Published in final edited form as:

Prostate. 2013 September ; 73(13): 1470–1482. doi:10.1002/pros.22694.

Prostate Cancer Cells Differ in Testosterone Accumulation, Dihydrotestosterone Conversion, and Androgen Receptor Signaling Response to Steroid 5 α -Reductase Inhibitors

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Abstract

BACKGROUND—Blocking 5 α -reductase-mediated testosterone conversion to dihydrotestosterone (DHT) with finasteride or dutasteride is the driving hypothesis behind two prostate cancer prevention trials. Factors affecting intracellular androgen levels and the androgen receptor (AR) signaling axis need to be examined systematically in order to fully understand the outcome of interventions using these drugs.

METHODS—The expression of three 5 α -reductase isozymes, as determined by immunohistochemistry and qRT-PCR, was studied in five human prostate cancer cell lines. Intracellular testosterone and DHT were analyzed using mass spectrometry. A luciferase reporter assay and AR-regulated genes were used to evaluate the modulation of AR activity.

RESULTS—Prostate cancer cells were capable of accumulating testosterone to a level 15–50 times higher than that in the medium. The profile and expression of 5 α -reductase isozymes did not predict the capacity to convert testosterone to DHT. Finasteride and dutasteride were able to depress testosterone uptake in addition to lowering intracellular DHT. The inhibition of AR activity following drug treatment often exceeded the expected response due to reduced availability of DHT. The ability to maintain high intracellular testosterone might compensate for the shortage of DHT.

CONCLUSIONS—The biological effect of finasteride or dutasteride appears to be complex and may depend on the interplay of several factors, which include testosterone turnover, enzymology

of DHT production, ability to use testosterone and DHT interchangeably, and propensity of cells for off-target AR inhibitory effect.

Keywords

prostate cancer; androgen receptor; steroid 5 α -reductase; testosterone; dihydrotestosterone; dutasteride; finasteride

INTRODUCTION

Testosterone is the major circulating androgen in the body. Once taken up into tissues, testosterone is converted to dihydrotestosterone (DHT) by 5 α -reductases [1,2]. The dissociation rate of DHT from the androgen receptor (AR) is much slower than that of testosterone, thus leading to a more stable AR-DHT complex. For this reason, DHT is considered the preferred ligand of AR [3,4]. Prostate cancer maintains the AR protein and an active AR signal in all stages of the disease, even after surgical or medical androgen deprivation [5,6].

Three isozymes of 5 α -reductase have been identified. Type 1 is expressed mainly in the liver and skin and type 2 is present primarily in the prostate. The enzymatic properties of 5 α -reductase-1 and -2 are well characterized [2]. On the other hand, not much is known about 5 α -reductase-3. In general, there appears to be an increased expression of 5 α -reductase-1 or a higher ratio of 5 α -reductase-1 to 5 α -reductase-2 in prostate cancer compared to either benign prostatic hyperplasia (BPH) or benign prostate [7]. Recently, 5 α -reductase-3 also has been shown to be expressed at higher levels in prostate cancer than BPH [8]. No information, however, is available on how changes in isozyme profile may affect the ability of cells to produce DHT from testosterone.

Dutasteride and finasteride are competitive inhibitors of 5 α -reductase. Dutasteride is a dual inhibitor of 5 α -reductase-1 and -2, with an IC₅₀ of 7 and 6 nM, respectively [7]. Finasteride is more specific for 5 α -reductase-2, with an IC₅₀ of 69 nM, which contrasts to an IC₅₀ of 360 nM for 5 α -reductase-1 [9,10]. These differences in specificity help explain the more complete suppression of serum and prostatic DHT levels by dutasteride when used clinically. Finasteride reduces serum or prostate DHT by 71% or 85%, respectively, while dutasteride reduces both serum and prostate DHT by 95% or more [11–14].

