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THE ROLE OF 5-ALPHA REDUCTASE 3 IN STEROID METABOLISM

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A

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THE ROLE OF 5-ALPHA REDUCTASE 3 IN STEROID METABOLISM

Zahi I Mitri, M.D.

Advisor Professor: Mark Titus, Ph.D.

Background

The growth and development of prostate cancer is largely driven by androgen mediated signaling. The 5-alpha reductase family of enzymes plays an essential role in the conversion of testosterone to the more potent androgen, 5-alpha dihydrotestosterone (DHT), which is capable of binding the androgen receptor to activate gene targets and downstream signaling. This study aimed to evaluate the role of 5-alpha reductase 3 (SRD5A3), a novel member of the 5-alpha reductase family, in steroid metabolism and prostate carcinogenesis.

Materials and Methods

HEK293 cells were transfected with the SRD5A3 human cDNA ORF Clone from OriGene. Steroid substrates (testosterone, progesterone, androstenedione, epitestosterone, 11-ketotestosterone, 11α-hydroxytestosterone, 11β-hydroxytestosterone, 6β-hydroxytestosterone, and cortisol) were added to transfected HEK293 cells. The dual 5-alpha reductase 1 and 2 inhibitor, dutasteride, was added to evaluate its inhibitory activity on SRD5A3 in the presence of each steroid. Mass spectrometry analysis was used to detect and quantify the conversion of steroid substrates to their 5-alpha reduced products.

Results

SRD5A3 plasmid was successfully transfected into HEK293 cells. Mass spectrometry analysis confirmed that SRD5A3 converted testosterone to DHT. This reaction was completely inhibited

by dutasteride. SRD5A3 did not convert progesterone or androstenedione to the 5-alpha reduced products. SRD5A3 had the capacity of reducing testosterone derivatives to their 5-alpha reduced products. This catalysis was concentration dependent, with higher substrate levels yielding higher amounts of the 5-alpha reduced steroid product. The inhibitory activity of dutasteride on SRD5A3 was substrate and concentration dependent.

Conclusion

SRD5A3 catalyzes the 5-alpha reduction of testosterone and testosterone analogues in the presence and absence of dutasteride. These 5-alpha reduced steroids are capable of binding and activating androgen receptor signaling. Further studies evaluating the role of SRD5A3 in prostate carcinogenesis are mandated to determine the role of therapeutic SRD5A3 inhibition across the spectrum of prostate cancer.

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Abbreviations

5-alpha dihydrotestosterone – DHT

Prostate Cancer Prevention Trial - PCPT

Reduction by Dutasteride of Prostate Cancer – REDUCE

5-alpha reductase-3 - SRD5A3

Dulbecco's Modified Eagle Medium – DMEM

Fetal Bovine Serum - FBS

Phosphate Buffered Saline – PBS

Luria Broth - LB Broth

Polymerase chain reaction – PCR

Sodium dodecyl sulfate – SDS

Tris-buffered saline with Tween - TBST

Liquid chromatography-mass spectrometry – LCMS

Standard - Std

CHAPTER 1: BACKGROUND

The growth and development of prostate cancer is largely driven by androgen mediated signaling (1). Testosterone, the predominant circulating androgen, is produced in the testis under the control of luteinizing hormone. The 5-alpha reductase enzyme family plays a key role in the conversion of testosterone to the more potent androgen, 5-alpha dihydrotestosterone (DHT), in target organs such as the prostate (2). Given this pathophysiology, there has been interest in the development of 5-alpha reductase inhibitors for the treatment and prevention of prostate cancer. Two large trials have evaluated 5-alpha reductase inhibitors as chemoprevention for patients at high risk for developing prostate cancer. The Prostate Cancer Prevention Trial (PCPT) randomized >18,000 men to finasteride, a predominantly 5-alpha reductase 2 inhibitor, or placebo. The trial showed a decreased incidence of prostate cancer overall, but also a slight increase in the number of more aggressive tumors in the finasteride arm (3). One hypothesis to explain the increase in high-grade tumors in the finasteride arm is that this drug does not inhibit the 5-alpha reductase 1 enzyme. This isoform is overexpressed in prostate cancer, and has the ability to 5-alpha-reduce testosterone to DHT (4-7). In contrast, 5-alpha reductase 2 expression appears to either decrease or stay the same between normal prostate tissue and prostate cancer (5, 8). These observations led to the Reduction by Dutasteride of Prostate Cancer (REDUCE) trial. This study compared dutasteride, a dual 5-alpha reductase 1 and 2 inhibitor to placebo in men at high risk of developing prostate cancer. There was a significant reduction in the development of prostate cancer in this study, but again was noted a trend for developing high-grade prostate cancer in the control arm. This was lower than the risk previously observed in the finasteride prevention study (2, 9). Both clinical trials failed to show a mortality difference between the intervention and the placebo arms. Currently, 5-alpha reductase inhibitors are indicated for the treatment of benign prostate enlargement and lower urinary symptoms. Conversely, the use of

finasteride or dutasteride as chemoprevention remains controversial (10-14), and mainly based on a risk/benefit conversation between patients and their physicians.

In addition, 5-alpha reductase inhibitors have been evaluated in the treatment of prostate cancer (2, 14). Androgen deprivation therapy is the standard of care for advanced prostate cancer; however this therapy is not curative, with most tumors evolving into a castrate-resistant, also termed androgen-independent phenotype. Preclinical and clinical data have shown that the androgen receptor axis continues to be active under androgen deprivation therapy (15-18), and can potentially drive prostate cancer growth in an autocrine fashion (19-23). Androgen deprivation therapy achieves large reductions in systemic testosterone and DHT levels, however seems to have a lesser effect on testosterone and DHT levels within the prostate, where they remain at sufficient concentrations to activate the androgen receptor (22, 24-28). In addition, prostate cancer cells have the ability to use adrenal androgens such as dehydroepiandrosterone (DHEA) as substrates to produce testosterone and DHT and drive the androgen receptor (23, 28-31). The 5-alpha reductase enzyme family appears to continue to play a role in the production of DHT from steroid precursors in this setting, especially with data showing that 5-alpha reductase 1 and 5-alpha reductase-3 (SRD5A3) levels to be higher in prostate cancer compared to benign prostate tissue (4, 6-8). Furthermore, Li et al. demonstrated that activation of the androgen receptor negatively regulates *SRD5A3* expression by binding a negative androgen response element on the *SRD5A3* promoter (32). The reduction in androgen receptor expression in the setting of androgen deprivation therapy can then potentially lead to an increase in the expression of *SRD5A3*, and magnify its role in the production of DHT and tumor growth (32). 5-alpha reductase inhibitors either as single agent or in combination with androgen deprivation therapy or androgen receptor antagonists have been evaluated in several settings of prostate cancer

therapy (2). In the preoperative setting, the use of dutasteride before radical prostatectomy was associated with reduction in tumor volume and increased apoptosis (33, 34). The use of 5-alpha reductase inhibitors in the setting of biochemical relapse (35-37), castrate-sensitive (38-40), or castrate-resistant (41-43) advanced prostate cancer, has resulted in various levels of PSA decline, however no clinically meaningful survival endpoints have been reported to date. To date no clinical study has evaluated androgen deprivation therapy alone compared to androgen deprivation therapy combined with 5-alpha reductase inhibitors (2). More recently, the combination of dutasteride and enzalutamide, a novel androgen receptor antagonist, in the preclinical setting has shown to be synergistic in suppressing castrate-sensitive (LAPC4) and castrate-resistant (DuCaP) prostate cancer cell lines (44). Based on these preclinical and early phase studies, trials are planned to evaluate the role of 5-alpha reductase inhibitors in the management of prostate cancer. Currently, there are no data to support the use of 5-alpha reductase inhibitors for the treatment of prostate cancer (2, 14).

The development of prostate cancer despite 5-alpha reductase inhibition in the prevention setting, and that of castrate resistant prostate cancer despite androgen blockade raised the hypothesis of an alternate pathway of androgen production to drive carcinogenesis. More recently, a new 5-alpha reductase isoform, SRD5A3, was discovered. The first report by Uemura et al. in 2008 identified this novel gene using gene expression profiling in hormone refractory prostate cancer (5). The *SRD5A3* gene is located on the long (q) arm of chromosome 4 at position 12 (4q12) (OMIM 611715). This novel enzyme's sequence aligned with known sequences of 5-alpha reductase 1 and 5-alpha reductase 2, including conservation of the C-terminal domains, which are thought to be important in the catalytic activity of the 5-alpha reductase family in converting testosterone to DHT (5). Uemura et al. showed that SRD5A3 was

overexpressed in hormone refractory prostate cancer compared to hormone sensitive prostate cancer and normal prostate tissue. In contrast, 5-alpha reductase 2, the predominant isoform in the prostate, is overexpressed in the normal prostate and in hormone sensitive prostate cancer, and is almost absent in hormone refractory prostate cancer. The SRD5A3 enzyme distribution was similar to 5-alpha reductase 1. In addition, the authors showed that SRD5A3 had the ability to convert testosterone to DHT. In contrast, mutated *SRD5A3* lost its ability to reduce testosterone to DHT (5).

Despite this observation, much remained unknown about this new enzyme that appeared to loosely fit in the 5-alpha reductase family. In 2010, Cantagrel et al. evaluated a large consanguineous family where several children were born with ocular defects, brain malformations, heart defects and developmental delays (45). Genome-wide linkage analysis localized the mutations to the region of the *SRD5A3* gene, mutations that were not found in any of the unaffected control subjects. Interestingly, unlike defects in 5-alpha reductases 1 and 2, these children had no sexual development abnormalities (OMIM #264600) to suggest a defect in steroid hormone production. Given the clinical findings for these patients, further functional studies revealed that SRD5A3 plays a role in N-linked glycosylation of surface proteins. *SRD5A3* encodes an enzyme that is required for the conversion of polyprenol to dolichol, an important molecule in the endoplasmic reticulum (45, 46). Following that, other reports linked congenital disorders of glycosylation with mutations in *SRD5A3*, with affected patients suffering from similar presentations, including cerebello-ocular syndromes. These studies showed that SRD5A3 was expressed in the brain, heart and retina in normal human tissues, and significantly reduced in individuals with *SRD5A3* defects. This may explain the clinical manifestations in individuals carrying mutations in this enzyme (45, 47, 48).

Despite the discovery of a surprising role of *SRD5A3* in protein glycosylation, this enzyme's localization and role in prostate cancer remained a mystery. Two studies by Godoy and Yamana analyzed the distribution of SRD5A3 in human tissues using immunohistochemical studies (49, 50). They found that SRD5A3 was overexpressed in several androgen regulated (prostate, skeletal muscle, skin) and non androgen regulated (pancreas, colon, kidney) benign tissues. This observation supports that SRD5A3 may play a role other than steroid reduction, potentially be involved in protein glycosylation as previously shown by Cantagrel et al. (45). Interestingly, SRD5A3 was overexpressed in prostate cancer tissues compared to benign prostate tissue. This result aligns with the findings by Uemura et al., who had shown that the expression of SRD5A3 seems to increase along prostate cancer carcinogenesis from benign tissue to castrate resistant cancer (5). With these findings regarding the localization of SRD5A3, Titus et al. evaluated SRD5A3 function in castrate resistant prostate cancer specimen homogenates. Using clinical specimens, they first confirmed previous reports that SRD5A3 was overexpressed in castrate resistant prostate cancer compared to 5-alpha reductase 1 and 5-alpha reductase 2 (51). In addition, Titus et al. expressed a HP-Thioredoxin-fusion SRD5A3 protein using a pBAD/Thio-TOPO vector in CHO-K1 cells (mammalian system), and TOP10 *E.coli* competent cells. The HP-Thioredoxin-fusion SRD5A3 protein in both systems was found to be catalytically active and 5α-reduced testosterone to DHT. However 5α-reduced steroids were produced in low amounts. This reaction was partially inhibited by dutasteride or abiraterone, suggesting that SRD5A3 can sustain DHT formation even in the presence of 5-alpha reductase 1 and 2 inhibitors, and potentially sustain prostate cancer development. Of note, this fusion protein also converted androstenedione and progesterone to their 5α-reduced products, androstanedione and dihydroprogesterone (51).

In contrast to these findings, a recent report looking at both hamster and human SRD5A3 showed that the enzyme was unable to reduce testosterone to DHT (52). In this study, Chaves et al. evaluated the 5-alpha reducing capacity of hamster SRD5A3 expressed as a fusion protein with green fluorescent protein from the pcDNA3.1-NT-GFP/hSrd5a3 plasmid and human SRD5A3 (OriGene, Maryland, USA) (52). Of note, the fusion protein localized to the cytoplasm, whereas the SRD5A3 enzyme has been described to be a membrane protein involved in protein glycosylation (45).

Several reports have now been published on SRD5A3; however its role in the pathogenesis of prostate cancer and in steroid metabolism has not been clearly defined. SRD5A3 is ubiquitously expressed in human tissues –both benign and malignant- and plays a role in N-linked protein glycosylation (45); with defects in this enzyme having been linked with congenital disorders of glycosylation (45, 47, 48). This study aims to evaluate the role of SRD5A3 in steroid metabolism, and elucidate its role in prostate carcinogenesis. Specifically, this study first assessed the ability of SRD5A3 to convert steroid precursors to their 5-alpha reduced products. Additionally, the effect of dutasteride on SRD5A3 function was also evaluated for each steroid precursor.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

The HEK293 cell line (cat# CRL-1573, ATCC, VA, USA) was used in this experiment. This is a permanent cell line established from primary embryonic human kidney and transformed with human adenovirus type 5 DNA (http://www.atcc.org/products/all/VR-1516.aspx). The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein. HEK293 cells were prepared from a clone selected for fast growth in serum-free medium (Figure 1), good adherence during plaque assays, superior transfection efficiencies and a high level of protein expression.

HEK293 cells that had been frozen in liquid nitrogen were removed and thawed on ice. When thawed, cells were added to a 10 cm plate containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM) (cat # SH30022.01, HyClone, Utah, USA) to which were added 10% Fetal Bovine Serum (FBS) (cat #10437-077, Thermo Fisher Scientific, Massachusetts, USA) and Antibiotic-Antimycotic (cat # 15240-062,Thermo Fisher Scientific, Massachusetts, USA). Plates become confluent and ready to split within 24-48 hours. To split HEK293 cells, media was removed and the cells were washed with Phosphate Buffered Saline (PBS). After that, trypsin (cat # 25-053-CI, Corning, VA, USA) was added to the plate and the cells were placed in the incubator for 2-3 minutes. Plates were removed and the cells observed to ensure they have detached. At that point, DMEM was added to neutralize the trypsin effect. The cells were then aliquoted into the desired number of plates, and to each plate was added DMEM to a total volume of 10 mL.

