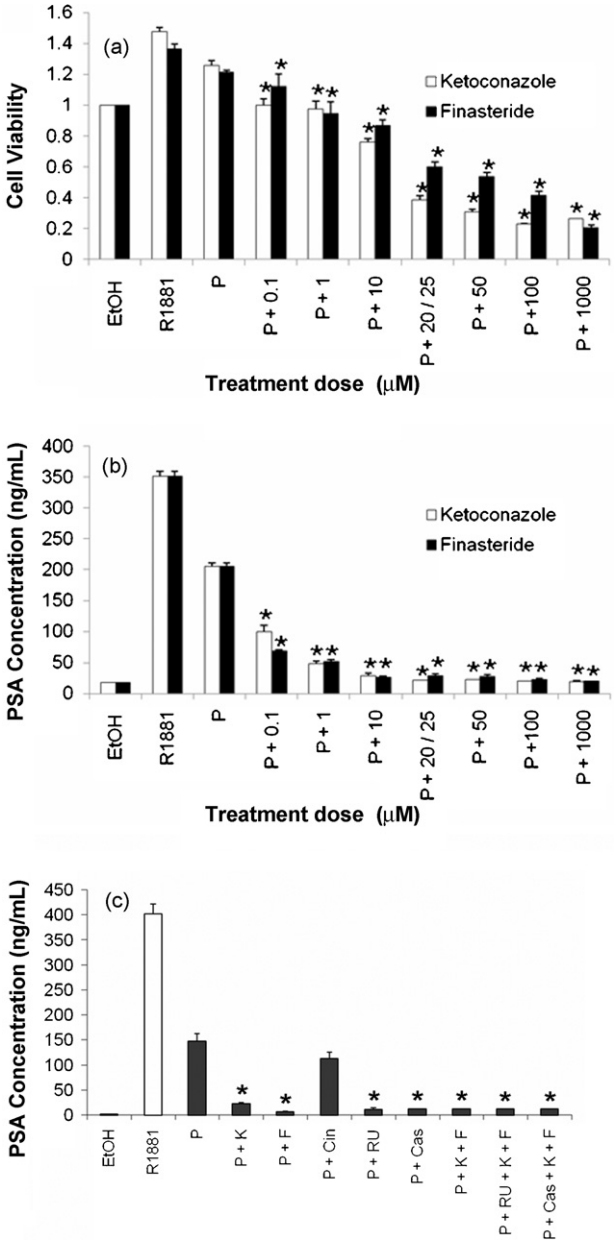

Fig. 4. (a) Effect of 0, 0.1, 1, 10, 20/25, 50, 100 and 1000 μM ketoconazole/finasteride on LNCaP cell viability and progesterone-induced secretion of PSA. *Statistically different from progesterone treated LNCaP cells ($P < 0.01$). All experiments were conducted in triplicate. (b) Effect of 1 nM R1881 and inhibitors: 20 μM ketoconazole, 25 μM finasteride, 50 μM cinnamic acid, 10 μM RU-486 (PR and AR) and 25 μM casodex (AR) on progesterone-induced secretion of PSA. Graph displayed as EtOH, R1881, Progesterone (P), P + ketoconazole (P + K), P + finasteride (P + F), P + cinnamic acid (P + Cin), P + RU-486 (P + RU) and P + casodex (P + Cas), P + ketoconazole + finasteride (P + K + F), P + RU-486 + ketoconazole + finasteride (P + RU + K + F) and P + casodex + ketoconazole + finasteride (P + Cas + K + F). *Statistically different from [^{3}H-Progesterone] treated LNCaP cells ($P < 0.01$). All experiments were conducted in triplicate.

to verify AR activation since PSA is androgen regulated target gene [37]. Progesterone treatment of LNCaP cells led to a significant increase in PSA secretion into media as compared to ethanol treatment ($P < 0.001$) (Fig. 4a and b).