Blocking testosterone conversion to DHT by either finasteride or dutasteride is the driving hypothesis behind two clinical trials: the Prostate Cancer Prevention Trial with finasteride [15] and the Reduction by Dutasteride of Prostate Cancer Events [16]. A number of issues have been raised by the results of the two trials and they have yet to be resolved. Certain factors affecting intracellular androgen turnover and the AR signaling axis must be taken into consideration in order to fully understand the outcome of intervention using these drugs. (i) The heterogeneity of 5 α -reductase isozymes in prostate cancer cells may alter their contribution to converting testosterone to DHT. (ii) Finasteride and dutasteride may affect differently the accumulation/retention of testosterone and DHT in prostate cancer cells. (iii) AR may function differently when the ratio of testosterone to DHT is perturbed from homeostasis and these differences may be magnified under different drug treatments. (iv)

Finasteride and dutasteride may have off-target effects that inhibit AR directly, and these effects may be independent of 5 α -reductase inhibition [17,18].

The present study was designed to investigate carefully the issues raised about androgen action and its alteration by 5 α -reductase inhibition. Five human prostate cancer cell lines were used to determine the distribution of the three 5 α -reductase isozymes, the capacity of the different cell lines to convert testosterone to DHT, the change in intracellular testosterone and DHT in response to finasteride or dutasteride treatment, and the consequence on AR signaling.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

One variant of LNCaP, designated as LNCaP (RPCI), was obtained from the laboratory at Roswell Park Cancer Institute that originally established the cell line [19]. A second variant of LNCaP, designated as LNCaP (ATCC), was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Both LNCaP lines are considered androgen-sensitive. LNCaP-C4-2, a castration-recurrent derivative of LNCaP established originally by Chung and coworkers [20], was purchased from MD Anderson Cancer Center, Houston, TX. LAPC-4, a castration-recurrent but androgen-sensitive cell line, was obtained from Dr. Charles Sawyer at Memorial Sloan-Kettering Cancer Center, New York, NY. 22Rv1, a castration-recurrent cell line, was purchased from ATCC. All cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). For LAPC-4 cells, the culture plates were coated with poly-L-lysine (Sigma, St. Louis, MO). Each cell line was seeded at the desired density and cultured for 48 hr, then switched to a phenol red-free RPMI-1640 medium containing 10% charcoal-stripped FBS. Cells were cultured in this condition for another 24 hr before beginning treatment. Testosterone was added at a concentration of 1 nM in the medium, either with or without dutasteride (a gift from Glaxo-Smith Kline, Clifton, NJ) or finasteride (Sigma).

Immunohistochemistry (IHC) for 5 α -Reductases

Cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Endogenous peroxidase was inhibited with 0.3% (v/v) H₂O₂ in methanol. Non-specific binding of antibodies was blocked with 3% (w/v) bovine serum albumin (BSA) as described previously [8]. Cells were incubated overnight with specific primary antibodies against 5 α -reductase-1 (1:200, Sigma), 5 α -reductase-2 (1:200, Sigma), or 5 α -reductase-3 (1:100), which was prepared by the Mohler laboratory [8]. All antibodies were diluted in 100 mM Tris-HCl buffer (pH 7.8) that contained 8.4 mM sodium phosphate, 3.5 mM potassium phosphate, 120 mM NaCl, and 1% (w/v) BSA. After washing three times with Tris-HCl buffer (pH 7.8), sections were incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibody (1:100, Dako, Carpinteria, CA) for 2 hr at room temperature. Peroxidase activity was developed using 100 mM Tris-HCl buffer containing 3,3-diaminobenzidine tetrahydrochloride (1 μ g/ml, Sigma) and H₂O₂ (1 μ l/ml). Immunohistochemistry in the absence of primary antibody was used as the negative control.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) Analysis of Medium and Intracellular Testosterone and DHT

Ten million cells on two 10-cm dishes were used for a single analysis of each treatment. Pre- and post-culture media also were collected. Cells were rinsed with phosphate-buffered saline (PBS), trypsinized, and harvested using centrifugation at 400g for 3 min. The procedure was repeated twice. The weight of each cell pellet was recorded for estimation of total cell volume. All cell pellet and medium samples were stored at -80°C before LC-MS/MS. Cell pellets were suspended in 1.0 ml of HPLC grade H_2O and sonicated to prepare cell lysates. One hundred microliters of cell lysates were reserved for protein determination. The remainder of the cell lysates was used for extraction and androgen quantitation. Intracellular testosterone or DHT was presented in the Results in two ways: as ng/ mg protein in cell lysate or as nM concentration based on total cell volume calculation.