Figure 1. HEK293 cell line growth time course

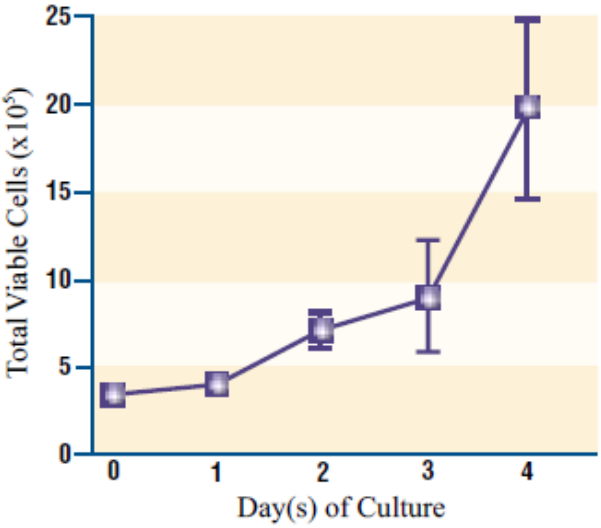

Figure 1. The average HEK-293 cell doubling time in shaker flask. *HEK293 suspension cells were seeded at* $3x10^5$*cells/mL in 125 mL shaker flasks containing 20 mL media. Results show average doubling time of 33 hours and average density approaching* $2x10^6$*cells/mL over a period of four days.*

2.2 SRD5A3 Expression Vector

DH5α E.coli competent cells were used to clone the SRD5A3 vector using a heat shock protocol. The SRD5A3 vector was diluted to 10 ng/µL, and then 2 µL were added to 4 tubes containing 45 µL DH5α cells. The cells were incubated on ice for 30 minutes, and then exposed to a heat shock for 20 seconds in a 42ºC water bath without shaking. The cells were then placed on ice for 2 minutes. Pre-warmed Luria Broth (LB Broth) was added to each tube and incubated at 37ºC for 1 hour in a heated shaker at 225 rpm. Next, 20 to 200 µL of broth from each tube were added to LB plates and incubated overnight at 37ºC. Subsequently, I picked bacterial colonies and inoculated LB broth. The SRD5A3 vector was isolated from E.coli and polymerase chain reaction (PCR) confirmed that the cDNA sequence matched the published sequence of the SRD5A3. Four different colonies were picked and the vector was quantified (ranged from 200-500 µg of DNA).

2.3 SRD5A3 Protein Expression - Creating a Transient Transfected Cell Line

The transfection experiment was run using the SRD5A3 (NM_024592) human cDNA ORF Clone from OriGene (cat # RC200970, Maryland, USA)**.** HEK293 cells were plated until they achieved 60-70% confluency in 10 cm plates. The OriGene application guide protocol for transient transfection was implemented during this experiment. Opti-Minimal Essential Medium (MEM, cat# 51985091, Thermo Fisher Scientific, Massachusetts, USA) 1.0 mL was added to each 10 cm plate. 10 µg of DNA was added to each plate as recommended by application guide (5-25 µg DNA per 10 cm plate), and then Turbofectin (cat# TF81001, OriGene, Maryland, USA) was added in a ratio of 3:1 (Turbofectin: DNA). The cells were then incubated for 30 minutes at room temperature. Following that, 9 mL of DMEM were added to the plates.

2.4 SRD5A3 Protein Expression - Creating a Stable Transfected Cell Line

Using a transiently transfected plate 24 hours after transfection, cells were passaged into fresh growth medium with the selective agent and Antibiotic-Antimycotic. After passaging the cells and adding fresh medium to allow for growth, in the presence of the selective agent, immunoblot analysis was performed to confirm that the cells continued to harbor the transfected plasmid. This process aimed to generate a stably transfected HEK293 cell line for future experiments.

2.5 Immunoblot Analysis

Acrylamide gels (12%) were prepared using the following reagents: water, 30% acrylamide mix (cat # 161-0158, Bio-Rad, California, USA), 1.5M Tries buffer (pH 8.8) (cat # 161-0798, Bio-Rad, California, USA), 10% sodium dodecyl sulfate (SDS) solution (cat # 161-0416, Bio-Rad, California, USA), 10% Ammonium Per sulfate (cat # 161-0700, Bio-Rad, California, USA), and TEMED (cat # 161-0800, Bio-Rad, California, USA). The lanes of each stacking gel were loaded with: molecular weight markers, negative control (cells without vector), and positive control **(**cat# RC200970, OriGene, Maryland, USA), positive control (cloned vector), and positive protein lysate (OriGene). The samples were run on the gel at 100 volts for 2 hours. Then, the separated proteins were transferred onto nitrocellulose for 2 hours. The nitrocellulose was removed from the transfer apparatus and immunoblot was performed using primary antibodies to DDK epitope (cat# AR100023, OriGene, Maryland, USA), SRD5A3 (cat# PA5-25200, Thermo Fisher Scientific, Massachusetts, USA), and actin (cat# ab8229, Abcam, Massachusetts, USA). Immunoblots were performed as described below:

1. The nitrocellulose was blocked for 30 minutes in 5% milk

2. Two washes in 10 mL Tris-buffered saline with Tween (TBST), 10 minutes each
3. Incubation with actin (3 µL in 5.0 mL BSA) and DDK (3 µL in 5.0 mL BSA) primary antibodies
4. Four washes in 10 mL TBST, 10 minutes each
5. A second block was applied using 5% milk in TBST for 30 minutes
6. Two washes with 10 mL TBST, 10 minutes each
7. Secondary antibody (3 µL in 5 mL of 3% BSA) for 45 minutes
8. Four washes with 10 mL TBST, 10 minutes each
9. The nitrocellulose was exposed for 2 minutes and developed in the dark room.

2.6 Protein Concentration

The optimal timing of protein expression following transfection was determined by quantifying amount of total cell protein (Bradford Protein Assay Kit, Bio-Rad (product #23208, Bio-Rad, CA, USA)) using lysates from fixed time intervals. The results were graphed to determine the time point of maximum protein expression.

2.7 SRD5A3 Enzyme Assays

The stably transfected HEK293 cell line was used to determine the enzymatic function of the SRD5A3 vector with the addition of the following C-13 labeled steroid compounds:

- Testosterone (cat# T1500, Sigma-Aldrich, Missouri, USA),
- Progesterone (cat# 10314, IsoSciences, Pennsylvania, USA),
- Dihydrotestosterone (cat# 6065, IsoSciences, Pennsylvania, USA),

- Androstenedione (cat# 9044, IsoSciences, Pennsylvania, USA),
- Epitestosterone (cat# E5878, Sigma-Aldrich, Missouri, USA),
- 11α-hydroxytestosterone (cat# 10008647, Cayman Chemicals, Michigan, USA),
- 6β-hydroxytestosterone (cat# H2898, Sigma-Aldrich, Missouri, USA),
- 11β-hydroxytestosterone (cat# A5760-000, Steraloids, Rhode Island, USA),
- 11-ketotestosterone (cat# A6720-000, Steraloids, Rhode Island, USA), and
- Cortisol (cat# 14465 IsoSciences, Pennsylvania, USA).

Each test steroid was added to DMEM containing 10% charcoal-stripped Fetal Bovine Serum (cat# F6765, Sigma-Aldrich, Missouri, USA) and Antibiotic-Antimycotic. Most endogenous steroids have been removed hence the effect of added steroids is monitored in the experiments. At 48 hours, the cell pellets and media were collected for steroid analysis using quantitative mass spectrometry.

2.8 Mass Spectrometry Analysis

Mass spectrometry analysis was performed using an Agilent 1290 UHPLC system (Agilent Infinity 1290 Autosampler, Binary Pump, Thermostatted Column Compartment), coupled to an Agilent 6490 Mass Spectrometer with Jet Stream Technology ESI source. Separation was achieved using a Chromolith Fast Gradient chromatography column, RP-18e, 50 x 2mm, 1.5 um particle size. The analysis was done using the MassHunter Workstation Quantitative Analysis Software (Version B.06.00 SP01/Build 6.0.388.1, Agilent, California, USA).

Cell pellets were reconstituted in 200 µL of UHPLC grade water and then spiked with 10 µL of a 10ng/mL C-13 steroid internal standard solution (C-13 Testosterone, C-13 Progesterone

and C-13 DHT, C-13 Pregnenolone). Media (200 uL) was also aliquoted and spiked with 10 µL of a 10ng/mL internal standard solution. Methyl t-butyl ether (MTBE) (3.0 ml) was then added and the sample was extracted with rocking for 15 minutes at room temperature. The organic layer was then separated by centrifugation, decanted and evaporated down to dryness under a steady stream of medical grade nitrogen. The residue was then reconstituted in 100 µL methanol. An aliquot (100 µL) of 1.5M Hydroxyalamine hydrochloride (cat# 159417, Sigma-Aldrich, Missouri, USA) was then added and the mixture was heated at 65ºC for 60 minutes. The samples were then transferred into liquid chromatography-mass spectrometry (LCMS) vials and analyzed by triple quadrupole mass spectrometry.

In addition, an 8 point standard (Std) calibration curve for the following 11 androgens was analyzed using mass spectrometry:

- Testosterone = 1.0 to 1000 pg/mL
- Epitestosterone = 1.0 to 1000 pg/mL
- Androstenedione = 1.0 to 1000 pg/mL
- Dihydrotestosterone = 10 to 1000 pg/mL
- Progesterone = 1.0 to 1000 pg/mL
- Dihydroprogesterone = 50 to 1000 pg/mL (cat# P2750-000, Steraloids, Rhode Island, USA)
- Pregnenolone = 10 to 1000 pg/mL (cat# Q5500-000, Steraloids, Rhode Island, USA)
- 17-hydroxyprogesterone = 1.0 to 1000 pg/mL (cat# Q3360-000, Steraloids, Rhode Island, USA)
- Cortisol = 10 to 1000 pg/mL
- Androstanedione = 10 to 1000 pg/mL (cat# A1630-000, Steraloids, Rhode Island, USA)

- 5-alpha pregnan-3-ol-20-one = 10 to 1000 pg/mL (cat# P3830-000, Steraloids, Rhode Island, USA)

This was done using the following stock solutions in methanol: Std1: 0.02 ng/mL (1 pg/mL); Std2: 0.1 ng/mL (5 pg/mL); Std3: 0.2 ng/mL (10 pg/mL) Std4: 1 ng/mL (50 pg/mL); Std5: 2 ng/mL (100 pg/mL); Std6: 5 ng/mL (250 pg/mL); Std7: 10 ng/mL (500 pg/mL); Std8: 20 ng/mL (1000 pg/mL). Two quality control (QC) solutions (representing both high and low concentrations) were also prepared using the following two solutions: QC1: 0.4 ng/mL (20 pg/mL); QC2: 15 ng/mL (750 pg/mL). The standard curve and QC samples were prepared with the same method used for sample extraction detailed above.

CHAPTER 3: RESULTS

3.1 Cloning the SRD5A3 plasmid

The SRD5A3 vector was successfully cloned in DH5α E.coli competent cells. PCR was used to confirm that our results matched the published sequence of the SRD5A3 vector with 98% concordance (Figure 2). Four colonies were obtained, yielding 496.4 µg, 266 µg, 466 µg, and 510 µg of vector respectively.

Figure 2. SRD5A3 Plasmid Sequence by PCR

Score	Expect	Identities	Gaps	Strand
1393 bits(1544)	0.0	805/821(98%)	8/821(0%)	Plus/Plus

```
Query  1    ATGGCTCCCTGGGCGGAGGCCGAGCACTCGGCGCTGAACCCGCTGCGCGCGGTGTGGCTC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  150  ATGGCTCCCTGGGCGGAGGCCGAGCACTCGGCGCTGAACCCGCTGCGCGCGGTGTGGCTC  209

Query  61   ACGCTGACCGCCGCCTTCCTGCTGACCCTACTGCTGCAGCTCCTGCCGCCCGGCCTGCTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  210  ACGCTGACCGCCGCCTTCCTGCTGACCCTACTGCTGCAGCTCCTGCCGCCCGGCCTGCTC  269

Query  121  CCGGGCTGCGCGATCTTCCAGGACCTGATCCGCTATGGGAAAACCAAGTGTGGGGAGCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  270  CCGGGCTGCGCGATCTTCCAGGACCTGATCCGCTATGGGAAAACCAAGTGTGGGGAGCCG  329

Query  181  TCGCGCCCCGCCGCCTGCCGAGCCTTTGATGTCCCCAAGAGATATTTTTCCCACTTTTAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  330  TCGCGCCCCGCCGCCTGCCGAGCCTTTGATGTCCCCAAGAGATATTTTTCCCACTTTTAT  389

Query  241  ATCATCTCAGTGCTGTGGAATGGCTTCCTGCTTTGGTGCCTTACTCAATCTCTGTTCCTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  390  ATCATCTCAGTGCTGTGGAATGGCTTCCTGCTTTGGTGCCTTACTCAATCTCTGTTCCTG  449

Query  301  GGAGCACCTTTTCCAAGCTGGCTTCATGGTTTGCTCAGAATTCTCGGGGCGGCACAGTTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  450  GGAGCACCTTTTCCAAGCTGGCTTCATGGTTTGCTCAGAATTCTCGGGGCGGCACAGTTC  509

Query  361  CAGGGAGGGGAGCTGGCACTGTCTGCATTCTTAGTGCTAGTATTTCTGTGGCTGCACAGC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  510  CAGGGAGGGGAGCTGGCACTGTCTGCATTCTTAGTGCTAGTATTTCTGTGGCTGCACAGC  569

Query  421  TTACGAAGACTCTTCGAGTGCCTCTACGTCAGTGTCTTCTCCAATGTCATGATTCACGTC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  570  TTACGAAGACTCTTCGAGTGCCTCTACGTCAGTGTCTTCTCCAATGTCATGATTCACGTC  629

Query  481  GTGCAGTACTGTTTTGGACTTGTCTATTATGTCCTTGTTGGCCTAACTGTGCTGAGCCAA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  630  GTGCAGTACTGTTTTGGACTTGTCTATTATGTCCTTGTTGGCCTAACTGTGCTGAGCCAA  689

Query  541  GTGCCAATGGATGGCAGGAATGCCTACATAACAGGGAAAAATCTATTGATGCAAGCACGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  690  GTGCCAATGGATGGCAGGAATGCCTACATAACAGGGAAAAATCTATTGATGCAAGCACGG  749

Query  601  TGGTTCCATATTCTTGGGATGATGATGTTCATCTGGTCATCTGCCCATCAGTATAAGTGC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  750  TGGTTCCATATTCTTGGGATGATGATGTTCATCTGGTCATCTGCCCATCAGTATAAGTGC  809

Query  661  CATGTTATTCTCGGCAATCTCAGGAAAAATAAAGCAGGAGTGGTCA-TTCACTGTAACCA  719
            ||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
Sbjct  810  CATGTTATTCTCGGCAATCTCAGGAAAAATAAAGCAGGAGTGGTCATTTCACTGTAACCA  869

Query  720  CAGGATCCCATTTGG-AGACTGGTTTG--AATATGTTTC-TTCCCCTAACTACTTAGCAG  775
            || |||||||||||| ||||||| ||    |||||||||| ||||||||||||||||||||
Sbjct  870  CANGATCCCATTTGGAAGACTGGNTTNGAAATATGTTTCTTTCCCCTAACTACTTAGCAA  929

Query  776  AGCTGATGATCTACGTTT--CCATGGCCGTC-ACCTTTGGG  813
            | ||||||||||||||||  | | ||||||| |||||| ||
Sbjct  930  ANCTGATGATCTACGTTTTCCAAGGGCCGTCAACCTTTNGG  970
```

Figure 2. PCR sequence of the cloned *SRD5A3* plasmid matched with the published sequence of *SRD5A3*

3.2 SRD5A3 Transfection

The HEK293 cells were initially transfected with the generated *SRD5A3* expression vector. Immunoblot analysis was performed using anti-DDK, anti-SRD5A3 and anti-actin antibodies. The results confirmed the presence of the SRD5A3 protein band at 37 kDA using the anti-DDK antibody. However, no band was detected on the immunoblot using the anti-SRD5A3 antibody. The immunoblot was repeated by adding increasing amounts of protein lysates to successive wells. Using the anti-DDK antibody, the immunoblot results showed that with increasing amounts of material, there is a commensurate increase in the size of the 37 kDa band corresponding to the SRD5A3 protein (Figure 3). Following confirmation of the transient transfection of the *SRD5A3* vector, HEK293 cells underwent a stable transfection protocol. The integration of the *SRD5A3* vector in these stably transfected cells was confirmed by immunoblot.