Initially we evaluated the effect of increasing doses of ketoconazole and finasteride on cell viability and progesterone-induced PSA secretion. As demonstrated in Fig. 4a at doses of 20 μM ketoconazole and 25 μM finasteride cell viability was reduced to 38.6 ± 0.03% and 60.0 ± 0.03%, respectively. Progesterone induced PSA secretion

was also inhibited by increasing doses of each drug. Both ketoconazole and finasteride treatment led to decreases in measured PSA levels even at a dose of 0.1 μM and at 1000 μM progesterone-induced PSA secretion was completely inhibited as compared to ethanol treated cells (Fig. 4a).

In Fig. 4b ketoconazole, finasteride, RU-486, casodex, finasteride + ketoconazole, RU-486 + finasteride + ketoconazole and casodex + finasteride + ketoconazole treatments significantly inhibited progesterone-induced PSA secretion into the media ($P_{ketoconazole} < 0.001$, $P_{finasteride} < 0.001$, $P_{RU\text{-}486} < 0.001$, $P_{Casodex} < 0.001$, $P_{finasteride+ketoconazole} < 0.001$, $P_{RU\text{-}486+finasteride+ketoconazole} < 0.001$, $P_{casodex+finasteride+ketoconazole} < 0.001$) but do not completely abrogate AR activation at the doses evaluated. The observed decrease in PSA secretion upon treatment with ketoconazole, finasteride, RU-486 and casodex suggests that progesterone in part induces PSA secretion through downstream conversion to androgens, and not entirely through direct binding to PR or AR in steroid starved LNCaP cells as suggested by Grigoryev et al. [38]. Grigoryev et al. previously showed that AR found in LNCaP cells contains a mutation in the form of T877A and with this mutation can bind and be activated by ligands such as progesterone in high concentrations [38,39]. Our result does not necessarily demonstrate that progesterone is mediating its effects solely through metabolism to DHT prior to AR activation but does show that at least some of the effect on PSA secretion is mediated through this mechanism. Furthermore, cinnamic acid did not significantly inhibit progesterone-induced PSA secretion suggesting also that this particular compound does not affect steroidogenesis leading to AR activation at the dose used (10 μM).

From this experiment it was determined that progesterone-induced PSA secretion (via AR activation) was decreased but not completed inhibited by the presence of steroidogenesis inhibitors ketoconazole and finasteride and anti-androgens RU-486 and casodex at the doses evaluated.

3.4. [^{3}H]-Progesterone metabolism in AD, N and CRPC LNCaP xenograft tumors cells occurs via different enzymatic reactions and steroidal intermediates

In order to determine whether tumors growing at different stages of progression to CRPC have differential abilities to synthesize androgens we evaluated the progesterone metabolism profiles in AD (pre-castration; $n = 3$), N (8 days post-castration, $n = 3$) and CRPC (upon PSA relapse or 35 days post-castration; $n = 3$) tumors obtained using the LNCaP xenograft model (see supplementary data section for PSA and tumor volume profiles). When we compare the chromatographic profiles of AD, N and CRPC tumors shown in Fig. 5a the AD and CRPC tumor metabolism of progesterone appears to be similar with only very subtle differences. In contrast, the N tumor metabolism of progesterone is significantly less extensive (~22% of AD or CRPC) ($P = 0.04$) and yields more hydrophobic metabolites (later retention times) (Fig. 5b). Furthermore, N tumors produce significantly more 17-OH progesterone (~5-fold, $P < 0.001$) and pregnan-3,17-diol-20-one (~62-fold, $P = 0.008$) and significantly less DHT ($P = 0.002$) than both AD and CRPC tumors. In fact, in N tumors there was no evidence of DHT formation while pregnan-3-17-diol-20-one was formed in such large quantities that it is likely to be the main end product of progesterone metabolism in these tumors. In contrast, metabolites 1 and 2 are likely to be the final end products of progesterone metabolism in AD and CRPC tumors. As demonstrated by this study N tumors obtained immediately after castration have a significantly hampered capacity to *de novo* metabolize progesterone as compared to AD tumors obtained prior to castration and CRPC tumors obtained once PSA had relapsed which we have previously shown have the potential ability to *de novo* synthesize androgens themselves [13]. In fact N tumors exhibit several different steroidal intermediates likely undergoing alternative enzymatic biotransformation than those seen in both AD and CRPC tumors.