Cell pellet and media samples were analyzed using a validated LC-MS/MS assay. Calibration samples (prepared in 75% MeOH) and plasma-spiked quality control (QC) samples were extracted in each run. QC samples were prepared in charcoal-stripped, heparinized female human plasma. A 250 μl aliquot of a calibrator, QC, plasma blank, or media sample, or a 900 μl aliquot for re-suspended cell pellets, was diluted with 750 μl of HPLC water, 250 μl 25% MeOH, 100 μl of IS solution (75.0/225 pg/ml $\text{d}_3\text{-T}$ / $\text{d}_3\text{-DHT}$ in 75% methanol), and extracted with 4.0 ml of methyl-*tert*-butyl ether (MTBE) in a glass screw-top tube. Tubes were rotated 15 min and centrifuged 3,000 rpm and 4°C for 15 min. The aqueous phase was frozen in a dry ice/acetone bath and the upper layer poured into a glass conical tube. MTBE was evaporated with nitrogen at 37°C , and the residue was reconstituted with 60.0 μl of 60% methanol. The suspension was filtered through a 96-well filter plate (0.45 μm) into a collection plate using centrifugation (3,300 rpm for 1.25 min). A 20 μl aliquot of filtrate was injected.

HPLC analysis was performed using a Shimadzu Prominence UFLC System containing two pumps, an autosampler, a column oven, a system controller, and two 10-port switching valves. The first switching valve was mounted in the column oven and used for inline sample cleanup, and the second valve was located between the column oven and mass spectrometer and functioned as a waste divert valve. Chromatographic separation was achieved using a Luna C18(2) column (3 μm , 2.0 mm $\times$ 150 mm, part number 00F-4251-B0, Phenomenex, Torrance, CA) preceded by a Phenomenex SecurityGuard cartridge (C18). The analytical column was maintained at 60°C and sample elution carried out at flow rate 175 $\mu\text{l}/\text{min}$ with a biphasic gradient. Mobile phase A was 65% methanol with 0.4 ml of 1.0 M ammonium formate and 62.0 μl of concentrated formic acid per liter; mobile phase B was 100% methanol with 0.4 ml of 1.0 M ammonium formate and 62.0 μl of concentrated formic acid per liter.

Androgens were detected using multiple reaction monitoring (MRM) and an AB SCIEX QTRAP® 5500 mass spectrometer (Framingham, MA) with an electro-spray ionization source in positive ion mode controlled by AB SCIEX Analyst® software, version 1.5.1. Mass spectrometer conditions were ion spray voltage 5250 V, turbo gas temperature 700°C , nebulizer gas 55, turbo gas 65, curtain gas 16, CAD gas medium, and unit mass resolution

for Q1 and Q3. Nitrogen was used for nebulizer, turbo, curtain and collision gases, and voltages for maximum parent/fragment ion pair intensities were optimized using direct infusion and flow injection analysis (FIA).

MMTV-Luciferase Reporter Assay for AR Activity

An adenoviral MMTV-luciferase reporter assay was used for the evaluation of AR activity as described previously [21]. Cells were incubated with viral particles for 2 hr and harvested in 1× Reporter Lysis Buffer (RLB) from Promega (Madison, WI) following the manufacturer's instructions. Luciferase activity was determined using the Promega Luciferase 1000 Assay System on a Clarity Luminescence Microplate Reader (BioTek Instruments, Winooski, VT). Protein concentration of the RLB lysate was determined using the Coomassie Plus Protein Assay Reagent (Thermo Scientific, Rockford, IL). Luciferase activity (relative light unit, RLU) was normalized against the amount of protein.

Reverse Transcription and Quantitative Real Time Polymerase Chain Reaction (PCR)

Total RNA was prepared using the QiaShredder Kit and the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time PCR probes for prostate-specific antigen (PSA), kallikrein-2 (KLK2), 5 α -reductase 1, 5 α -reductase 2, 5 α -reductase 3, and β -actin were obtained from Invitrogen. Real time PCR reactions were set up using the Taqman Universal PCR Master Mix (Invitrogen), and run on an Applied Biosystems 7900HT Fast Real Time-PCR System (Foster City, CA).

Statistical Analysis

Experiments were performed in triplicates. Data were presented as mean $\pm$ standard deviation. The Student's *t*-test was used to determine statistical significance, and *P*-values 0.05 were considered significant.