Figure 3. SRD5A3 Immunoblot

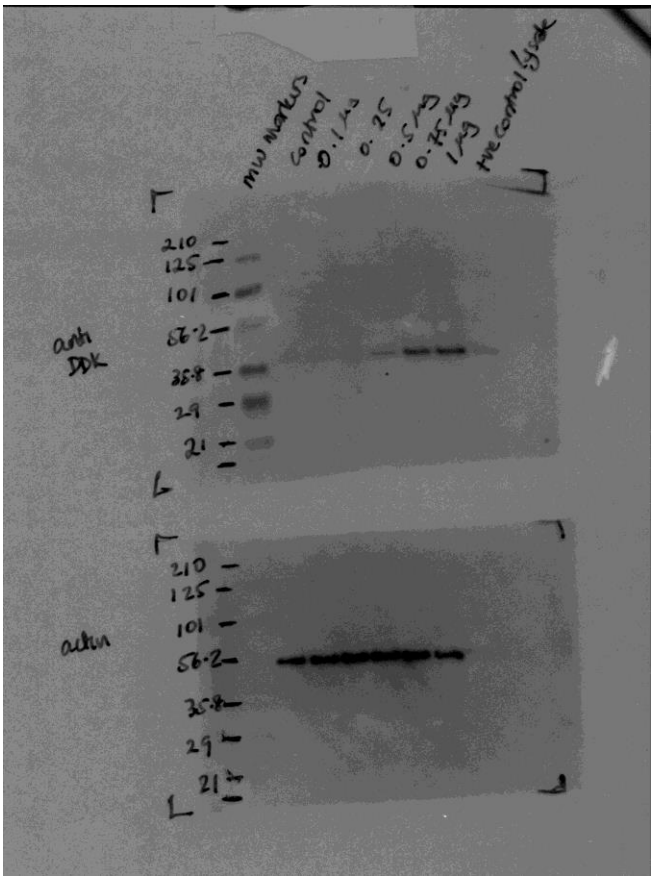

Figure 3. Immunoblot using anti-DDK antibody to detect the SRD5A3 protein. There is an increase in the size of the 37kDa band corresponding to SRD5A3 with increasing amounts of protein lysate loaded into the wells (Top panel). Actin control for the immunoblot (Bottom panel).

3.3 Estimation of SRD5A3 Protein Concentration

The amount of SRD5A3 present in transfection was evaluated at successive intervals following transfection. There was an increase in the optical density, and subsequently of the protein expression in the system over the first 72 hours, after which the expression started decreasing. The time point of 72 hours post transfection was identified to correspond to the highest SRD5A3 protein expression (Figure 4). This time point was used for all experiments to evaluate the enzymatic activity of SRD5A3 in steroid metabolism.

Figure 4. Estimation of SRD5A3 Protein Concentration

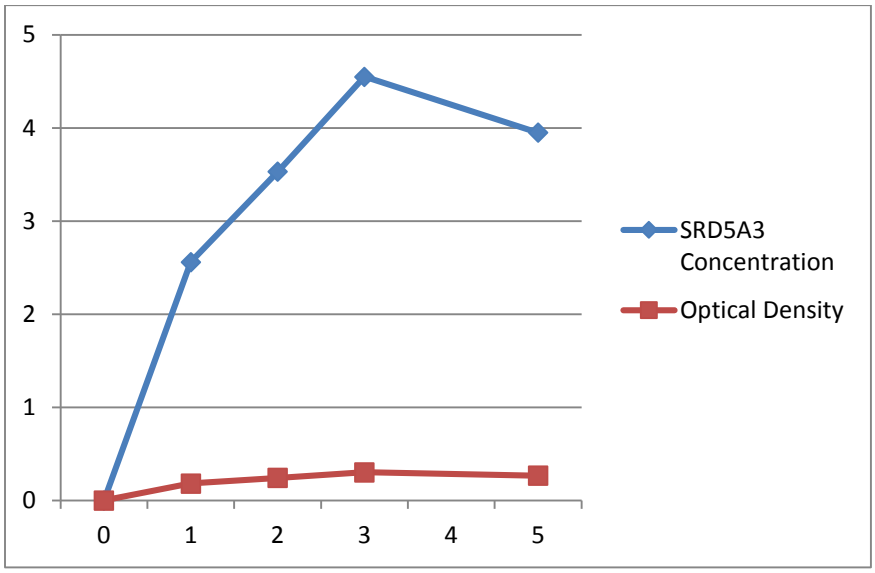

Figure 4. SRD5A3 protein concentration shows increasing protein expression after transfection (D0) that peaks around day 3.

Table 1. The increase in protein expression is measured by an increased in the optical density of the sample

DAY	OPTICAL DENSITY	CONCENTRATION (µg/µl)
0	0	0
1	0.182	2.561
2	0.242	3.532
3	0.302	4.55
4	N/A	N/A
5	0.267	3.95

3.4 Quantitative Mass Spectrometry

Liquid chromatography mass spectrometry analysis was used to determine the presence or absence of reduced products following the addition of C-13 labeled precursor steroids to HEK293 cells transfected with the SRD5A3 vector. Dutasteride, a known 5-alpha reductase 1 and 2 inhibitor, was added to each steroid precursor to determine its effect on the enzymatic activity of SRD5A3.

3.41. Testosterone

C-13 labeled testosterone at 1 and 10 µM was added to HEK293 control cells (non-transfected) and HEK293 transfected cells. C-13 labeled testosterone was converted to DHT only in the transfected system (Figure 5). Notably, the baseline steroid profile of non-transfected HEK293 cells without the addition of any steroids did not contain any DHT.

In addition, a standard curve for DHT using C-13 internal standards was created to quantify the amount of DHT produced following the addition of testosterone (Figure 6). The amount of DHT produced was quantified by mass spectrometry and showed a positive correlation with the amount of precursor C-13 labeled testosterone introduced. The addition of 1 µm C-13 labeled testosterone yielded 379.7 +/- 19.4 pg/mL of DHT; 10 µm C-13 labeled testosterone yielded 2129.7 +/- 177.4 pg/mL of DHT. These findings confirmed that the SRD5A3 enzyme catalyzes the conversion of testosterone to DHT.

Furthermore, we generated a cell free lysate of the HEK293 transfected cells, to which we added C-13 labeled testosterone. In this setting, we did not detect evidence of conversion of C-13 labeled testosterone to DHT by mass spectrometry (Figure 7).

The addition of 1 nM dutasteride completely inhibited the conversion of C-13 labeled testosterone to DHT. DHT was not detected by mass spectrometry following the addition of 1 µm or 10 µm of C-13 labeled testosterone (Figures 8, 9).

Figure 5. Conversion of Testosterone to DHT by SRD5A3

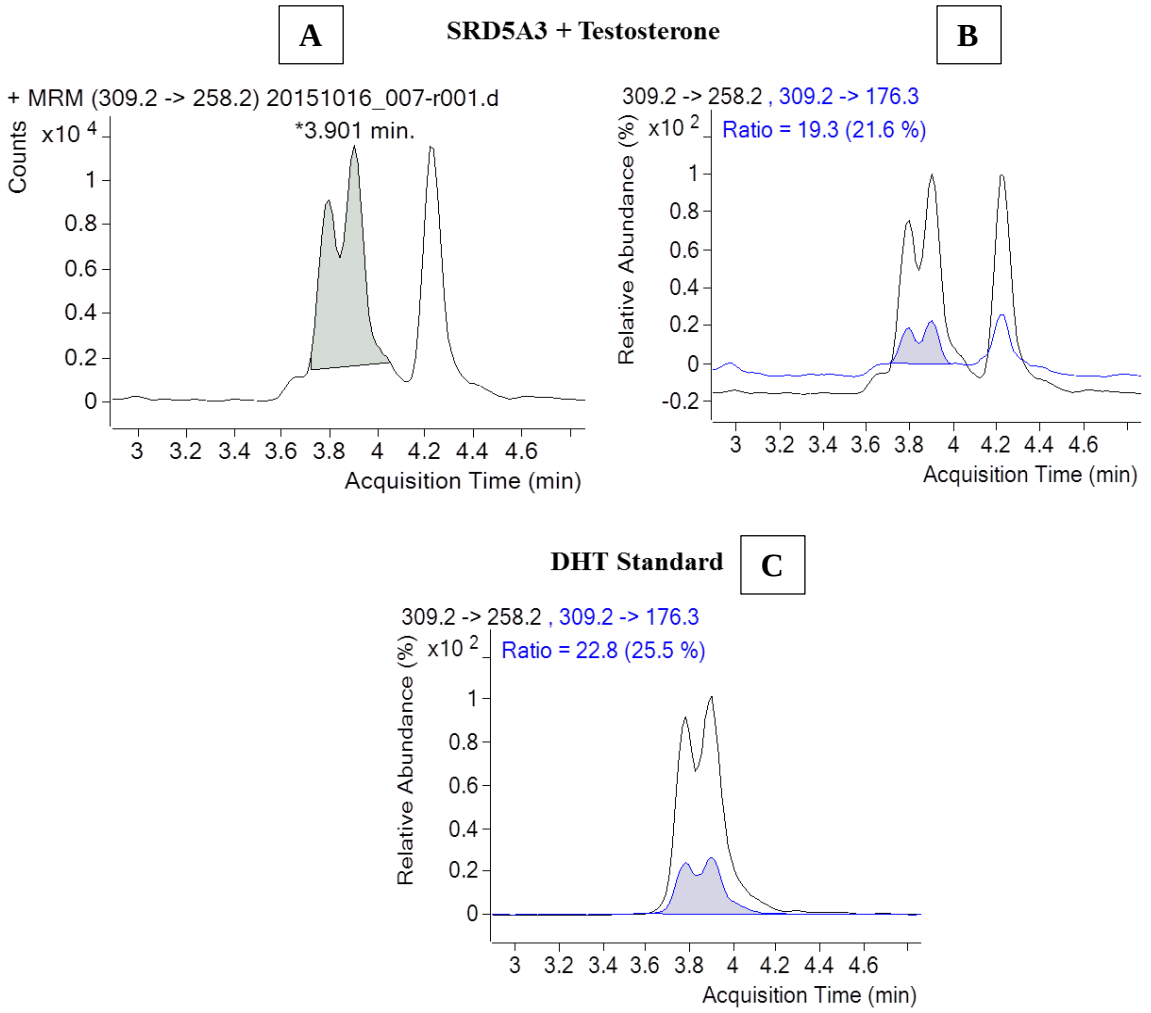

Figure 5. HEK293 cells transfected with SRD5A3 vector reduced testosterone to DHT. Testosterone added to HEK293 cells showed that DHT was detected and quantified using mass spectrometry. The double peak eluting at 3.901 minutes (Panel A) was fragmented into a qualifier and quantifier ion (Panel B). The DHT retention time, ratio of qualifier/quantifier ion corresponds to results obtained with the DHT internal standard (Panel C).

Figure 6. DHT Standard Curve

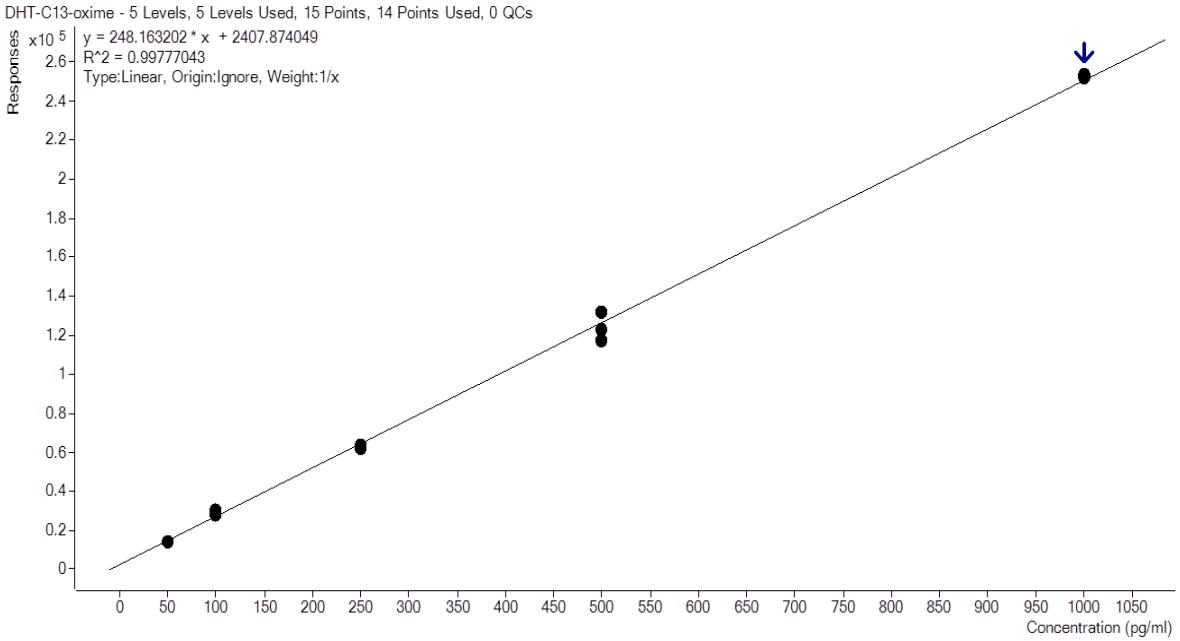

Figure 6. DHT standard curve was created using the following DHT concentrations: 50, 100, 250, 500, and 1000 pg/mL.