Upon comparison of the AD and CRPC tumor progesterone metabolism profiles (Fig. 2a) they appear relatively similar with perhaps more testosterone produced by AD tumors than CRPC tumors (not statistically different) (Fig. 2b). This suggests that the enzymatic systems utilized by AD and CRPC tumors are similar.

In summary, it appears that steroid intermediates and enzymatic reactions in both classical and backdoor steroidogenesis pathways are utilized by AD, N and CRPC tumors. However, because more testosterone (classical pathway) was produced by the AD tumors than the N and CRPC tumors, prior to castration tumors utilize the classical pathway more predominantly. The predicted steroidal end product (pregnan-3,17-diol-20-one) in N tumor progesterone metabolism is principally observed in the backdoor pathway and because it forms in such a large amount it appears to act as a sink. This may indicate an inability of N tumor cells to utilize CYP17A1 lyase to produce downstream steroids. Furthermore, both AD and CRPC tumors were able to *de novo* produce DHT (albeit in small amounts compared to other steroid intermediates). Likely this low production reflects the cell's need for only minimal androgen for AR activation.

This work uniquely demonstrates using the LNCaP CRPC progression model that tumors of different stages of classical disease progression possess differential abilities to synthesize androgens and do so using different steroidal intermediates and enzymatic reactions.

4. Discussion

Increasing lines of evidence indicate that androgens remain important mediators of CRPC progression despite the low levels of androgens observed in serum after castration therapy [10,12,15,40–42]. It has recently been shown that DHT synthesis can occur intratumorally from both adrenal steroid precursors and *de novo* from cholesterol [8,10,12–15]. We identified progesterone as an important intermediate steroid that can be metabolized by CRPC LNCaP xenograft tumors through both the classical and backdoor pathways (Fig. 1) [13].

We further demonstrate here that inhibitors targeting the androgen synthesis axis alter the metabolism of progesterone to downstream androgens in steroid starved LNCaP cells and CRPC LNCaP xenograft tumors. Using progesterone as a steroidal precursor we demonstrate that inhibitors of enzymes CYP17A1 (ketoconazole) and SRD5A2 (finasteride), alter the levels of given intermediates in these two pathways and thereby the steroidogenesis profile observed in CRPC cells. In contrast, anti-androgens targeting AR (casodex and RU-486) did not alter progesterone metabolism profiles significantly. Furthermore, the steroidogenesis inhibitors used did not completely eliminate progesterone-induced PSA secretion at the doses evaluated suggesting that DHT synthesis from progesterone is not completely inhibited and can occur via alternative pathways in a compensatory manner. Survival and proliferation of evading tumor cells is therefore a likely event and we propose that inhibition of steroidogenesis enzymes in patient's displaying CRPC disease might result in disease relapse through mechanisms such as these described. Using the LNCaP progression model we also compared the ability of tumors at different stages of disease progression to synthesize steroids from progesterone and found that immediately after castration tumor cells utilize different enzymatic reactions to produce different steroid metabolites compared to progressing CRPC tumors. Because of these dramatic differences observed immediately post-castration as compared to when they have become CRPC, targeting the