RESULTS

Expression of 5 α -Reductase Isozymes in Prostate Cancer Cells

The expression of 5 α -reductase isozymes was evaluated using IHC for cells that had been cultured with or without 1 nM testosterone (Fig. 1). Both 5 α -reductase-1 and -3 were expressed highly in all cell lines, while 5 α -reductase-2 expression was lower and more variable. In the majority of cases, 5 α -reductase was detected in the cytoplasm, although nuclear/perinuclear localization also was observed. In the presence of testosterone, 5 α -reductase-2 appeared to be up-regulated in LNCaP and LNCaP-C4-2 cells. No striking difference in the intensity of immunostaining was noted with 5 α -reductase-1 and -3, either in the presence or absence of testosterone, in any cell line. IHC results were verified at the mRNA level using qRT-PCR (Fig. 2). In every cell line, only the transcripts of 5 α -reductase-1 and -3 were found. All cell lines except LNCaP (RPCI) expressed more 5 α -reductase-3 mRNA than 5 α -reductase-1 mRNA. Testosterone treatment had little or no effect on the message level of either 5 α -reductase-1 or -3. In general, qRT-PCR and IHC showed that 5 α -reductase-1 and -3 are the major isozymes in prostate cancer cells. However, the qRT-PCR results do not match IHC results in every detail; for example, 5 α -

reductase-2 mRNA was not detected but protein was found by IHC in LNCaP and LNCaP-C4-2. mRNA levels in these related cell lines may have been too low to be quantified under the RT-PCR conditions, whereas translation of the message was high enough to produce an amount of protein that was recognized by the antibody. Indeed, real-time PCR of 5 α -reductase 2 using a set of customer-designed primers with improved sensitivity detected very low levels of transcripts in LNCaP and LNCaP-C4-2 cells, and the levels of 5 α -reductase 5 in these cell lines were much lower compared to the level in LAPC-4 cells (data not shown).

LNCaP Cells Lack the Ability to Maintain Intracellular Testosterone and to Convert Testosterone to DHT

LNCaP cells were cultured in the presence of 1 nM testosterone in the medium with increasing doses of either dutasteride or finasteride and, 24 hr later, testosterone and DHT levels in the cell pellet were determined using LC-MS (Fig. 3A). No DHT was recovered in LNCaP cells under any condition, even in the absence of drug treatment. The testosterone data were normalized to protein concentration in the cell pellet. Between 0.004 and 0.005 ng testosterone/mg protein was found inside the cells when they were cultured with 1 nM testosterone in the medium. Dutasteride reduced intracellular testosterone to essentially non-detectable levels. Finasteride blocked testosterone accumulation equally well in each cell line. A dose of 0.5 μ M finasteride eliminated any trace of testosterone inside the cells. The dutasteride and finasteride results suggest that these 5 α -reductase inhibitors also may block the uptake of testosterone into cells.

Analysis of LNCaP (RPCI) cells at earlier time points showed an accumulation of intracellular testosterone of about 15 nM after 1 hr (Fig. 3B). This represented a 15-fold enrichment since the medium contained 1 nM testosterone. The amount of testosterone inside the cells decreased gradually over time and fell by 50% at 8 hr. DHT was not detected at any time point.

Testosterone and DHT in the pre-culture and post-culture media were analyzed (Fig. 3C). DHT was not detected in any of the samples. Testosterone was not found in the post-culture medium. However, treatment of the medium with glucuronidase, an enzyme that hydrolyzes glucuronide conjugate, allowed recovery of nearly all the testosterone present originally in the medium. In summary, the data suggest that LNCaP cells proficiently take up testosterone, but they also export testosterone very quickly via glucuronidation that prevents testosterone conversion to DHT.

Variability in the Capacity of C4-2, LAPC-4, and 22Rv1 Cells to Reduce Testosterone to DHT

Intracellular androgens were determined after 24 hr of culture with 1 nM testosterone in the medium. Dutasteride or finasteride was added at various concentrations. Low levels of testosterone and DHT were detected in control LNCaP-C4-2 cells which were not treated with testosterone (Fig. 4A). Both testosterone and DHT were accumulated by LNCaP-C4-2 cells in the presence of 1 nM testosterone. About 0.1 ng testosterone/mg protein was found in the cell pellet. The amount of DHT was about 20% of that of testosterone, the results

suggest a modest 5 α -reducing activity in these cells. As little as 0.02 μ M dutasteride or 0.05 μ M finasteride completely blocked the production of DHT. Increasing the dose of either drug reduced greatly the level of testosterone inside the cells.