Figure 7. Conversion of Testosterone to DHT by SRD5A3 Cell Lysate

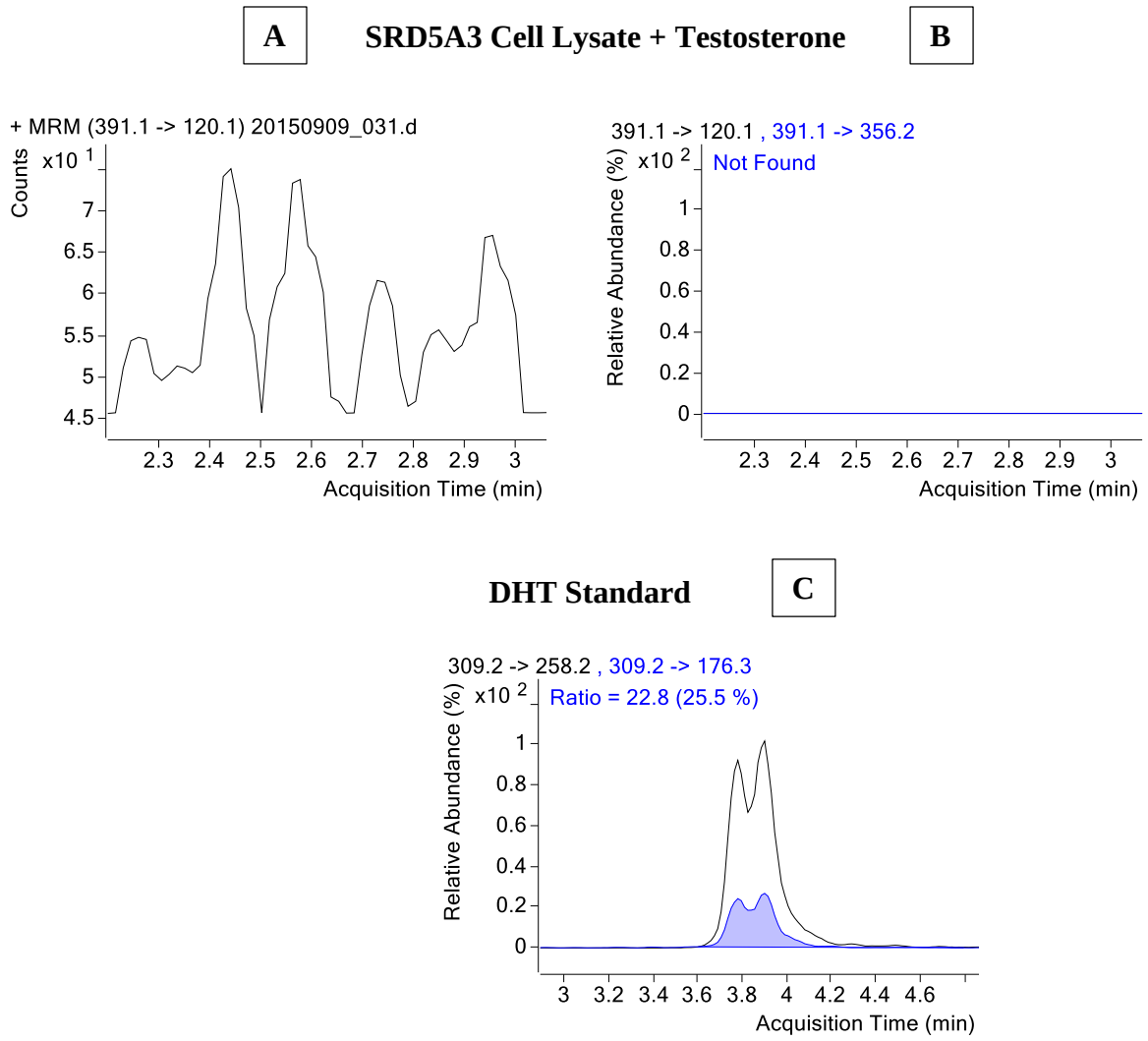

Figure 7. HEK293 cell lysate did reduce testosterone to DHT. The addition of testosterone to HEK293 cells did not result in the detection of DHT by mass spectrometry (Panels A, B). The DHT retention time, ratio of qualifier/quantifier ion, does not correspond to results obtained with the DHT internal standard (Panel C).

Figure 8. Conversion of Testosterone to DHT with Addition of Dutasteride

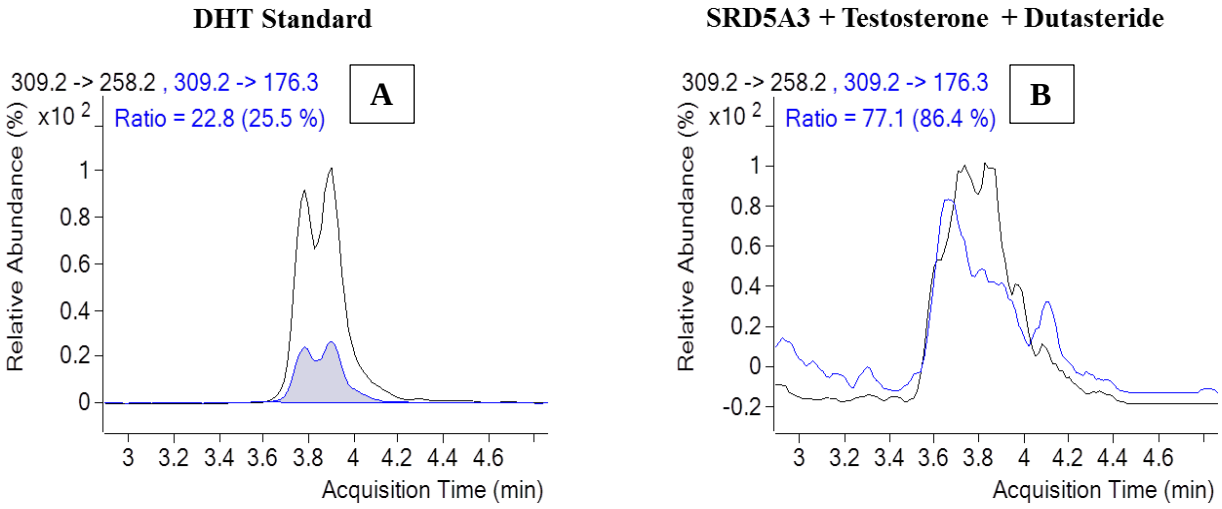

Figure 8. DHT internal standard showing a double peak at an elution time of 3.901 minutes (Panel A). No DHT peak was detected following the addition of 10µM testosterone with 1 nM dutasteride to HEK293 cells transfected with the *SRD5A3* expression vector.

Figure 9. SRD5A3 Protein Inhibition by Dutasteride

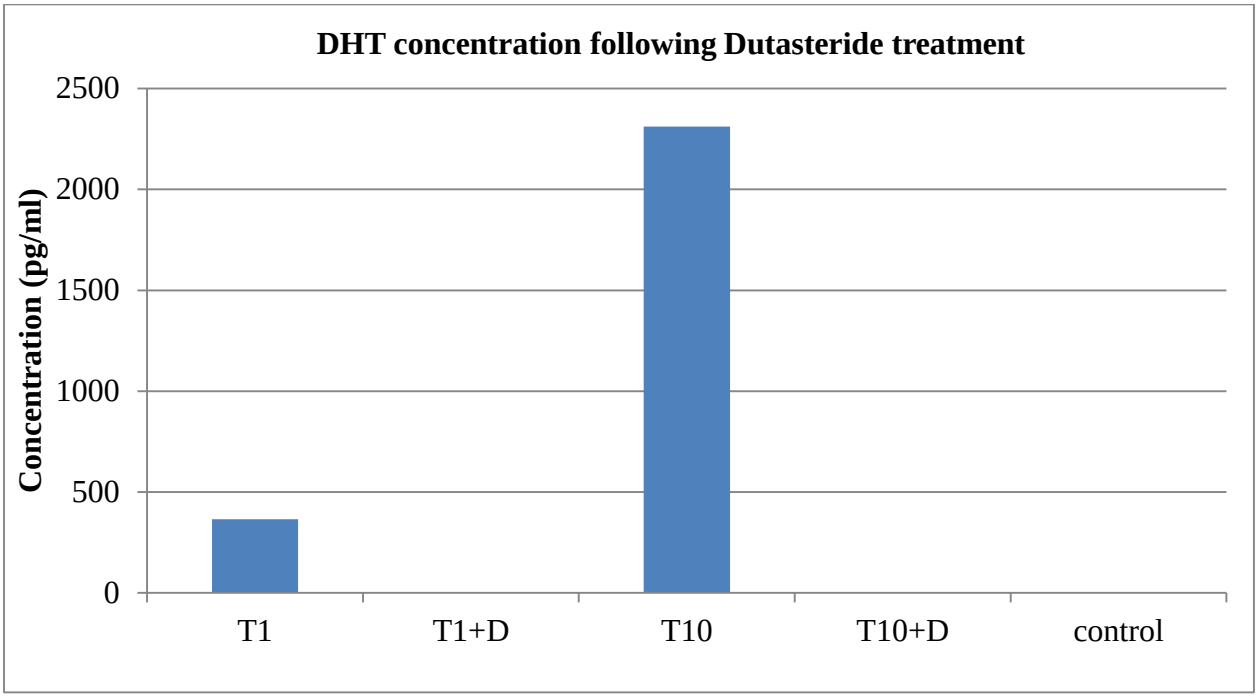

Figure 9. The addition of testosterone to HEK293 cells transfected with the *SRD5A3* vector resulted in the production of DHT. The addition of 10 µM of testosterone (T10) resulted in a significantly higher amount of DHT detected compared to 1 µM of testosterone (T1). The addition of dutasteride (D) to T1 and T10 inhibited the conversion of testosterone to DHT, and no DHT was detected or quantified. The control cells had no detectable DHT.

3.42. Progesterone

C-13 labeled progesterone at 1 and 10 µM was added to HEK 293 transfected cells. The conversion of progesterone to its 5-alpha reduced product, dihydroprogesterone, was not detected at either concentration by mass spectrometry (Figure 10).

Figure 10.Conversion of Progesterone to Dihydroprogesterone by SRD5A3

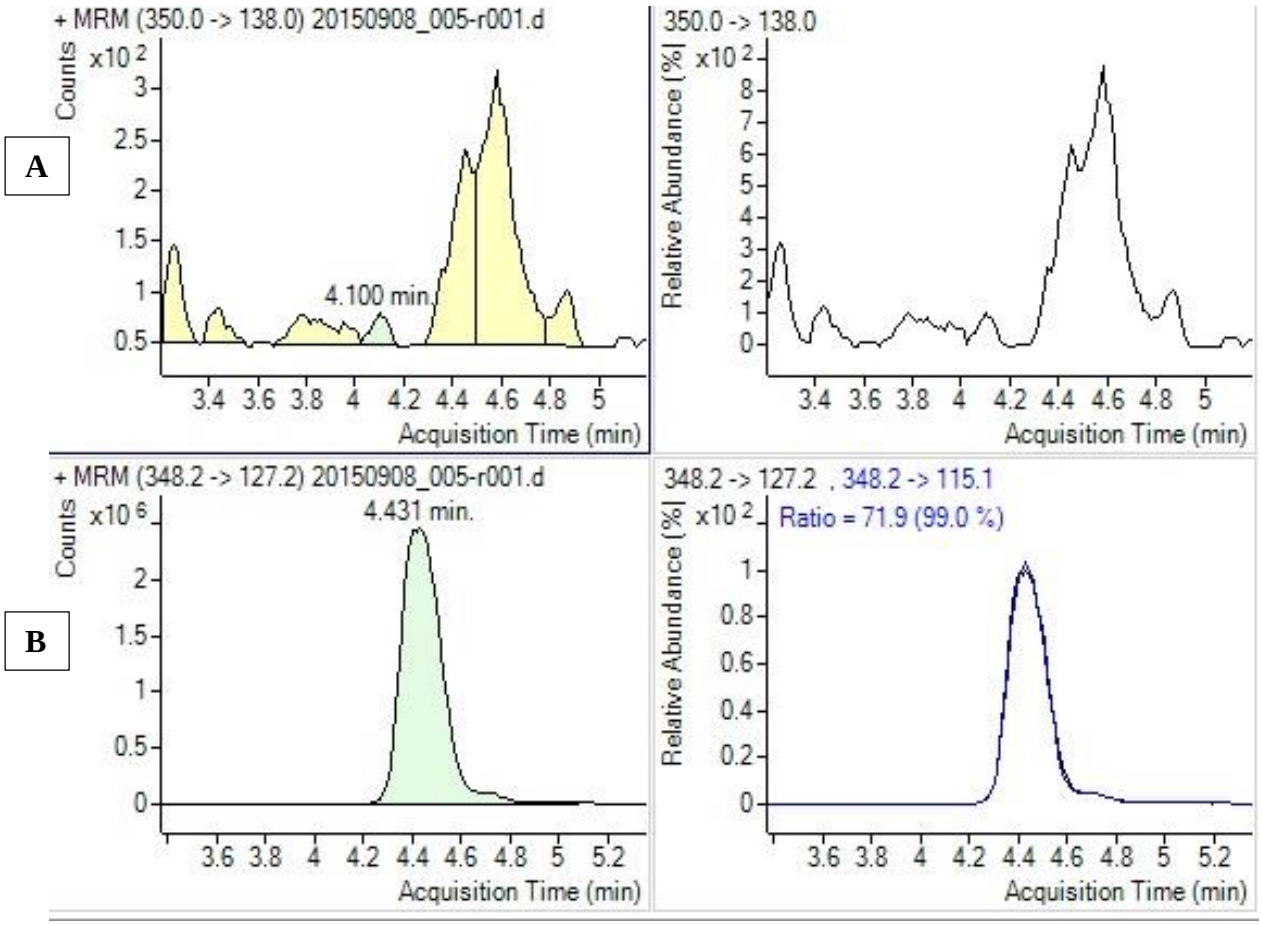

Figure 10. HEK293 cells transfected with SRD5A3 vector did not convert progesterone to the 5-alpha reduced product, dihydroprogesterone. The addition of progesterone to HEK293 cells did not result in the detection of dihydroprogesterone (Panel A). The internal standard for progesterone shows a peak eluting at 4.2-4.6 minutes (Panel B).