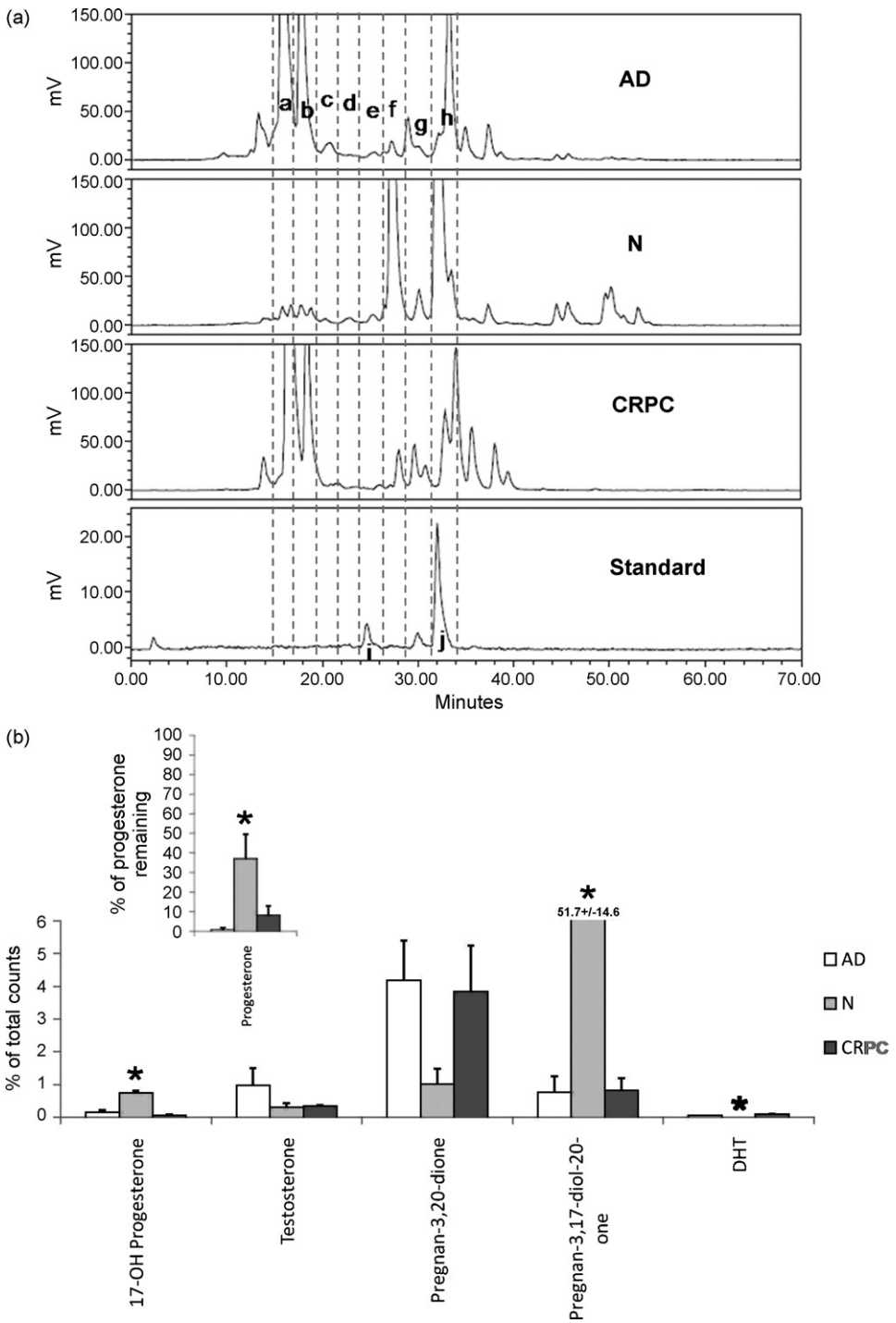

Fig. 5. (a) Example chromatographic profile of metabolites from LNCaP xenograft androgen-dependent (AD), nadir (N) and castration-resistant (CRPC) tumor cells + [^{3}H]-Progesterone by HPLC-radiometric detection. (b) Levels of steroidal intermediates measured in androgen-dependent (AD), nadir (N) and castration-resistant (CRPC) tumor progesterone metabolism profiles as a % of total counts (Mean + SEM). *Statistically different from AD tumor ($P < 0.01$). All experiments were conducted in triplicate.

CaP tumors in patients prior to PSA relapse with steroidogenesis inhibitors may offer a more effective method in prolonging the progression of the disease and improving overall survival of patients.