In LAPC-4 cells, very low levels of DHT were detected in the control, that is, without testosterone added to the medium (Fig. 4B). High levels of testosterone and DHT were recovered in the cell pellet when LAPC-4 cells were treated with testosterone. The amount of DHT was about 50% of that of testosterone. Assuming all the DHT was produced from testosterone, as much as one-third of testosterone taken up by LAPC-4 cells was reduced to DHT. Dutasteride and finasteride were equally effective in decreasing the level of intracellular DHT. Only dutasteride was able to lower testosterone levels inside the cells, and even then, the magnitude of inhibition was not nearly as great as observed in LNCaP-C4-2 cells. Finasteride did not appear to affect testosterone uptake in LAPC-4 cells.

A trace amount of testosterone, but not DHT, was detected in control 22Rv1 cells (Fig. 4C). 22Rv1 cells accumulated the highest level of testosterone of the five cell lines examined. On the other hand, the amount of DHT was below the detection limit. Neither dutasteride nor finasteride had much of an effect on the uptake of testosterone in 22Rv1 cells.

Post-culture media from the LNCaP-C4-2, LAPC-4, and 22Rv1 experiments were analyzed for testosterone (Fig. 4D). Glucuronidase treatment of LNCaP-C4-2 post-culture medium recovered additional testosterone, with increase in concentration from 0.21 to 0.64 nM, suggesting that ~66% of the total testosterone was in the form of glucuronide conjugate. In contrast to LNCaP-C4-2, neither LAPC-4 nor 22Rv1 cells appear to export testosterone via the glucuronidation mechanism.

A number of conclusions can be drawn from the data of Figures 3 and 4. (i) Prostate cancer cells differ in their capacity to accumulate testosterone. In the presence of 1 nM testosterone in the medium, LNCaP cells retained <0.01 ng testosterone/mg protein, LNCaP-C4-2 and LAPC-4 cells reached approximately 0.1 ng testosterone/mg protein, while 22Rv1 cells showed the highest level of nearly 1 ng testosterone/ mg protein. (ii) The amount of DHT as a fraction of the total androgen pool (testosterone + DHT) varies greatly among prostate cancer cells from none or minimal in LNCaP and 22Rv1 cells, to ~17% in LNCaP-C4-2 cells, and ~30% in LAPC-4 cells. (iii) Both dutasteride and finasteride, at the appropriate doses, inhibit testosterone conversion to DHT, regardless of the 5 α -reductase profile of the cells. (iv) Dutasteride and finasteride also may inhibit the uptake of testosterone in LNCaP and LNCaP-C4-2 cells.

Interpreting the Concentration Gradient Between Extracellular and Intracellular Testosterone

Testosterone and DHT in the cell pellet were recalculated in nM concentrations to better appreciate the extent of testosterone enrichment inside the cells relative to the concentration of testosterone in the medium. The calculation was based on the assumption that water constitutes about 90% of cell volume. Intracellular concentrations of testosterone and DHT were computed after exposure to 1 nM testosterone (Table I). LNCaP (RPCI) and LNCaP (ATCC) attained levels of 0.20 and 1.1 nM testosterone, respectively. Biologically

significant levels remained inside the cells after 24 hr even though LNCaP exported nearly all the testosterone it managed to accumulate initially. In contrast, the concentration gradient of testosterone increased in LNCaP-C4-2, LAPC-4, and 22Rv1 by 29-, 34-, or 51-fold, respectively. Intracellular DHT concentration also rose from 1.3 to 6.2 nM in LNCaP-C4-2 cells, and from 1.5 to 14.4 nM in LAPC-4 cells.

Inhibition of Testosterone-Stimulated AR Activity by Dutasteride or Finasteride

The effect of dutasteride or finasteride on testosterone-stimulated AR activity was evaluated in the prostate cancer cell lines using the MMTV-luciferase assay (Fig. 5). The testosterone-stimulated luciferase value was set at 100%. Since both LNCaP (RPCI) and LNCaP (ATCC) cells responded similarly to the various treatments, only the LNCaP (RPCI) data were presented (Fig. 5A). The addition of 1 nM testosterone to the medium stimulated AR activity, despite the fact that these cells retained a relatively low level of testosterone and produced no detectable DHT (Table I). The AR activity was sensitive to dutasteride. A dose of 0.1 μ M dutasteride eliminated the stimulatory effect of testosterone. Finasteride was not as potent as dutasteride; a dose of 10 μ M was needed to depress AR activity to baseline values.

