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Studies on Regulation of Dihydrotestosterone Binding to and Releasing from the Androgen Receptor

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STUDIES ON REGULATION OF DIHYDROTESTOSTERONE BINDING TO AND RELEASING FROM THE ANDROGEN RECEPTOR

A Thesis

by

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ABSTRACT

Resto Santos, Tahyra M., Studies on Regulation of Dihydrotestosterone Binding to and Releasing from the Androgen Receptor. Masters of Science (MS), May, 2019, 50pp, 4 tables, 26 figures, references, 20 titles, appendix.

The androgen receptor (AR) is a nuclear receptor that responds to testosterone and 5α-dihydrotestosterone (DHT) by modulating gene expression in a variety of cells, including prostate and muscle tissues. Studies of small molecule interaction with AR have shown diphenyl compounds inhibiting through a binding site labeled Binding Function 3 (BF3).

The goal of this research is to analyze the interaction of the BF3 site with DDT and related compounds. Amino acids that are exposed on the surface of the BF3 that might interact with different compounds were mutated. Mutant proteins were tested experimentally for their impact on the inhibition the AR. Results show that mutations in F673, G724, and L830 prevent the inhibition of DDE, suggesting that DDE and related compounds act by interacting at the BF3 site of the AR. Understanding how the BF3 site binds with these compounds may lead to the development of new anticancer medications.

DEDICATION

My thesis is dedicated first of all to God who allowed me to complete this work successfully. I also dedicate it to my father Gonzalo Resto and mother Ada Santos, who inspired, motivated and encouraged me every day to pursue my dreams. Finally, I dedicate it to my husband Rafael Soto who supported me all the way and always believed that I could accomplish this work. Thank you for your patience, your love and sacrifices.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

This chapter introduces the main problem and purpose for conducting this thesis research. Also, general information for understanding the androgen receptor structure and function will be discussed as well as the function of the BF3 site.

1.1 Prostate Cancer

According to the American Cancer Society, 174,650 new cases of prostate cancer will be diagnosed in the US during 2019. [Siegel] Although rates of death for prostate cancer have decreased over the years, prostate cancer is still considered one of the leading causes of cancer death among men of all races and Hispanic origin populations. The first case of prostate cancer was described as “a very rare disease” in 1853 by J. Adams, a surgeon at The London Hospital. [Denmeade] The development of prostate cancer has been linked to old age, black ethnicity, and family history of the disease. However, it has also been associated with eating habits, early sexual intercourse, and sexually transmitted infections such as herpes and *Neisseria gonorrhea*. [Felgueiras] Unfortunately, prostate cancer is still labeled as one of the main causes of death among men worldwide. Therefore, identification of new molecular markers and therapeutic targets for prostate cancer are needed.

Prostate cancer develops due to an uncontrolled growth of cells in the prostate gland. Studies have shown that proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) are the main precursors of prostate cancer. [Felgueiras] Most cases correspond to acinar adenocarcinomas that arise from prostatic epithelial cells which express AR. [Felgueiras] This means that the AR signaling pathway is essential for the physiological development and maintenance of the prostate gland. [Tan]

1.2 Androgen Receptor

The androgen receptor (AR) is a nuclear receptor (NR) that responds to testosterone and 5α- dihydrotestosterone (DHT) by modulating gene expression in a variety of cells, including prostate and muscle tissues. The AR is found in the cytoplasm of the cell, until testosterone or DHT binds to it and promotes the translocation of the AR into the nucleus of the cell as shown in Figure 1. It has been proven that the AR is essential for the physiological development and maintenance of the prostate gland. Therefore, intervening with this signaling pathway is one of the main targets in anti-cancer drug development. The AR gene encodes a 110kDa protein consisting of 919 amino acids. [Tan] The AR structure consist of 3 major functional domains: N-terminal domain (NTD), the DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD).