Steroidogenesis drugs such as ketoconazole and aminoglutethimide and anti-androgens such as flutamide, nilutamide and casodex have been widely used in treating patients with CRPC disease because of their demonstrated PSA responses even after androgen deprivation therapies has become exhausted [2,16,43–46]. The development and evaluation of several other steroidogenesis inhibitors such as statins [47], abiraterone acetate [6], VN/124-1 [48,49], cinnamic acid [34], finasteride and dutasteride [22] as well as anti-androgens such as MDV3100 (Medivation, Inc., San Francisco, CA) and BMS-641988 (Bristol-Myers Squibb, New York, NY) [50–52] and AR chaperone proteins like Hsp27 [53] are on the rise. Therapeutic responses demonstrated in this study using the LNCaP progression model for CaP suggest that CRPC tumors that respond initially to steroidogenesis inhibitors such as these are likely to develop resistance and the disease will ultimate progress. We demonstrate that inhibitors targeting

CYP17A1 (ketoconazole) and SRD5A2 (finasteride) do indeed alter the metabolism of progesterone to downstream androgens but do not completely inhibit it as other alternative steroidal pathways to DHT synthesis become utilized. Furthermore, progesterone-induced PSA response, although decreased by these inhibitors, is not completely eliminated even at their IC_{50} doses. Previously, it has been shown that CRPC patients who initially respond to ketoconazole display reduced amounts of CYP17A1 produced steroids in their serum and when they develop resistance to ketoconazole these steroids once again increase in the serum [48,54,55]. In this manuscript we provide mechanistic rationale as to how some CRPC patients on ketoconazole treatment might become resistant to therapy as tumor cells develop an ability to produce these androgens by alternative mechanisms. Upon ketoconazole treatment CRPC LNCaP tumor cells produce more 17-OH progesterone and pregnan-3,17-diol-20-one than in the absence of this CYP17A1 targeting inhibitor. In humans 17-OH progesterone is a poorer substrate for CYP17A1 lyase activity than pregnan-3,17-diol-20-one [56] and in the presence of 20 μM ketoconazole it appears that the CRPC cells convert progesterone via 17-OH progesterone to pregnan-3,17-diol-20-one for further bioconversion to DHT, thus demonstrating an escape mechanism after ketoconazole treatment. Furthermore, although finasteride has not yet been evaluated in a large population of CRPC patients as a potential treatment, according to our metabolism study CRPC cells will likely develop a steroid synthesis escape mechanism similar to that observed with ketoconazole treatment. In fact, we have found that significantly greater amounts of pregnan-3,17-diol-20-one and testosterone are produced in the presence of finasteride in steroid starved LNCaP cells and CRPC LNCaP xenograft tumors as compared to ethanol control or ketoconazole treatment. Interestingly, DHT levels do not appear altered by finasteride treatment suggesting that 17-OH progesterone is converted to pregnan-3,17-diol-20-one and through step-wise reactions in the backdoor pathway to DHT for AR activation, even in the presence of inhibitors blocking the SRD5A2 conversion of testosterone to DHT in the classical pathway. Conversion of 17-OH progesterone to pregnan-3,17-diol-20-one is predominantly mediated by SRD5A1 (Fig. 1) [57] while finasteride is known to predominantly target SRD5A2 [22,58] suggesting that CRPC cells find alternative pathways to produce DHT by utilizing more readily available steroid substrates such as pregnan-3,17-diol-20-one and enzymatic reactions such as those mediated by SRD5A1 rather than SRD5A2. SRD5A1 and SRD5A2 are both known to be expressed in LNCaP cells [58], however SRD5A2 to a much lower degree suggesting that the backdoor pathway may be the predominant (but not sole) route utilized in LNCaP cells. Furthermore, although SRD5A2 expression is the predominant isoform found in the healthy human prostate, SRD5A1 tissue expression has been shown to increase and surpass SRD5A2 expression during progression of the disease to CRPC [59,60]. Clinical trials evaluating treatment inhibition of SRD5A2 with finasteride in CRPC patients may yield further insight into whether this mechanistic hypothesis is valid in human CaP disease progression.