The AR activity responded similarly to dutasteride or finasteride in LNCaP-C4-2 cells compared to LNCaP cells (Fig. 5B). A dose of 0.2 μ M finasteride did not inhibit AR activity, although the same dose blocked testosterone conversion to DHT (Fig. 4). The results suggest that testosterone, in the absence of DHT, was capable of maintaining AR activity in LNCaP-C4-2 cells. However, AR activity was inhibited when the dose of finasteride was increased to 5 or 10 μ M. This effect of finasteride could not be attributed to blocking testosterone conversion to DHT.

The AR activity in LAPC-4 cells also was inhibited by dutasteride or finasteride (Fig. 5C). Since a dose of 0.1 μ M dutasteride was sufficient to block testosterone reduction, greater inhibition of AR activity by higher doses of dutasteride suggest that the drug might inhibit AR activity by a mechanism other than limiting the availability of DHT. The same inference could be extended to the finasteride results. A dose of 2 or 5 μ M finasteride reduced AR activity by about 50%. This inhibitory effect was unlikely to be caused by a shortage of DHT because DHT production was already reduced to baseline level by treatment with lower doses of finasteride.

The 22Rv1 data were similar (Fig. 5D). A dose of 0.1 μ M dutasteride or 1 μ M finasteride did not affect AR activity significantly, although the same dose of each drug blocked testosterone reduction to DHT (Fig. 4). AR activity was depressed further when dutasteride or finasteride doses were increased. The results again suggested that the effect on AR was independent of 5 α -reductase inhibition.

Inhibition of AR-Regulated Genes by Dutasteride or Finasteride

The mRNA level of 2 AR-regulated genes, PSA and KLK2, was determined using qRT-PCR (Fig. 6). The expression level in the presence of 1 nM testosterone was set at 100%. The transcriptional induction of the two genes differed between the two LNCaP lines (Fig. 6A). The expression of both PSA and KLK2 was not responsive to testosterone in LNCaP

(RPCI), but was stimulated in LNCaP (ATCC). In LNCaP (RPCI) cells, PSA and KLK2 expression was not affected by dutasteride and was decreased slightly by finasteride. In LNCaP (ATCC) cells, PSA and KLK2 expression was inhibited by 1 μ M dutasteride and by finasteride at all doses tested. In LNCaP-C4-2 and LAPC-4 cells (Fig. 6B, C), only higher doses of dutasteride or finasteride inhibited PSA and KLK2 expression. Both PSA and KLK2 were induced poorly by testosterone in 22Rv1 cells (Fig. 6D). Consequently, the effect of drug treatment was difficult to assess. The expression of PSA decreased to control values by dutasteride treatment, but KLK2 expression was less sensitive to drug treatment. In summary, in those cell lines in which PSA and KLK2 responded to testosterone stimulation, both dutasteride and finasteride depressed expression of AR target genes at concentrations that were higher than those required to block testosterone reduction.

DISCUSSION

A major finding of the present study is that the distribution and levels of 5 α -reductase isozymes in prostate cancer cells did not predict the capability to convert testosterone to DHT. All five cell lines share similar 5 α -reductase isozymes profiles. However, their ability to reduce testosterone differs greatly. About 30% or 15% of testosterone was converted to DHT in LAPC-4 or LNCaP-C4-2 cells, respectively. Little DHT was produced in LNCaP and 22Rv1 cells, which suggests that testosterone might not be a substrate of 5 α -reductase in the two cell lines. The data suggest a disconnection between 5 α -reductase protein level and enzymatic activity. Although the underlying cause for this phenomenon is unclear, the clinical implication is apparent: prostate cancer microenvironmental androgen biology may be highly variable and unpredictable based on 5 α -reductase IHC performed on clinical specimens. The effects of dutasteride or finasteride in the presence on 1 nM DHT on a panel of cell lines, which included LNCaP, 22Rv1, LAPC-4, and VCaP were examined (data not shown). The ability of testosterone to activate AR was comparable to that of DHT, and there did not seem to be a significant effect of AR variants on the response to testosterone versus DHT. However, the role of AR variants in responsiveness to testosterone and DHT needs to be examined carefully.