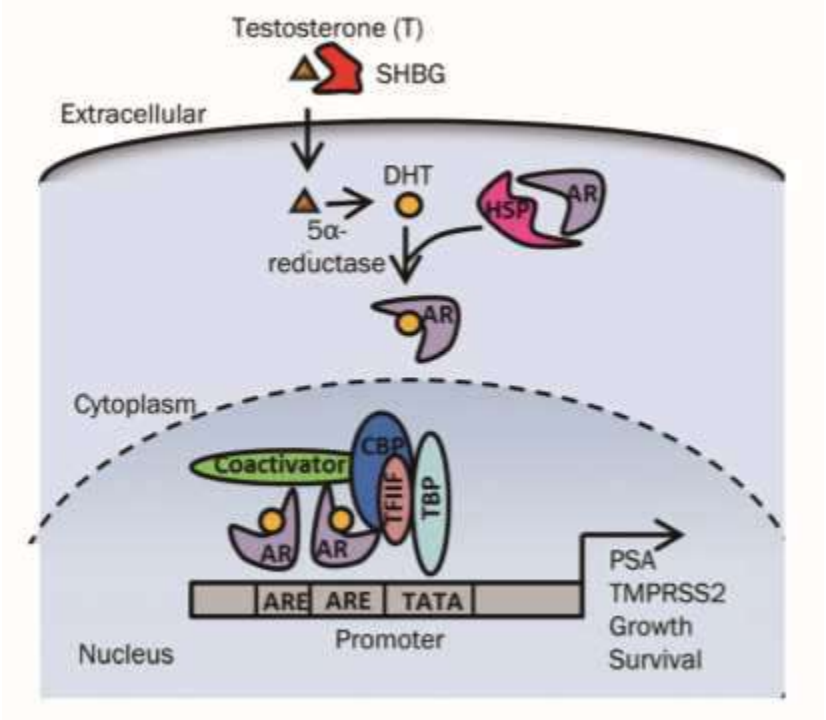

Figure 1: *Androgen receptor mechanism in prostate cells. Testosterone is transported into targeted tissues and converted to DHT by 5-α- reductase. DHT binds to the LBD of AR and promotes the dissociation of regulatory proteins from the AR. AR is then translocated into the nucleus to bind to the promoter region and allow recruitment of transcription factors and coregulators to further assist gene expression in the growth of the cells.* [Tan]

1.3 Binding Function 3 Site

In recent years, a new way of targeting AR was discovered through the search of new inhibitors acting at the AF-2 site, which led to the identification of the binding function 3 site (BF30 [Estebanéz-Perpiña]. It was found that compounds that bind to the BF3 site would allosterically regulate the AF-2 coactivator binding. Interestingly, several of such compounds which bound to the BF3 site share a diphenyl structure, such as that found in DDT and related compounds.

1.4 DDT and Related Compounds

Endocrine disrupting chemicals (EDCs) can interfere with the function of endocrine systems, leading to birth defects, cancer, and disruptions of development. The endocrine

disruptions include a wide range of effects, including both pro- and anti- hormonal effects. DDT (dichlorodiphenyltrichloroethane, 4,4'-DDT) is an insecticide that has been identified as an EDC and a persistent organic pollutant with a wide range of effects on wildlife and human health. DDT has an interesting diphenyl structure, specifically a diphenyl ethane (Figure 3A). A binding site has been identified on the androgen receptor (AR) protein surface, BF3. [Estebanéz-Perpiña] The androgen receptor (AR) is a nuclear receptor (NR) [Adrenal Cancer, Estebanéz-Perpiña] that responds to testosterone and 5α-dihydrotestosterone (DHT) by modulating gene expression in a variety of cells, including prostate and muscle tissues. [He] Both testosterone and DHT are ligands for the androgen receptor and bind to the ligand binding domain (LBD), which has been the focus of AR-based drug discovery. [Osguthorpe] DHT has been shown to have strong interactions with AR by binding on the inside of the AR, and not being exposed on the surface of the AR, as shown in Figure 2.

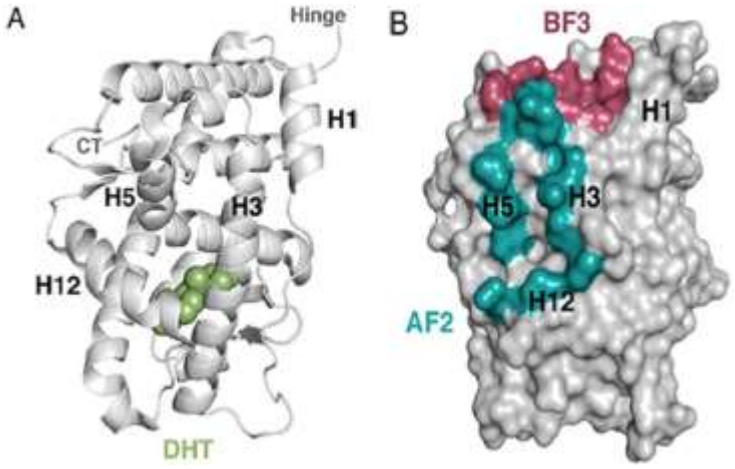

Figure 2: Structure of the androgen receptor and its domains. Figure 1: (A) Schematic of AR LBD with DHT (green) binding on the inside of the receptor. (B) Space-filling model showing residues in AF-2 (blue) and BF3 (purple). [Estebanéz-Perpiña]

Compounds that act at BF3 allosterically regulate the binding of a steroid receptor coactivator (SRC) peptide at AF-2. Several of the compounds that interact with the site have a

diphenyl structure, either a diphenyl amine or diphenyl ether. [Estebanéz-Perpiña] However, in DDT the two phenyl rings are bridged by a carbon atom instead of nitrogen or oxygen, making a diphenyl ethane, (Figure 3A). Since compounds with a diphenyl structure binding to BF3 have an allosteric effect on the protein, leading to loss of SRC binding, we tested the ability of DDT to inhibit binding of radioactively labeled dihydrotestosterone (^{3}H-DHT) to AR. We also tested the effects of three structurally related compounds shown in figure 3: dichlorodiphenyldichloroethane (4,4'DDD) which is also an insecticide, dichlorodiphenyldichloroethylene (4,4'DDE), which is a breakdown product of DDT, and Mitotane (2,4'DDD), which is an antineoplastic medication used in the treatment of adrenocortical carcinoma. [Hahner]