3.43. Androstenedione

C-13 labeled androstenedione at 1 and 10 µM was added to HEK 293 transfected cells. Mass spectrometry analysis did not detect the conversion of androstenedione to its 5-alpha reduced product, androstanedione, at either concentration. (Figure 11).

Figure 11. Conversion of Androstenedione to Androstanedione by SRD5A3

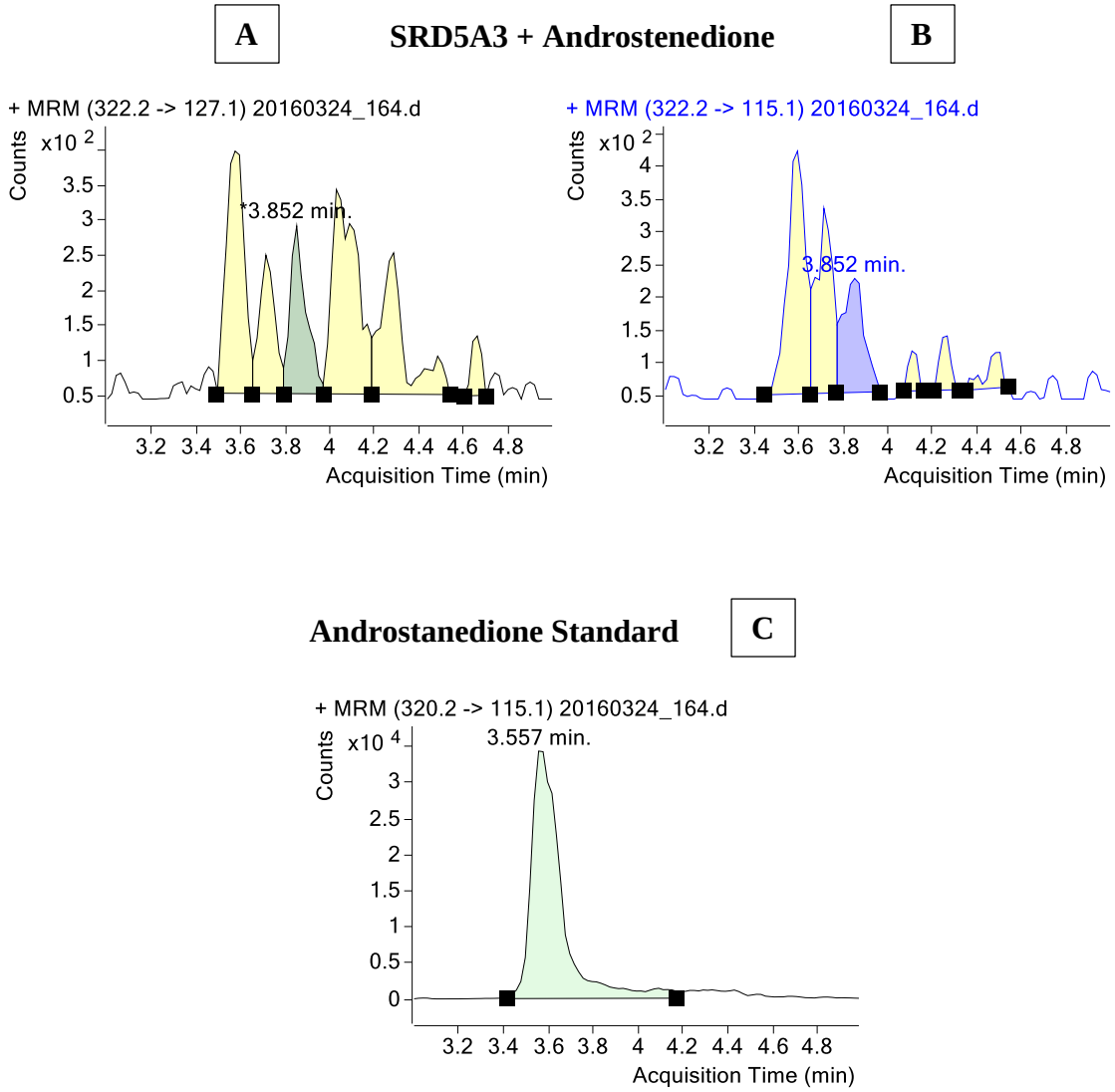

Figure 11. HEK293 cells transfected with SRD5A3 vector did not convert androstenedione to the 5-alpha reduced product, androstanedione. The androstanedione retention time (Panel A), qualifier and quantifier ratio (Panel B), does not correspond to the androstanedione standard (Panel C).

3.44. Epitestosterone

Epitestosterone at 1 and 10 µM was added to HEK293 transfected cells. Mass spectrometry analysis revealed a peak associated with the formation of the 5-alpha reduced product of epitestosterone at both concentrations. This is based on the correct transition similar to testosterone, and correct retention time based on the testosterone to DHT standard available (Figure 12). The addition of 10 µM epitestosterone resulted in a two-fold increase in the conversion to the reduced product compared to the addition of 1 µM of epitestosterone. The addition of dutasteride significantly decreased the conversion of epitestosterone to its 5-alpha reduced product at both 1 µM and 10 µM concentrations ($p<0.05$) (Figure 13).

Figure 12. 5-alpha Reduction of Epitestosterone by SRD5A3

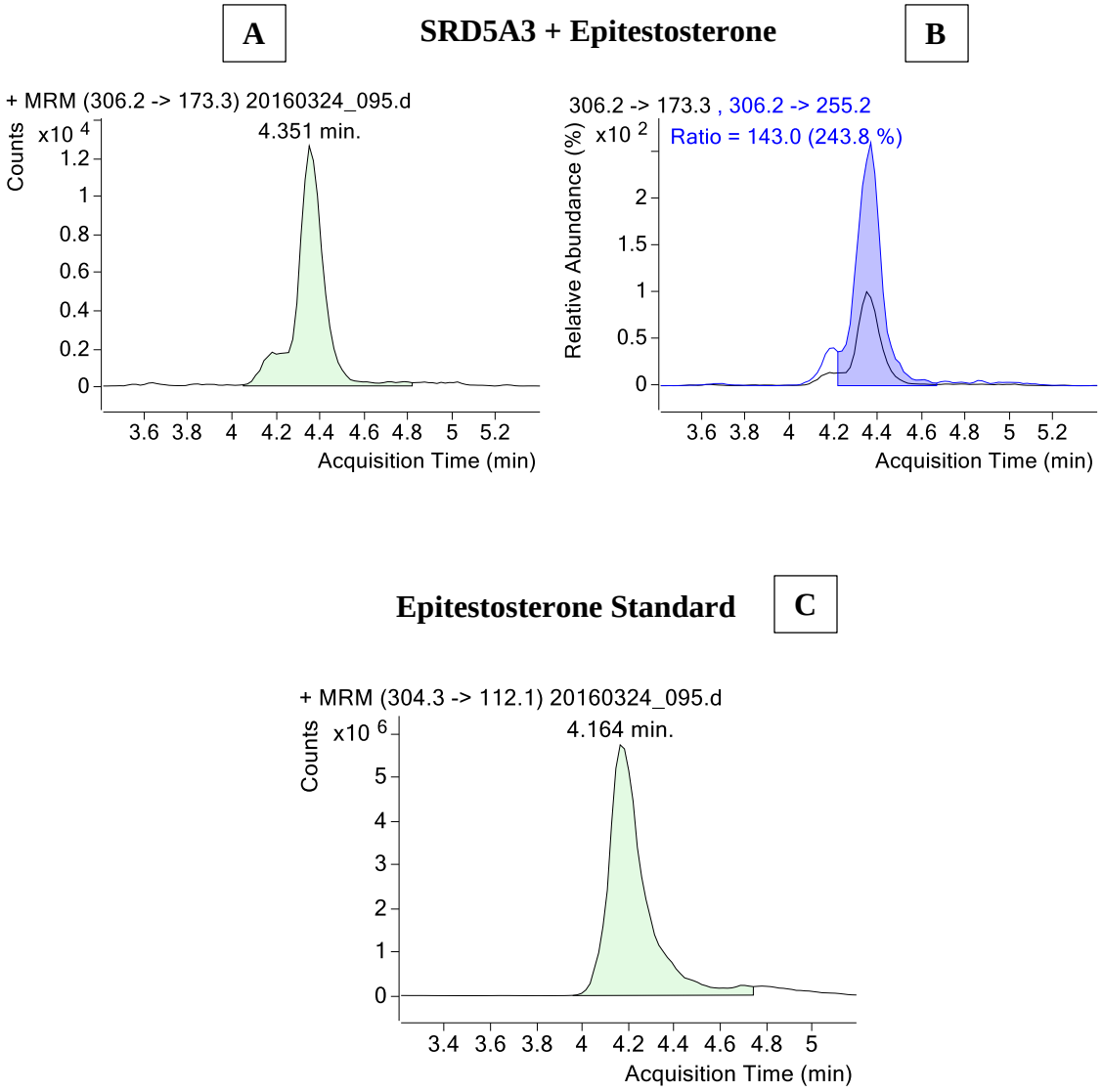

Figure 12. HEK293 cells transfected with SRD5A3 vector did convert epitestosterone to the 5-alpha reduced product. The resulting 5–alpha reduced product was detected and quantified using mass spectrometry. The peak eluting at 4.351 minutes (Panel A) was fragmented into a qualifier and quantifier ion (Panel B). The epitestosterone standard is shown in Panel C, with a peak eluting at 4.164 minutes.

Figure 13. SRD5A3 Protein Inhibition by Dutasteride – Epitestosterone

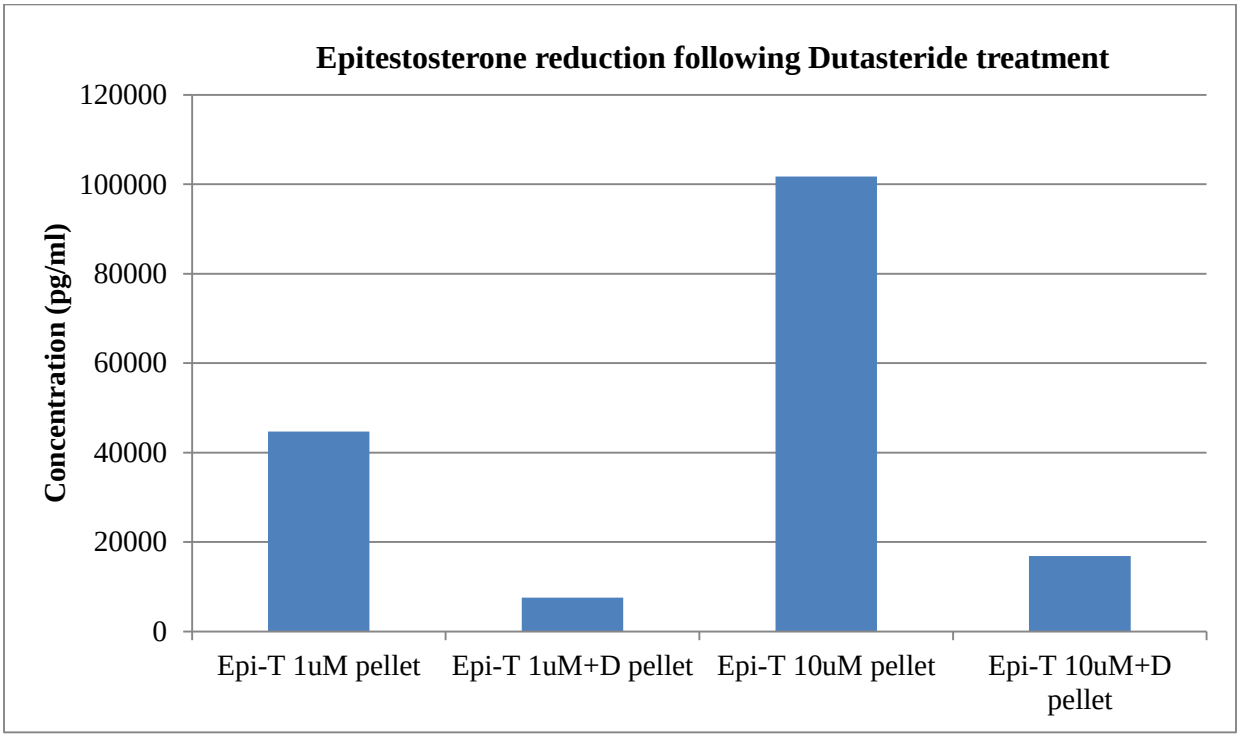

Figure 13. The addition of epitestosterone to HEK293 cells transfected with the *SRD5A3* vector resulted in the production of the corresponding 5-alpha reduced product. The addition of 10 µM of epitestosterone resulted in a higher amount of product detected compared to 1 µM of epitestosterone . The addition of dutasteride (D) to 1 and 10 µM of epitestosterone resulted in a significant decrease in the conversion of epitestosterone to its 5-alpha reduced product.

3.45. 6-β-hydroxytestosterone

6-β-hydroxytestosterone at 1 and 10 µM was added to HEK293 transfected cells. Mass spectrometry analysis revealed a peak associated with the formation of the 5-alpha reduced product of 6-β-hydroxytestosterone (Figure 14). There was a two-fold increase in the amount 5-alpha reduced product formed between the 1 µM and 10 µM concentrations of 6-β-hydroxytestosterone added. The addition of dutasteride significantly decreased the reduction of 6-β-hydroxytestosterone at 1 µM concentration ($p<0.05$), but not at 10 µM concentration ($p>0.05$) (Figure 15).