An equally important finding is that the capacity to produce DHT from testosterone does not predict the AR activity response to finasteride or dutasteride. The lack of a relationship is best illustrated by comparing what happened in LNCaP and LAPC-4 cells. LNCaP did not convert testosterone to DHT, but showed the strongest inhibitory effect on AR activity in response to finasteride or dutasteride. LAPC-4 converted most testosterone to DHT, and the conversion was decreased significantly by treatment with finasteride or dutasteride. However, the AR signal was much less sensitive to the two drugs. In other words, the depression of AR activity did not seem to be in lockstep with the lower availability of DHT. A direct, off-target effect of finasteride and dutasteride on AR inhibition may contribute in part to the dichotomy [17,18], especially when this off-target effect is determined primarily by cell line specificity. The data also suggested that some prostate cancer cells use testosterone and DHT interchangeably and that dutasteride or finasteride inhibits testosterone-stimulated AR activity.

Prostate cancer cells can accumulate testosterone to a level that is at least 15–50 times higher than levels in the medium. Members of the organic anion transporter superfamily are known to be involved in steroid hormone uptake [22]. Selective transporters of this family, such as SLCO1B3 and SLCO2B1, have been shown to be up-regulated in androgen-stimulated prostate cancer or castration-recurrent prostate cancer [23–26]. Better understanding of the regulatory mechanism of androgen uptake could impact prostate cancer management. Levels of intracellular testosterone in LNCaP or LNCaP-C4-2 cells were reduced by increased doses of dutasteride or finasteride (Figs. 3A and 4A). The reason for this reduction is under investigation. The structural similarity between steroids and dutasteride/finasteride, raises the possibility that these drugs may block steroid transporters used by LNCaP or LNCaP-C4-2 cells. Although circulating testosterone and DHT levels are reduced greatly by surgical or medical castration [27–30], residual androgens may be sufficient to activate AR signaling especially if the androgens can be taken up efficiently by transporters.

Intracellular testosterone concentration is a balance between influx and efflux. Testosterone is exported from cells after glucuronidation that makes testosterone more hydrophilic. LNCaP cells do not accumulate any significant amount of testosterone; these cells also have a high glucuronosyl transferase (UGT) activity [31]; UGT2B15 and UGT2B17 are the two enzymes known to glucuronidate androgens [32–34]. The regulation of UGT expression and the role of this enzyme in prostate cancer biology require further investigation. This area of research is more than just academic, since genetic polymorphisms that cause lower UGT activity have been reported to be associated with more aggressive prostate cancer [35–38].

The clinical use of finasteride and dutasteride for prostate cancer chemoprevention has engendered a great deal of controversy, which should not be surprising based on this preclinical work. The biological effect of finasteride or dutasteride appears to be extremely complex. The down-regulation of AR activity by either drug does not necessarily correlate with the ability of the drug to inhibit conversion of testosterone to DHT. Part of the reason may be due to the fact that knowledge of the distribution of 5 α -reductase isozymes and their level of expression cannot be used to predict the degree of testosterone metabolism to DHT. Prostate cancer cells that are susceptible to off-target AR inhibitory effect of finasteride or dutasteride are likely to be controlled successfully by these drugs regardless of their 5 α -reducing capability. Cells that have a low capacity to reduce testosterone may not be inhibited, unless they also are sensitive to the off-target effect of the drug on AR. The response of cells with a robust capacity to reduce testosterone to DHT will depend on their ability to maintain AR activity with testosterone in the absence of DHT. Other factors may impact the interplay between 5 α -reductase enzymology and androgen biology. These factors may include population-based differences in polymorphisms in 5 α -reductase and androgen transporters, subcellular differences in finasteride and dutasteride pharmacokinetics, androgen degradation, and AR-regulated gene expression. Further studies are required since millions of men have been or will be placed on finasteride or dutasteride for treatment of lower urinary tract symptoms or chemoprevention of prostate cancer.