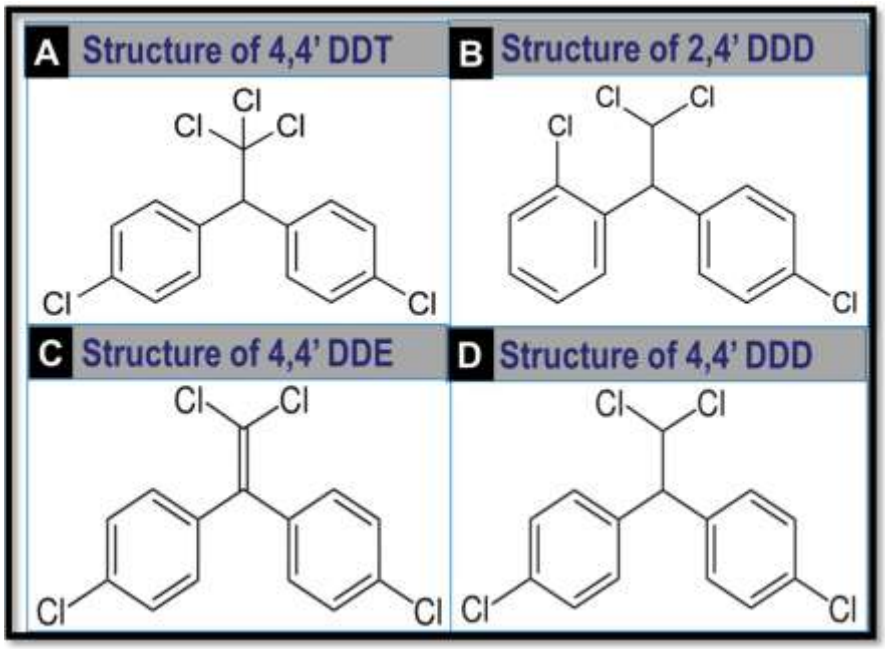

Figure 3: Structure of DDT and Related Compounds. A) Structure of dichlorodiphenyltrichloroethane, 4,4'- DDT. B) Structure of Mitotane, 2,4'DDD C) Structure of dichlorodiphenyldichloroethylene, 4,4'DDE. D) Structure of dichlorodiphenyldichloroethane, 4,4'DDD.

CHAPTER II

REVIEW OF LITERATURE

This chapter examines previously published knowledge and data regarding androgen receptor (AR) and its signaling mechanism of action. The biological role AR in our system, and where BF3 is located in the AR. The rationale for performing this research will also be explained.

2.1 AR Inhibition Pathways

The AR is required for development of the male reproductive system and secondary sexual characteristics, making it a target for the prevention of prostate cancer in men. [Davey] The AR is mainly responsible for maintaining libido, spermatogenesis, muscles mass and strength, and bone mineral density. [Gao] The role of AR in these target tissues such as prostate, skeletal muscle, liver, and central nervous system makes it of interest to target and inhibit.[Gao] To better understand how DDT and related compounds could inhibit the AR, the structure of the sites of interactions in the AR must be analyzed.

The AR is composed of three main domains: the N-terminal transcriptional regulation domain, the DNA binding domain (DBD) and the ligand binding domain, to which the testosterone and DHT bind. [Davey] The first transcriptional activation function, AF-1, as shown in Figure 4, is composed of the N-Terminal and DBD domains, which are ligand- independent,

making it a difficult site to use for regulation of AR activity. The N-terminal domain is required for maximal activity of the AR, and the ligand-dependent AF-2, located in the ligand binding domain, is important for forming the coregulator binding site. The DBD lies between the N-terminal and ligand binding domains and is important for mediating direct interactions between them. Due to its interaction with the transcriptional co-activator factors, AF-2 has been studied and identified as a coregulator site of the AR.

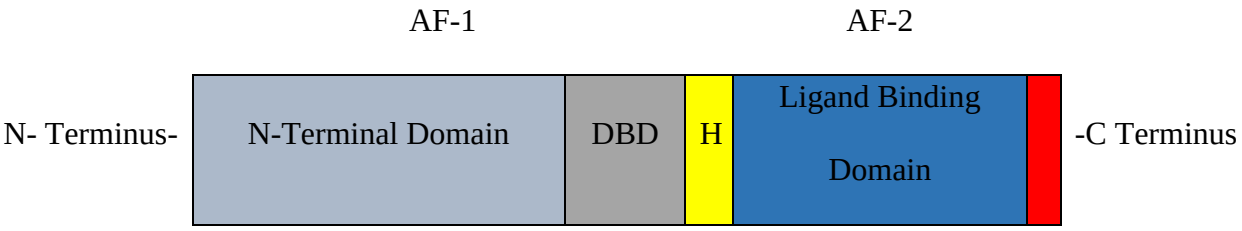

Figure 4: Functional domains of the AR. N-terminal domain, DNA binding domain (DBD), Ligand binding domain. (H – hinge region, AF-1 – transcriptional activating function 1, AF-2 – transcriptional activating function 2).

The AF-2 is a highly ordered hydrophobic surface in the LBD that requires androgen binding for its structural integrity. [Askew] Due to its specificity to the LBD, AF-2 has been studied as an AR coregulator and has been successfully used for targeting the AR to regulate degeneration in Spinal Bulbar Muscular Atrophy. [Badders] Also, in 2015 endostatin was found to function as an endogenous AR inhibitor that impairs receptor function through protein–protein interactions.[Lee] Most common prostate cancer treatments, such as Enzalutamide, target the signaling pathway of the AR. [Alex] [Lee] However, the development of additional inhibitors with more selective effects or having concomitant inhibitory actions on the AR activation remains an important goal.