Figure 14. 5-alpha Reduction of 6-β-hydroxytestosterone by SRD5A3

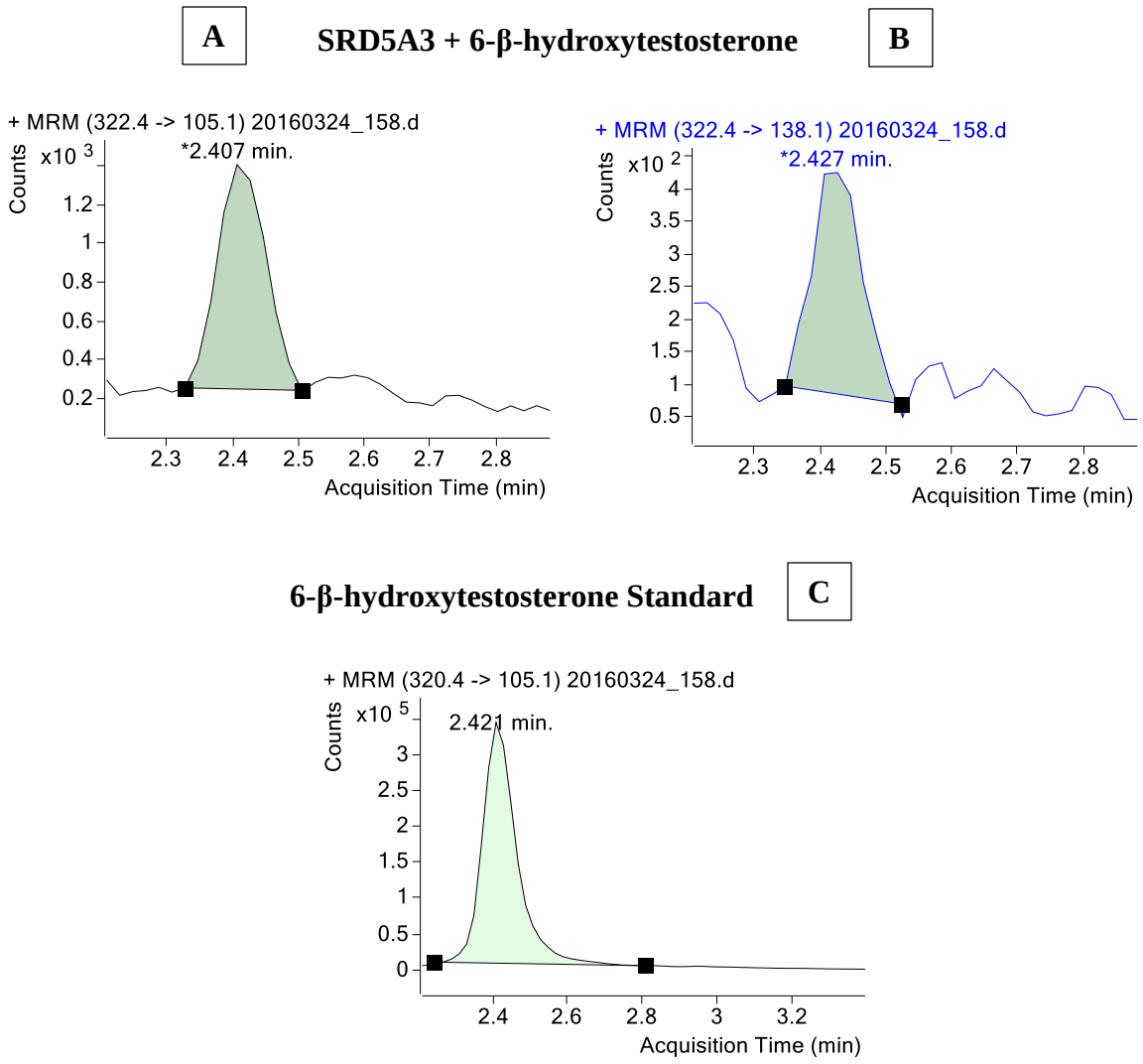

Figure 14. HEK293 cells transfected with SRD5A3 did convert 6-β-hydroxytestosterone to the 5-alpha reduced product. The resulting 5–alpha reduced product was detected and quantified using mass spectrometry. The peak eluting at 2.407 minutes (Panel A) was fragmented into a qualifier and quantifier ion (Panel B). The 6-β-hydroxytestosterone standard is shown in Panel C, with a peak eluting at 2.421 minutes.

Figure 15. SRD5A3 Protein Inhibition by Dutasteride - 6-β-hydroxytestosterone

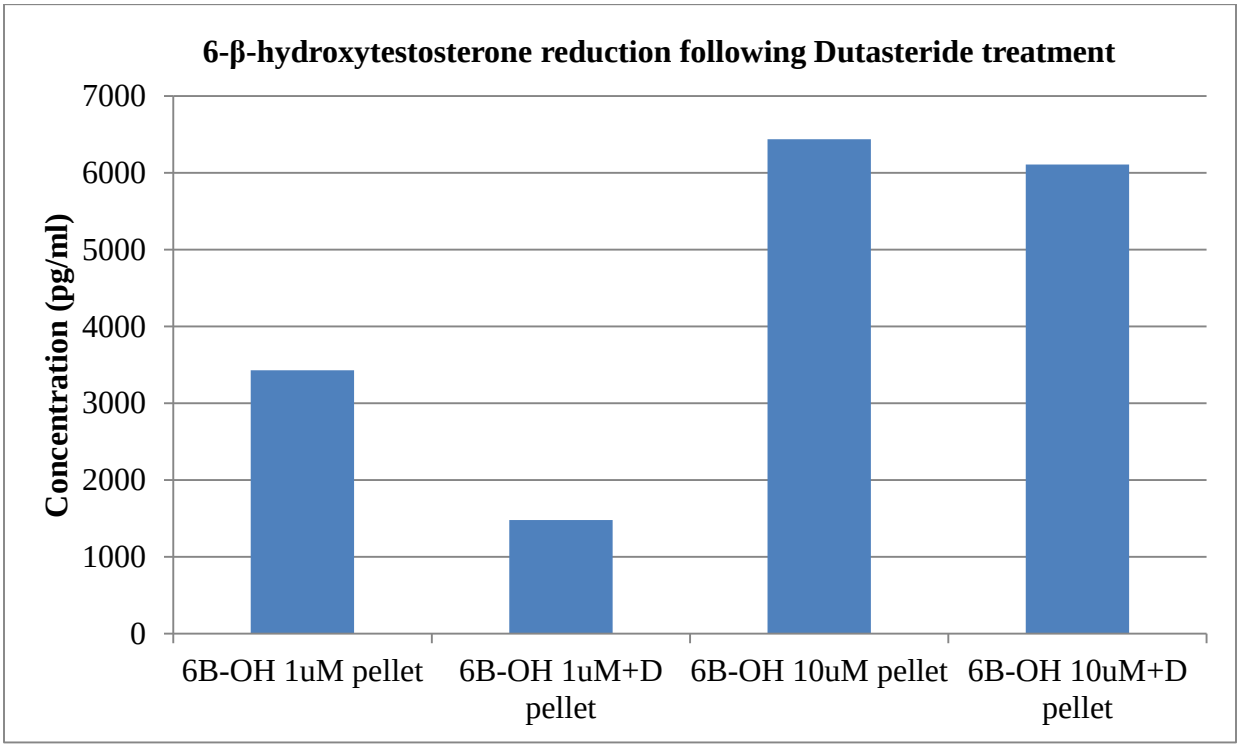

Figure 15. The addition of 6-β-hydroxytestosterone to HEK293 cells transfected with the *SRD5A3* vector resulted in the production of the corresponding 5-alpha reduced product. The addition of 10 µM of 6-β-hydroxytestosterone resulted in a higher amount of product detected compared to 1 µM of 6-β-hydroxytestosterone. The addition of dutasteride (D) significantly decreased the reduction of 6-β-hydroxytestosterone at 1 µM, but not at 10 µM.

3.46. 11-β-hydroxytestosterone

11-β-hydroxytestosterone at 1 and 10 µM was added to HEK293 transfected cells. Mass spectrometry analysis revealed a peak associated with the formation of the 5-alpha reduced product of 11-β-hydroxytestosterone (Figure 16). There is an almost three-fold increase in the amount of 5-alpha reduced product formed between the 1 µM and 10 µM concentrations of 11-β-hydroxytestosterone. The addition of dutasteride (D) significantly decreased the reduction of 11-β-hydroxytestosterone at 1 µM concentration ($p<0.05$), but not at 10 µM concentration ($p>0.05$) (Figure 17).

Figure 16. 5-alpha Reduction of 11-β-hydroxytestosterone by SRD5A3

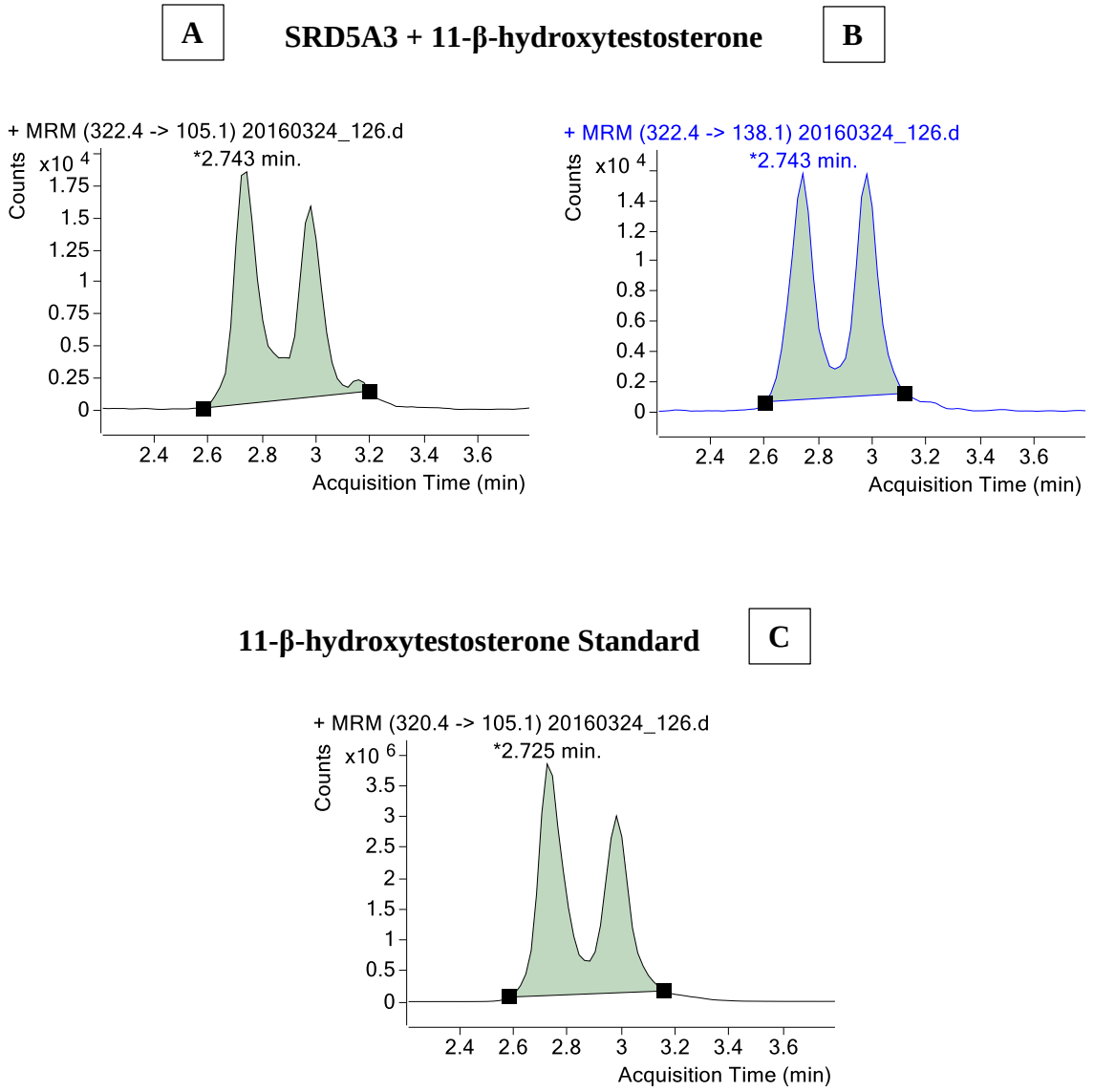

Figure 16. HEK293 cells transfected with SRD5A3 vector did convert 11-β-hydroxytestosterone to the 5-alpha reduced product. The resulting 5–alpha reduced product was detected and quantified using mass spectrometry. The peak eluting at 2.743 minutes (Panel A) was fragmented into a qualifier and quantifier ion (Panel B). The 11-β-hydroxytestosterone standard is shown in Panel C, with a peak eluting at 2.725 minutes.

Figure 17. SRD5A3 Protein Inhibition by Dutasteride - 11-β-hydroxytestosterone

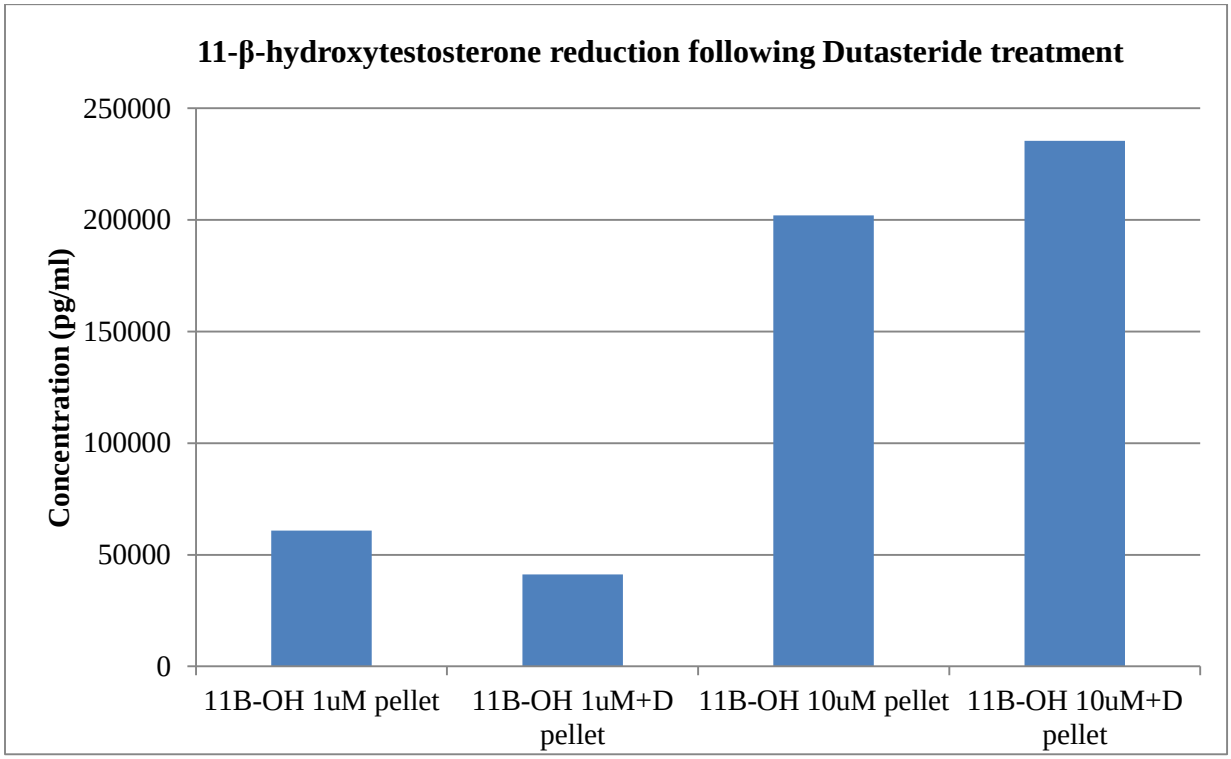

Figure 17. The addition of 11-β-hydroxytestosterone to HEK293 cells transfected with the *SRD5A3* vector resulted in the production of the corresponding 5-alpha reduced product. The addition of 10 µM of 11-β-hydroxytestosterone resulted in a higher amount of product detected compared to 1 µM of 11-β-hydroxytestosterone. The addition of dutasteride (D) significantly decreased the reduction of 11-β-hydroxytestosterone at 1 µM, but not at 10 µM.