CONCLUSIONS

The biological effects of finasteride or dutasteride appear to be complex and depend on the interplay of several factors, which may include testosterone turnover, effects of subcellular compartmental pH, cofactor availability and other unknown factors on 5 α -reductase enzyme activity, the ability to use testosterone and DHT interchangeably, and the propensity of cells to manifest off-target AR inhibitory effects.

Acknowledgments

Grant sponsor: National Cancer Institute; Grant numbers: P01CA126804; P01CA77739; Grant sponsor: National Cancer Institute, Cancer Center; Grant number: P30CA16056.

This work was supported by NCI/NIH P01CA126804 (to C. Ip) and P01CA77739 (to J.L. Mohler); and NCI Cancer Center Support Grant P30CA16056 to Roswell Park Cancer Institute.

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Fig. 1.

IHC for 5α-reductase-1, -2, and -3 in prostate cancer cells cultured in the presence or absence of 1 nM testosterone (T). Note: the pink hue in row 2 was an image acquisition error that did not affect analysis and was not affected by image processing.

Fig. 2.

Message level of 5α-reductase-1, -2, and -3 as determined using qRT-PCR in LNCaP (A), LNCaP-C4-2 (B), LAPC-4 (C), and 22Rv1 (D) cultured either in the presence or absence of 1 nM testosterone (T).

Fig. 3.

Lack of ability of LNCa P cells to maintain intracellular testosterone and to convert testosterone to DHT. **A:** Testosterone concentration in LNCaP (RPCI) or LNCaP (ATCC) cells cultured for 24 hr with 1 nM testosterone, with or without dutasteride (Dut) or finasteride (Fin). Control cells were not treated with testosterone. No DHT was recovered in the cell pellet in these experiments. * $P < 0.05$ compared to T (1 nM) alone value. **B:** Testosterone concentration in LNCaP (RPCI) cell lysate as a function of time of incubation. Analysis of medium testosterone showed a concentration of 1 nM at the 0 hr time point. * $P < 0.05$ compared to 1 hr control value; # $P < 0.05$ compared to 1 hr testosterone treatment value. **C:** Testosterone concentration in pre- and post-culture media of LNCaP (RPCI) cells treated with 1 nM testosterone (T). Control cells were not treated with testosterone. The post-culture medium was incubated with glucuronidase to hydrolyze any glucuronide conjugate.

Fig. 4.

Testosterone or DHT concentration in LNCaP-C4-2 (A), LAPC-4 (B) or 22Rv1 (C) cells cultured for 24 hr with 1 nM testosterone, with or without dutasteride (Dut) or finasteride (Fin). Control cells were not treated with testosterone. Testosterone concentration in post-culture media was determined in the presence or absence of glucuronidase (D). * $P < 0.05$ compared to non-treated control. # $P < 0.05$ compared to T (1 nM) alone value. ^ $P < 0.05$ compared to non-glucuronidase treated value.

Fig. 5.

Luciferase activity in LNCaP (RPCI) (A), LNCaP-C4-2 (B), LAPC-4 (C), or 22Rv1 (D) cells cultured with 1 nM testosterone, with or without dutasteride (Dut) or finasteride (Fin). Control cells were not treated with testosterone. Testosterone-stimulated activity was set at 100%. * $P < 0.05$ compared to testosterone (1 nM) alone value.

Fig. 6.

PSA or KLK2 message levels in LNCaP (**A**), LNCaP-C4-2 (**B**), LAPC-4 (**C**), or 22Rv1 (**D**) cells cultured with 1 nM testosterone, with or without dutasteride (Dut) or finasteride (Fin). Control cells were not treated with testosterone. Testosterone-stimulated message levels of PSA and KLK2 were set at 100%. * $P < 0.05$ compared to PSA value of treatment with testosterone (1 nM) alone. # $P < 0.05$ compared to KLK 2 value of treatment with testosterone (1 nM) alone.

TABLE I

Intracellular Testosterone (T) and Dihydrotestosterone (DHT) Concentrations (nM) After 24 hr Culture in the Presence of 1 nM T

	Intracellular concentration (nM)			
	<u>Without T in medium</u>		<u>With T in medium</u>	
	T	DHT	T	DHT
LNCaP (RPCI)	0	0	0.20	0
LNCaP (ATCC)	0	0	1.09	0
C4-2	1.63	1.25	29.02	6.24
LAPC-4	0.39	1.53	34.30	14.40
22Rv1	1.22	0	50.70	0