2.2 Identification of the BF3 Site

Although AF-2 has functioned as a great target to modulate AR activity, over the years prostate cancer has developed resistance to these treatments, making it harder to regulate the

metastasis of prostate cancer. This has led to the search of other methods or pathways to target the AR and inhibit the signaling pathway. As stated in chapter I, a new possible allosteric regulatory site has been identified as the Binding Function 3 (BF3). The BF3 site has been found as a hydrophobic cleft at the junction of H1, the H3–H5 loop of AR, and H9 that is almost as large as AF-2 (Figure 5) and exhibits characteristics of protein interaction surfaces. [Estebanez-Perpina]

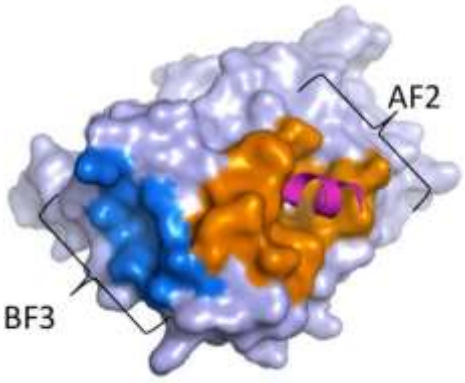

Figure 5: Surface rendering of AR showing AF-2 and BF3 residues side by side. [Estebanez-Perpina]

Due to the location and behavior described by Estebanez-Perpina et al., it has been proposed that BF3 serves as an allosteric regulatory site on the AR by binding to diphenyl compounds such as flufenamic acid, triiodothyronine, and 3,3′,5 –triiodo thyroacetic acid. [Estebanez-Perpina] Several researchers have followed up on this idea by targeting a different site on the AR, AF-2, and BF3 inhibitors can be taken concurrently with antiandrogens to prolong time to cancer remission.[Tan]

2.3 Published Effects of AR Inhibition at the BF3 Site

Inhibition of the AR has been a target of our medical research for a number of years. However, discovery of the BF3 regulatory site has opened another pathway with which we can inhibit and target AR activity. Studies have focused on the function and purpose of BF3 on the AR. Virtual screening was performed by workers at the University of British Columbia, who conducted a large-scale *in-silico* screen followed by experimental evaluation. They discovered

four high affinity compounds that were specifically binding to the BF3 site.[Lack] These compounds were identified in co-crystal structures as shown in figure 6.[Lack]

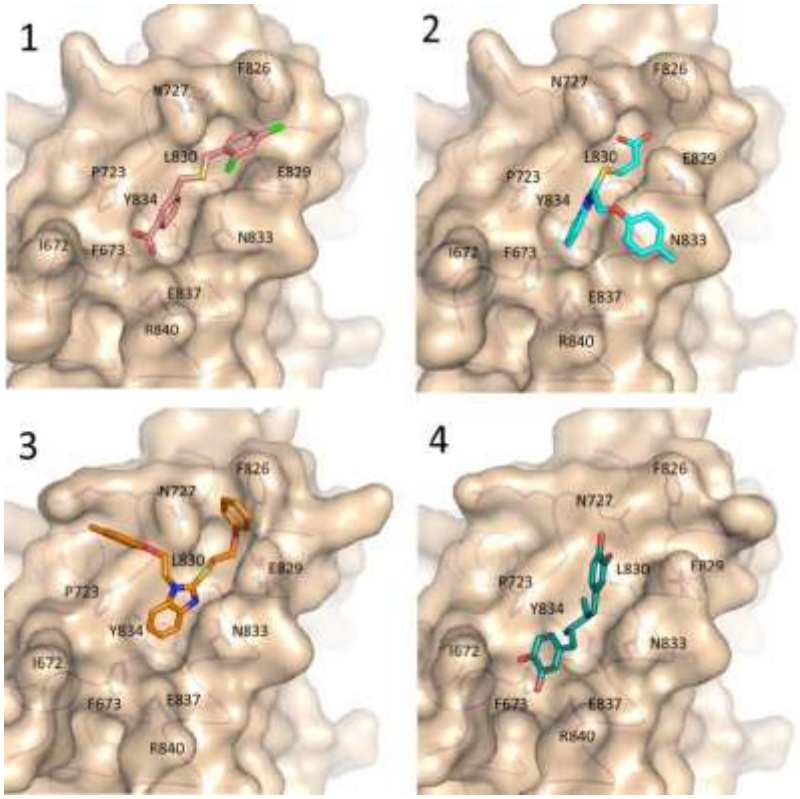

*Figure 6: Crystallographically determined conformation of the identified BF3 binders **1-4** inside the BF3 target site.* [Lack]

Such research was followed by site-directed mutagenesis to confirm the affinity of compounds 1-4 towards the BF3 site. Results showed that BF3 can act as a regulatory binding site to prevent the development of prostate cancer. [Lack]

The research of Estebanez-Perpina and Lack are similar in that in both studies, action at BF3 has a directly inhibiting effect on the binding function of the AR. Also compounds that were tested or used in both studies share a diphenyl structure, suggesting that the BF3 site has special affinity for diphenyl structure compounds. In this study, diphenyl compounds of DDT were tested to see if they could act as inhibitors of the AR, and if they had affinity towards the BF3

site. The results supports the idea that BF3 functions as an allosteric regulatory binding site on the AR, and suggest that diphenyl compounds structurally similar to DDT might serve as inhibitors of the AR signaling pathway, leading to possible new treatments for prostate cancer.