3.47. 11-α-hydroxytestosterone

11-α-hydroxytestosterone at 1 and 10 µM was added to HEK293 transfected cells. Mass spectrometry analysis revealed a peak associated with the formation of the 5-alpha reduced product of 11-β-hydroxytestosterone (Figure 18). The addition of 10 µM of 11-α-hydroxytestosterone resulted in an increased quantity of reduced product formed. The addition of dutasteride did not decrease 5-alpha reduced product formation at either 1 or 10 µM of 11-α-hydroxytestosterone.

Figure 18. 5-alpha Reduction of 11-α-hydroxytestosterone by SRD5A3

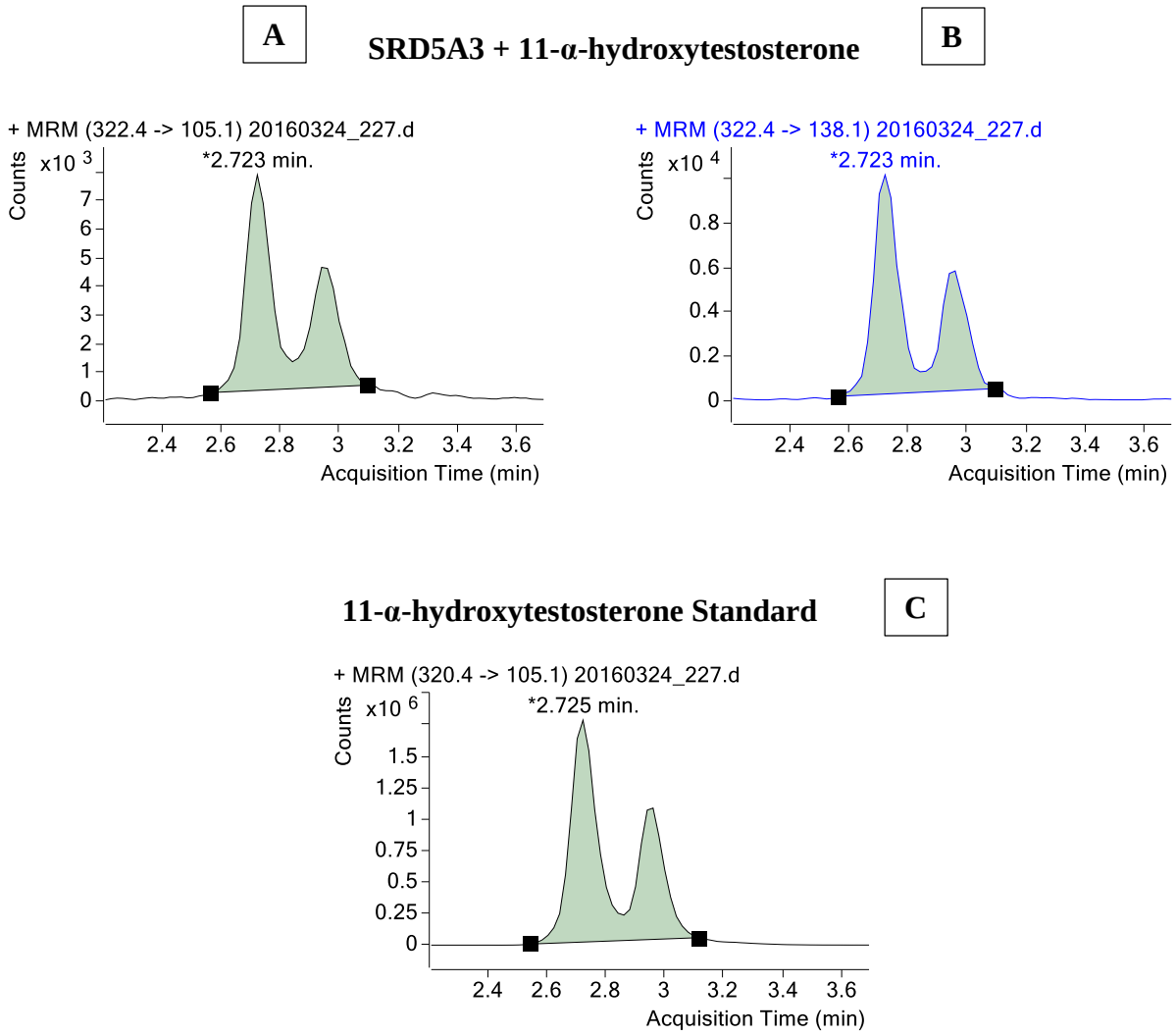

Figure 18. HEK293 cells transfected with SRD5A3 vector did convert 11-α-hydroxytestosterone to the 5-alpha reduced product. The resulting 5–alpha reduced product was detected and quantified using mass spectrometry. The peak eluting at 2.723 minutes (Panel A) was fragmented into a qualifier and quantifier ion (Panel B). The 11-α-hydroxytestosterone standard is shown in Panel C, with a peak eluting at 2.725 minutes.

Figure 19. SRD5A3 Protein Inhibition by Dutasteride - 11-α-hydroxytestosterone

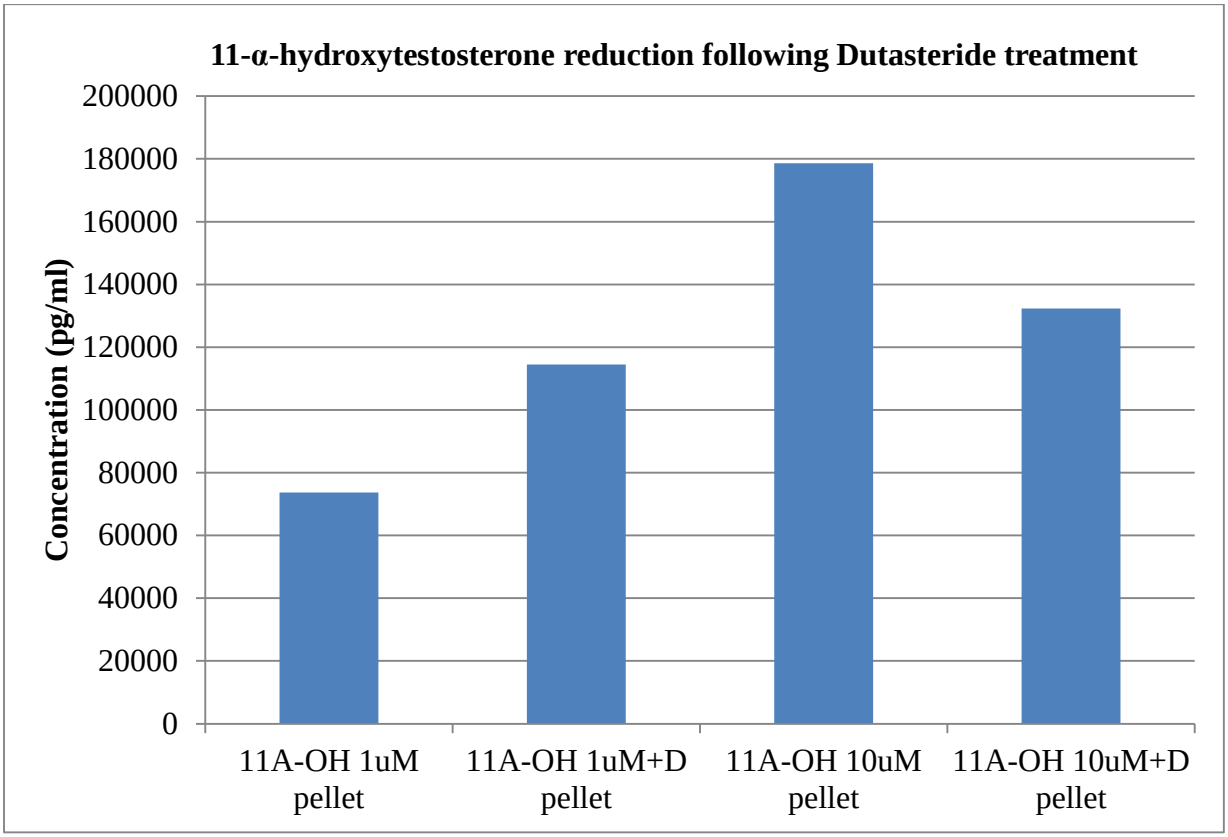

Figure 19. The addition of 11-α-hydroxytestosterone to HEK293 cells transfected with the *SRD5A3* vector resulted in the production of the corresponding 5-alpha reduced product. The addition of 10 µM of 11-α-hydroxytestosterone resulted in a higher amount of product detected compared to 1 µM of 11-α-hydroxytestosterone. The addition of dutasteride (D) did not consistently affect the conversion of 11-α-hydroxytestosterone to the 5-alpha reduced product.

3.48. 11-ketotestosterone

11-ketotestosterone at 1 and 10 µM was added to HEK293 transfected. Mass spectrometry analysis revealed a peak corresponding to 11-ketoDHT, the 5-alpha reduced product of 11-ketotestosterone (Figure 20). The addition of dutasteride to both the 1 and 10 µM concentrations of 11-ketotestosterone did not result in a significant difference in the amount of 11-ketoDHT formed (Figure 21).

Figure 20- 5-alpha Reduction of 11-ketotestosterone by SRD5A3

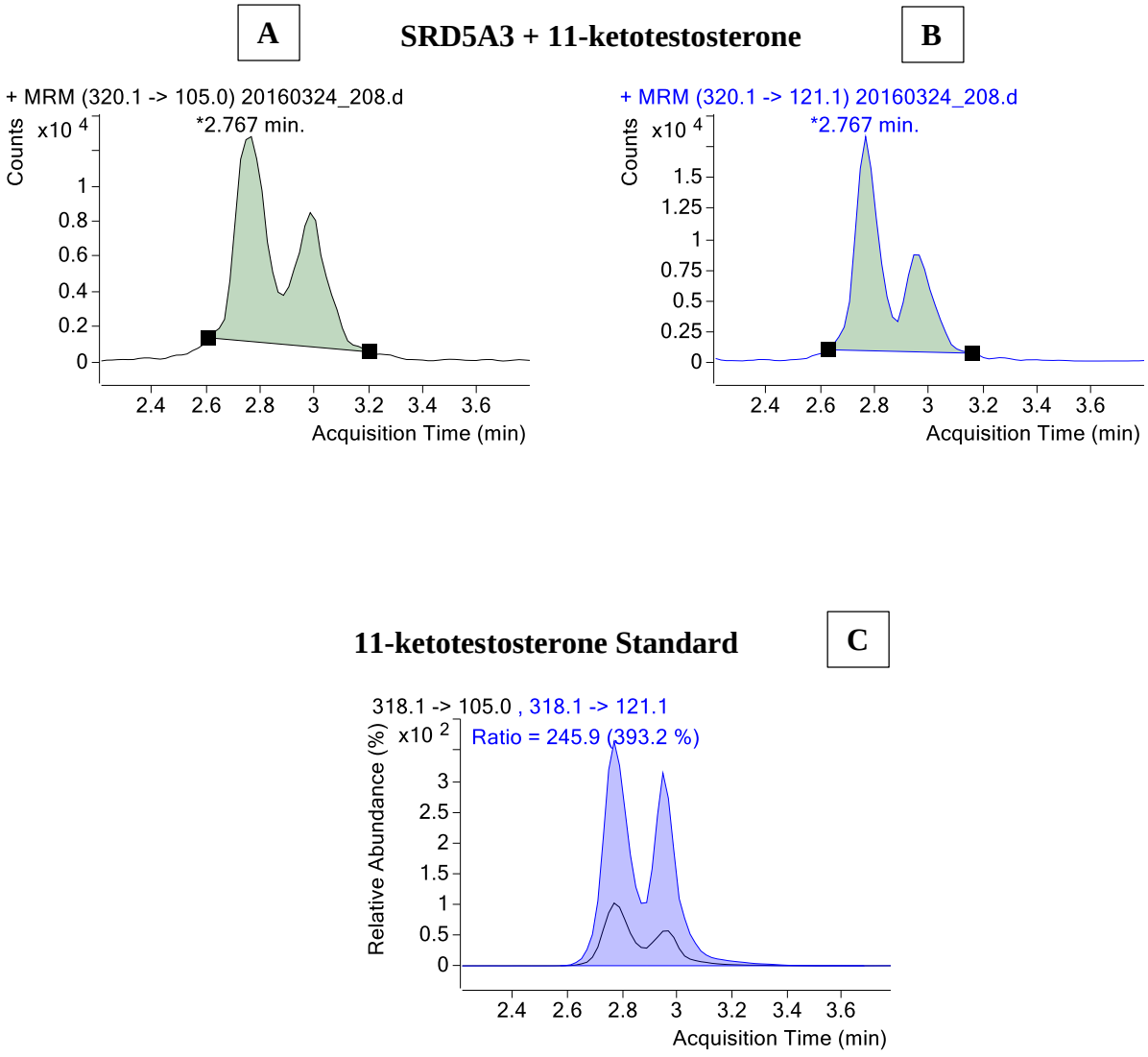

Figure 18. HEK293 cells transfected with SRD5A3 vector did convert 11-ketotestosterone to the 5-alpha reduced product, 11-ketoDHT. 11-ketoDHT was detected and quantified using mass spectrometry. The peak eluting at 2.767 minutes (Panel A) was fragmented into a qualifier and quantifier ion (Panel B). The 11-ketotestosterone standard is shown in Panel C.