Androgen signaling may be eliminated by the development of more potent steroidogenesis inhibitors like abiraterone acetate. Interestingly CRPC patients who developed resistance following initial response to abiraterone acetate treatment did not have increased levels of CYP17A1 mediated steroid production in their serum as previously observed in the ketoconazole relapsing patients [48]. Furthermore in a Phase I clinical trial reported by Ryan et al. 52% of patients (total 19) who previously became resistant to ketoconazole treatment displayed further PSA response (>50% decline) to abiraterone acetate treatment despite the fact that both drugs target the same enzyme [61]. Abiraterone acetate is a 20 times more potent inhibitor of CYP17A1 than ketoconazole [55] which is a broad spectrum inhibitor of steroid drug metabolism and this may explain why ketoconazole resistance can occur through alternative synthesis mechanisms while abiraterone acetate may completely block all androgen synthesis pathways downstream of CYP17A1. Furthermore, as we demonstrated a potential resistant mechanism of CRPC tumors to finasteride whereby the cells potentially divert to SRD5A1 driven metabolism upon SRD5A2 inhibition, dutasteride (targets both SRD5A1 and 2) may be able to eliminate androgen synthesis by blocking both classical and backdoor metabolism to DHT [22,23,58,62]. Assessment of more potent and targeted steroidogenesis inhibitors and anti-androgens may provide more effective "maximal androgen blockade" than the drugs studied here. These detailed metabolism studies also provide rationale for the use of combination therapies targeting steroidogenesis enzymes and AR in CRPC patients. Ketoconazole combined with finasteride treatment as well as in both/either drug combined with anti-androgens RU-486 and casodex was observed to alter androgen synthesis through progesterone metabolism to a much greater extent than ketoconazole or finasteride alone. Perhaps by utilizing these inhibitors or other inhibitors targeting rate-limiting CYP17A1, SRD5A1/2 and AR in combination "maximal androgen blockade" can be facilitated [63]. In support of this, a Phase II clinical trial in 57 CRPC patients investigating ketoconazole and dutasteride combination treatment versus ketoconazole alone previously demonstrated a prolonged time to relapse in the combination treated patients (13.7 months) as compared to ketoconazole alone treated patients (8.6 months) [64].

Lastly, we propose that the timing of treatment with steroidogenesis inhibitors and anti-androgens might be better optimized and further evaluation of the timely emergence of *de novo* steroidogenesis mechanisms is warranted during disease progression in humans. Our data demonstrating a significant difference in the ability of tumors at different stages of the disease to synthesize androgens suggests that targeting the androgen axis with potent inhibitors such as abiraterone acetate or combinations of these inhibitors may be more optimally administered in patients prior to PSA relapse than when they have already reached CRPC. Increased PSA levels after castration is a measure of AR activation [7,65] and patients displaying PSA relapse likely exhibit tumors that are already capable of androgen synthesis. Since we demonstrate that immediately after castration tumors are significantly worse at producing androgens (testosterone and DHT) from progesterone than tumors that are already castration-resistant perhaps targeting the androgen axis immediately after castration with steroidogenesis inhibitors and anti-androgens will prevent acquired mechanisms of *de novo* steroidogenesis from developing.

In summary we demonstrate that current steroidogenesis inhibitors do alter androgen synthesis mechanisms in CRPC tumor cells. However, we also identify potential mechanisms by which tumor cells can evade these drug treatments. Based on these results we suggest that targeting the androgen axis with combination treatments before cells develop the ability to make their own androgens may be optimal for improving CaP patient survival rather than waiting to treat patients with these inhibitors individually once the cancer has relapsed. While future clinical trials evaluating these combination therapies and their effect on overall survival should also consider drug–drug interactions and their resulting side effects, this research provides rationale for the evaluation of combined steroidogenesis inhibitors concomitant to androgen deprivation therapy with a goal to preventing the emergence CRPC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.03.011.

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