CHAPTER III

EXPERIMENTAL METHODS

This chapter introduces the methods and experimental assays that were performed to test our hypothesis.

3.1 Inhibition of DDT Compounds

An 80μL reaction mixture was prepared for binding and releasing assays to the androgen receptor:

- 10mM potassium phosphate buffer
- 10mM sodium molybdate (Na_2MoO_4)
- 5mM dithiothreitol (DTT)
- 0.1mM ethylene dinitrilo tetra acetic acid (EDTA)
- 5nM androgen receptor (AR)
- 4μL 4,4' DDT, 4,4' DDE, 4,4' DDD, or 2,4' DDD diluted in DMSO
- 35nM Radioactive dihydrotestosterone (3H-DHT)

To measure inhibition of steroid binding to AR by 4,4' DDT, 4,4' DDE, 4,4' DDD, and 2,4' DDD, AR was first incubated in the presence of one of these compounds for 2 min at 20°C. Reactions were then placed on ice, ^{3}H-DHT was added, and the reactions were further incubated for 10 min at 20°C. After the second incubation, hydroxyapatite was added (30uL of a 50% slurry) to the reactions to allow the radioactive steroid-receptor complex to bind to

hydroxyapatite. The reaction mixtures were then transferred onto glass fiber filters and the amount of ^{3}H-DHT binding to AR was determined in a liquid scintillation counter.

To measure steroid release from AR by 4,4' DDT, 4,4' DDE, 4,4' DDD, and 2,4' DDD, AR was first incubated with ^{3}H-DHT for 10 min at 20°C. Rebinding of the steroid to the AR after the release of DHT was prevented with 4μM of non-radioactive Estradiol. The test compound was then added, and reactions were incubated for 10 min at 20°C. After the second incubation, hydroxyapatite was added to the reactions as above, and the amount of ^{3}H-DHT bound to AR was determined in a liquid scintillation counter.

The binding and releasing assays were performed at 1500 μM, 500 μM, 150 μM, 50 μM, 15μM, 5 μM, 1.5 μM, 0.5μM, 0.15μM, 0.05μM, 0.015μM, and 0.005μM final concentrations of 4,4' DDT, 4,4' DDE, 4,4' DDD, or 2,4' DDD.

3.2 Molecular Studies of AR

Computational methods for modeling of the AR receptor (AR 2PIT) and the BF3 site were employed to predict what amino acids in the BF3 site would be predicted to be in contact with DDT bound at the site. Mutations on such amino acids will lead to a change in the shape of the surface of the BF3 site and interfere with the binding of the DDT and similar compounds to AR. Mutations were selected based on their conformational effect on the structure of the BF3 site (See Chapter IV Results).

3.3 Mutant Primer Design and Synthesis

Primers used for site-directed mutagenesis were synthesized and HPLC purified by Integrated DNA Technologies and ranged between 34-40 base pairs (bp) length, 35-57% GC content, 72.90 -80.16 °C melting temperature and a less than 10% bp mismatch. Sequences of

the forward and reverse primers for each mutation are listed in Table1. Mutagenesis primers were resuspended in 1X TE buffer (10mM Tris, pH 7.5, 0.1mM EDTA) and final primer concentrations ranged between 177-191ng/ul.

G724R **Wild Type G (GGC)**	
Forward Primer 5'	**GG GCC AAG GCC TTG CCT AGA TTC CGC AAC TTA CA 3'**
	K A L P R F R N L
Reverse Primer 5'	**TG TAA GTT GCG GAA TCT AGG CAA GGC CTT GGC C**

G724M **Wild Type G (GGC)**	
Forward Primer 5'	**GG GCC AAG GCC TTG CCT ATG TTC CGC AAC TTA CA 3'**
	K A L P M F R N L
Reverse Primer 5'	**TG TAA GTT GCG GAA CAT AGG CAA GGC CTT GGC C**

F673W **Wild Type F (TTT)**	
Forward Primer 5'	**C TAT GAA TGT CAG CCC ATC TGG CTG AAT GTC CTG GA**
	E C Q P I W L N V L E
Reverse Primer 5' 3'	**GC TTC CAG GAC ATT CAG CCA GAT GGG CTG ACA TT**

F673K **Wild Type F (TTT)**	
Forward Primer 5'	**C TAT GAA TGT CAG CCC ATC AAG CTG AAT GTC CTG GAA**
	E C Q P I K L N V L E
Reverse Primer 5'	**GC TTC CAG GAC ATT CAG CTT GAT GGG CTG ACA TTC**

L830D **Wild Type L (CTT)**	
Forward Primer 5'	**CAA AAA TTC TTT GAT GAA GAC CGA ATG AAC TAC ATC**
	Q K F F D E D R M N Y I
Reverse Primer 5'	**C CTT GAT GTA GTT CAT TCG GTC TTC ATC AAA GAA TTT**

Table 1: List of Primers Created for Use in Site-Directed Mutagenesis Experiment. Primers listed introduce single or amino acid mutations into the AR protein sequence.

3.4 Site Directed Mutagenesis

In site-directed mutagenesis, a PCR reaction is used to introduce desired mutations into template DNA. The reaction was performed on double stranded, mini-prepped plasmid DNA containing the human AR gene sequence (RC215316 from Origene), as shown in Figure 7.