Figure 21. SRD5A3 Protein Inhibition by Dutasteride - 11-ketotestosterone

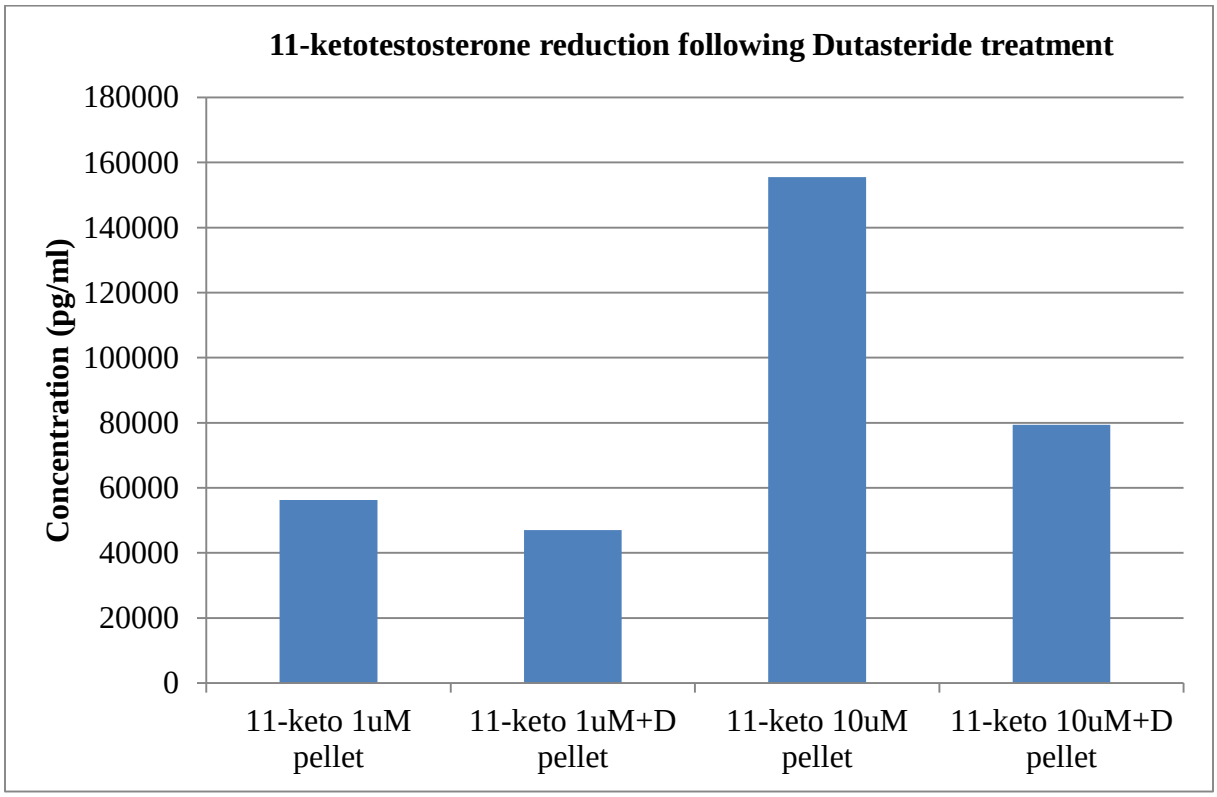

Figure 19. The addition of 11-ketotestosterone to HEK293 cells transfected with the *SRD5A3* vector resulted in the production of the corresponding 5-alpha reduced product, 11-ketoDHT. The addition of 10 µM of 11-ketotestosterone resulted in a higher amount of product detected compared to 1 µM of 11-ketotestosterone. The addition of dutasteride (D) did not significantly change the amount of 11-ketoDHT produced at 1 µM, but did significantly decrease the conversion at 10 µM of 11-ketotestosterone.

CHAPTER 4: DISCUSSION

The 5-alpha reductase enzyme family plays an important role in the conversion of steroid precursors to their 5-alpha reduced products, such as the conversion of testosterone to the more potent androgen, DHT (16, 53). Androgen deprivation therapy aimed at reducing levels of circulating testosterone, and subsequently DHT, is well established as first line therapy for prostate cancer (54). 5-alpha reductase inhibitors, such as finasteride and dutasteride, have the capacity of inhibiting the conversion of testosterone to DHT (55). However, despite showing activity in reducing the incidence of prostate cancer development in preventive studies, the role of 5-alpha reductase inhibitors in prostate cancer prevention remains controversial (3, 9, 13). In addition, 5-alpha reductase inhibitors are not currently included in the treatment guidelines for prostate cancer, as they have not shown convincing clinical activity in this setting (2). The distribution and function of 5-alpha reductase 1 and 2 have been well characterized in the normal prostate as well as prostate cancer (4). This study aimed at shedding light on the enzymatic activity of a newer member of the 5-alpha reductase family of enzymes, SRD5A3, in steroidogenesis and prostate carcinogenesis.

SRD5A3 cDNA ORF was transfected into HEK293 cells using the OriGene vector system. This is different from the HP-Thioredoxin-fusion SRD5A3 protein using a pBAD/Thio-TOPO vector used by Titus et al. (51), and the FuGENE 6 vector used by Uemura et al.(5). Using this vector, the SRD5A3 plasmid was successfully transfected into HEK293 cells. Transient and stable transfections were verified by immunoblot assays using an anti-DDK antibody revealing a band at 37 kDa, corresponding to the SRD5A3 protein (Figure 3). Conversely, the anti-SRD5A3 antibody did not yield a positive immunoblot result, despite the addition of increasing concentrations of SRD5A3 into the immunoblot wells. These results

suggest that the anti-DDK antibody may be a more sensitive tool to detect successfully SRD5A3 transfection using the OriGene vector with an anti-DDK tag.

Mass spectrometry analysis revealed that HEK293 transfected cells did convert C-13 labeled testosterone to DHT (Figure 5). This activity was concentration dependent, with higher levels of testosterone yielding a higher DHT levels (Figure 9). This report confirms prior observations by Uemura et al. using a different transfection system (5), and Titus et al. using a fusion SRD5A3 protein (51). The conversion of C-13 labeled testosterone to DHT was only observed in intact HEK293 transfected cells. Mass spectrometry analysis of a cell lysate of HEK293 transfected cells did not detect any DHT production following the addition of C-13 labeled testosterone (Figure 7). This is in contrast to the study by Titus et al. where HP-Thioredoxin-fusion SRD5A3 protein using a pBAD/Thio-TOPO vector showed 5-alpha reducing activity in both intact cells and cell lysate (51). Conversely, another study evaluated hamster SRD5A3 expressed as a fusion protein with green fluorescent protein from the pcDNA3.1-NT-GFP/hSrd5a3 plasmid and human SRD5A3. In that study, the fusion protein localized to the cytoplasm and did not show steroid reducing capacity (52). These findings in addition to the known role of SRD5A3 role in in N-linked glycosylation of surface proteins (45), suggest that SRD5A3 exerts its 5-alpha reducing activity mainly when intact in the plasma membrane.

This study also aimed at evaluating the ability of SRD5A3 to reduce testosterone analogues. The development of castrate resistant prostate cancer despite low testosterone levels has generated interest in looking for other steroid substrates able to active the androgen receptor under castrate conditions. Castrate resistant prostate cancer appears to possess the machinery to bypass testosterone and use adrenal androgens, such as dehydroepiandrosterone and androstenedione, to produce intratumoral DHT through a “backdoor steroid pathway” (16, 20,

56). Additionally, more recent studies have expanded the list of adrenal androgens that may potentially be involved in the development of castrate resistant prostate cancer (31, 57). 11-hydroxyandrostenedione is an abundant adrenal androgen which has limited ability to activate the androgen receptor. Through the action of 17β-hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase enzymes, 11-hydroxyandrostenedione is converted to 11-ketotestosterone. Subsequently, 11-ketotestosterone is 5-alpha reduced to 11-ketoDHT, a steroid as potent as DHT in its ability to active the androgen receptor (31, 57). These studies have shed light on the role of 5-alpha reductases 1 and 2 in this "backdoor steroid pathway". However, the role of SRD5A3 in this setting has not been studied yet. In our study, the addition of testosterone analogues (epitestosterone, 11-hydroxytestosterone, 6-β-hydroxytestosterone, 11-β-hydroxytestosterone, 11-α-hydroxytestosterone, and 11-ketotestosterone) resulted in the production of the respective 5-alpha reduced counterparts. These findings, in addition to SRD5A3 being almost ubiquitous in the body (49, 50), suggests that this enzyme may be involved in the conversion of circulating steroid precursors to more potent androgens capable of activating the androgen receptor. Developing a model containing SRD5A3, the androgen receptor gene, as well as linked reporter assay such as luciferase would demonstrate activation of the androgen receptor. In addition, such a reporter assay can potentially allow quantification of the androgen receptor activation depending on the precursor steroid added to the system.

Additionally, SRD5A3 is upregulated under androgen deprivation therapy (32), potentially magnifying its role in the reduction of circulating androgens capable of binding the androgen receptor to activate gene targets and downstream signaling. This potentially implicates SRD5A3 in the non-canonical steroid pathway which is hypothesized to drive castrate resistant prostate cancer even in the presence of castrate levels of testosterone. Mass spectrometry

analysis can be performed to compare the levels of circulating adrenal androgens between castrate-sensitive and castrate-resistant prostate cancer samples, in the setting of castrate levels of testosterone. Circulating adrenal androgens levels, along with their respective potencies in activating the androgen receptor, can be helpful in determining their role in driving androgen receptor signaling.

These findings, along with previous studies linking adrenal androgens to the production of potent androgen receptor ligands suggest that measuring peripheral blood testosterone levels alone may not fully reflect the circulating steroid burden that can potentially drive prostate cancer growth. A more accurate assessment of castrate androgen levels may be obtained by evaluating a panel of circulating androgens shown to bind and androgen receptor signaling.

Furthermore, this study aimed to evaluate the effect of dutasteride, a known 5-alpha reductase 1 and 2 inhibitor, on the enzymatic activity of SRD5A3. Titus et al. previously reported that dutasteride partially inhibited the conversion of testosterone to DHT in a HP-Thioredoxin-fusion SRD5A3 enzyme system (51). Using an SRD5A3 cDNA ORF transfected into HEK293 cells using the OriGene vector system, our findings suggest that the inhibitory effect of dutasteride on SRD5A3 is substrate dependent. Dutasteride completely inhibited the conversion of testosterone to DHT at both 1 and 10 μM concentrations of testosterone. When added to epitestosterone, dutasteride significantly decreased but did not completely eliminate the conversion of epitestosterone to the 5-alpha reduced product at both the 1 and 10 μM concentrations. For 6-β-hydroxytestosterone and 11-β-hydroxytestosterone, dutasteride significantly decreased the conversion to their respective 5-alpha reduced products at 1 μM, but not at the 10 μM concentration. Finally, dutasteride did not inhibit the conversion of 11-α-hydroxytestosterone or 11-ketotestosterone at either the 1 or 10 μM concentrations.

These results suggest that the interaction between the steroid substrate and the SRD5A3 enzyme affects the inhibitory activity of dutasteride. The androgen substrate conformation and location of hydroxyl and ketone groups may dictate the potential for drugs such as dutasteride to bind SRD5A3 and inhibit its catalytic 5-alpha reducing function. Similarly to previously described activity with 5-alpha reductases 1 and 2 (55), dutasteride inhibited the conversion of testosterone to DHT by SRD5A3. Interestingly, dutasteride reduced but did not fully eliminate the conversion of epitestosterone, an epimer of testosterone, to its 5-alpha reduced product. Furthermore, with variations in the location of hydroxyl and ketone groups in testosterone analogues, the inhibitory potential of dutasteride on SRD5A3 appeared to be weaker. A higher concentration (10 μM) of 6-β-hydroxytestosterone and 11-β-hydroxytestosterone was able to overcome the inhibitory activity of dutasteride on SRD5A3. This suggests that the mechanism of action of dutasteride with these specific substrates may be through competitive inhibition. Finally, dutasteride had no effect on the 5-alpha reducing capacity of SRD5A3 when 11-α-hydroxytestosterone or 11-ketotestosterone were added to HEK293 transfected cells.

These findings suggest that further investigation is warranted to understand the structure activity relationship of the SRD5A3-dutasteride enzyme-substrate complex. This knowledge may shed light on the reasons underlying the variation in dutasteride inhibitory activity on SRD5A3 based on the type and polarity of androgen substrates. Additionally, given the differences in properties between various 5-alpha reductase enzymes, and between 5-alpha reductase inhibitors, it is imperative to determine if dutasteride is the optimal 5-alpha reductase inhibitor to target SRD5A3.

The mass spectrometry findings suggest that SRD5A3 has the capacity of reducing steroid substrates into potent androgens that have been shown to activate the androgen receptor

(31, 57). In this closed system, dutasteride lacks the capacity to inhibit SRD5A3 5-alpha reducing capacity across all androgen substrates. In vivo studies measuring circulating adrenal androgens prone to becoming 5-alpha reductase substrates are mandated to evaluate the overall androgen burden that may be contributing to prostate carcinogenesis. Findings from such studies, in conjunction with ubiquitous distribution of SRD5A3 in the body (49, 50), can potentially magnify this enzyme's role in the development of castrate resistant prostate cancer. This would provide a basis for the discovery of targeted SRD5A3 inhibiting drugs, to be used in rational therapeutic combinations across the spectrum of prostate cancer. This would include, but not limited to prostate cancer prevention, preoperative therapy for prostate cancer, and the prevention and treatment of castrate resistant prostate cancer.

CHAPTER 5: CONCLUSION

In conclusion, this study confirmed that, within an in-vitro system, SRD5A3 has the capacity to convert testosterone and its derivatives to their more potent, 5-alpha reduced, products, capable of binding and activating androgen receptor signaling. The inhibitory activity of dutasteride, a known 5-alpha reductase 1 and 2 inhibitor, on this enzyme, appears to be substrate and concentration dependent. These findings suggest a role for SRD5A3 in androgen metabolism and the development of the lethal phenotype of castrate resistant prostate cancer. In-vitro assessment of SRD5A3 is mandated to determine the potential role of therapeutic SRD5A3 inhibition across the spectrum of prostate cancer.

CHAPTER 6: LIMITATIONS

There are several limitations to this study. First, SRD5A3 was evaluated in a closed in-vitro system. This likely doesn't replicate the intricate steroidogenic pathways and regulatory mechanisms in the human body, and less so in the presence of prostate cancer. Additionally, each steroid substrate was added separately into the system. This approach does not allow for the assessment of relative substrate affinity to SRD5A3. Furthermore, mass spectrometry internal standards for the 5-alpha reduced products of certain testosterone derivatives are not commercially available. Mass spectrometry analysis was carried out using standards extrapolated from the substrate compounds, or from previously published work in the literature (57). Finally, liquid chromatography-mass spectrometry is a highly sensitive tool that can detect compounds at minute quantities. This sensitive approach may lead to the detection of background noise which may contaminate the results, and lead to false positive conclusions.

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