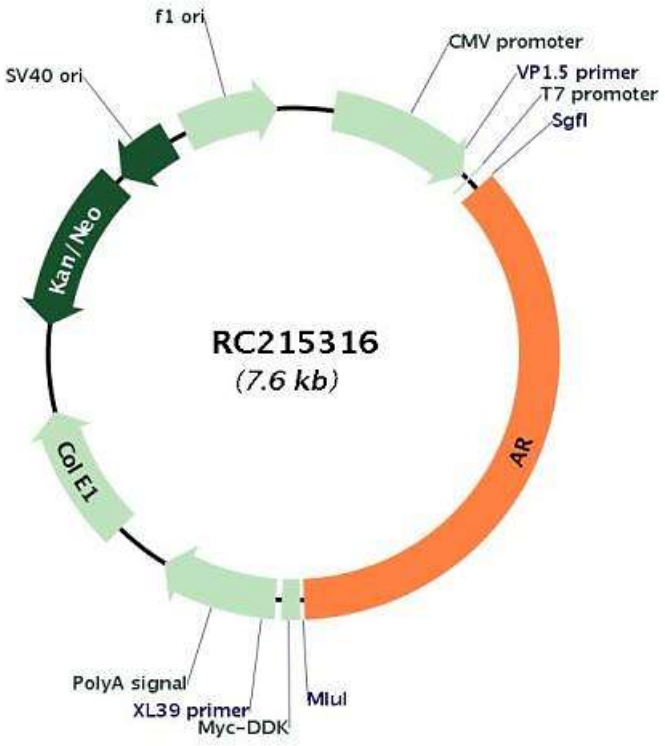

Figure 7: Human AR Gene Vector RC215316. The androgen receptor codes for a protein that has 3 major functional domains: the N-terminal domain, DNA-binding domain, and androgen-binding domain. The protein functions as a steroid-hormone activated transcription factor. Upon binding the hormone ligand, the receptor dissociates from accessory proteins, translocates into the nucleus, dimerizes, and then stimulates transcription of androgen responsive genes.

From modeling studies using the program Schrodinger, residues Ile 672, Phe 673, Pro723, Gly 724, Asn 727, Phe 826, Glu 829, Leu 830 Asp 833, Thr 834, Glu 837, and Arg 840 were identified as amino acids which are exposed to the surface at the BF3 site. To obtain a significant conformational change at the protein surface, the following mutations were made:

F673W, F673K, G724M, G724R, and L830D. These mutations were chosen, because they would make the largest and most significant changes in the BF3 site. Using the QuikChange II Site-Directed Mutagenesis Kit from Agilent Technologies. Mutagenic primers were designed following manufacturer's protocol as described above and the mutations were made

3.4.1 PCR Mutagenesis Reaction.

A PCR reaction was performed for each mutant using a BIO RAD T100 Thermal Cycler and mutagenesis primers specific for each desired mutation. Each PCR reaction was mixed using the following reagents:

1) 5µl 10x reaction buffer
2) 2µl purified template GPR119 DNA (200ng)
3) 1µl forward primer (191ng)
4) 1µl reverse primer (191ng)
5) 2µl dNTPs (25mM)
6) 6µl of 25% dimethyl sulfoxide (DMSO)
7) 32µl double distilled H_2O
8) Lastly 1µl of Pfu Ultra HF DNA polymerase (2.5U/µl) was added to the reaction

Thermal Cycler parameters were fixed and utilized at 94°C for 1 min and 94°C for 50 sec for template strand denaturation, 56°C for 50 sec for primer annealing, 68°C for 28 min for mutant strand elongation. These steps were repeated 9 times to produce an abundant amount of mutated plasmid DNA. A final elongation step was then set at 72°C for 30 min and after cycle completion reactions were held at 4°C.

3.4.2 Digestion of PCR Reaction.

Digestion of PCR reactions is essential to remove parental methylated and hemimethylated DNA from the reaction mixture, because only the unmethylated product DNA has the mutation in it. To facilitate the degradation of parental DNA, 1µl of the restriction enzyme Dpn I was added to each PCR reaction and mixed gently. The digestion reactions were incubated in a 37°C water bath for 2½ hours.

3.5 Bacterial Transformation and Sequencing of AR Plasmid Mutant DNA

Transformation of the Dpn I digested mutant AR DNA into E. coli was performed using the following protocol:

1) XL Gold cells were thawed on ice for 20 minutes.
2) For each reaction 50µl of XL Gold cells were pipetted into a pre-chilled falcon tube.
3) 5µl of Dpn I digested mutant DNA was added to the cells.
4) Cell/DNA mixture was placed on ice for 20 minutes.
5) Reactions were then heat shocked in a 42°C water bath for 45 sec.
6) 500µl of SOC media preheated to 42°C was added to each reaction.
7) Reaction tubes were placed in a 37°C shaker at 200 rpm for 45 minutes.
8) 200µl of each reaction was plated on 2xYT Agar plates supplemented with kanamycin at a working concentration of 25 µg/ml.
10) Plated reactions were incubated overnight at 37°C and transformed colonies were picked the following day.
11) Picked colonies were placed in 3ml of 2xYT-Broth supplemented with kanamycin and were incubated overnight at 37°C with shaking.

12) Bacterial cells were harvested the next day by centrifugation at 6000 xg for 15 minutes at 4°C.

13) Plasmid DNA was purified using the Qiagen QIAprep Spin Maxiprep Kit following the manufacturer's protocol.

Purified plasmid DNA was sequenced by Functional Biosciences, Inc. using sequencing primers:

Fwd. 5' GGACTTTCCAAAATGTCG 3'

Rev. 5' ATTAGGACAAGGCTGGTGGG 3'

Resulting sequences were checked for desired mutations. Samples displaying the desired mutations were then maxi-prepped to bring mutant DNA concentrations to ~1ug/µl.

3.6 Mammalian Cell Culture

HEK 293 cells were obtained from Dr. Megan Keniry's lab (Biology Dept.UTRGV). Cells were grown in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U and 10mg/ml). Cells were cultivated at 37°C in a humidified 5% CO_2 incubator in 100mm plates until 80-90% confluent for experiments.

3.6.1 Cytotoxicity of DDT and Related Compounds

HEK 293 cells were plated per well in a 96 well plate using Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Solution for 24 hours before the assay. Cells were grown under standard tissue culture conditions (5% CO_2 and 37°C). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) + 10% Fetal Bovine Serum (FBS) medium in a tissue culture plate until cells were 70-90% confluent.

DDT and related compounds were diluted in DMSO to achieve 10uM concentration in figure 15.

Cells were treated with the compound dilutions or DMSO alone for 24 hours. After this, the MTT reagent from the Trevigen TACS MTT Cell Proliferation Assay Kit was added (10 µl per well) and the plate was incubated for 2 to 12 hours to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. After this, 100 µl of detergent reagent is added to each well to solubilize the formazan dye and cells were incubated on a shaker for 4 hours at room temperature. Absorbance of each sample was measured in a microplate reader at 550 - 600 nm, depending upon the filters available. Samples were done in triplicates.

3.6.2 Transfection of Cells with Mutant and Wild-Type AR DNA

Prior to transfection HEK 293 cells were plated in DMEM supplemented with 10% FBS without antibiotics in 6 well plates at a density of 250,000 cells/mL in DMEM supplemented with 10% FBS without antibiotics. Once cells were 70-90% confluent, cells were transfected with *Renilla Luciferase (RL) TK* (Figure 8), *Firefly* Luciferase Receptor (Figure 9), wild-type AR DNA or AR mutations using the Lipofectamine® 3000 Reagent kit purchased from ThermoFisher Scientific, Inc.

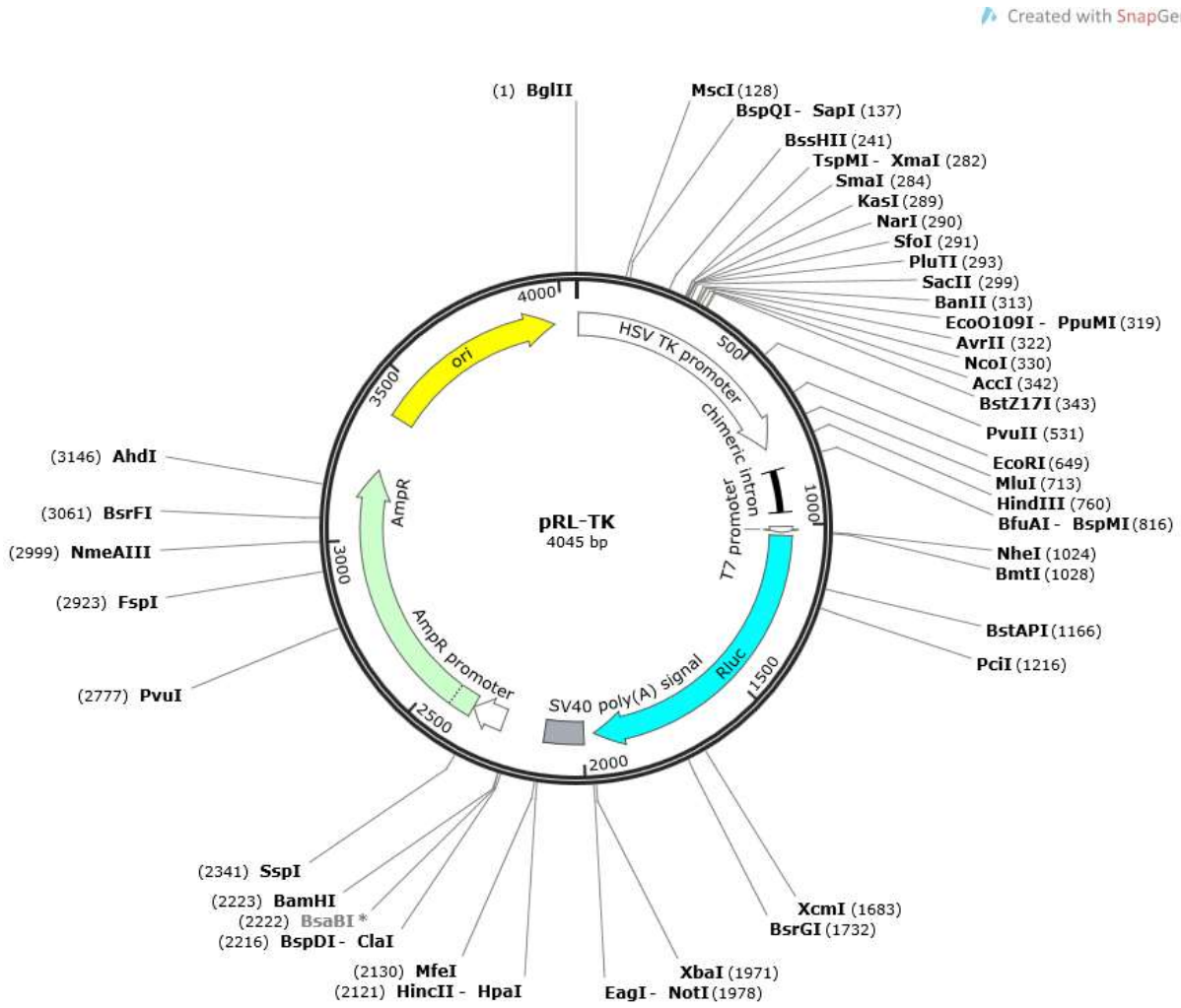

Figure 8: Renilla Luciferase(RL) TK. pRL-TK Vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of Renilla luciferase expression in co-transfected mammalian cells.

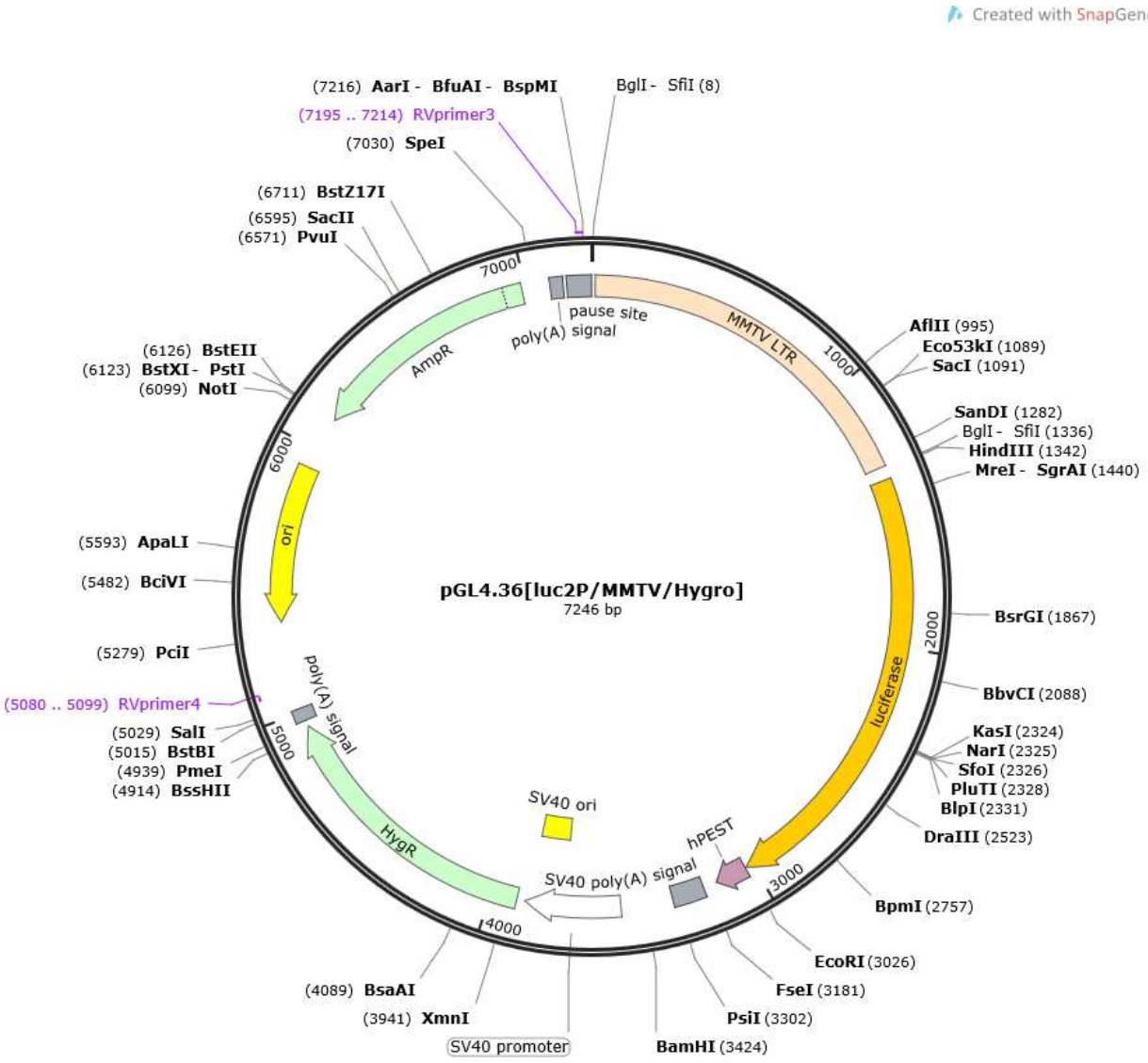

Figure 9: Firefly Luciferase Reporter. The pGL4.36[luc2P/MMTV/Hygro] Vector contains MMTV LTR (Murine Mammary Tumor Virus Long Terminal Repeat) that drives transcription of the luciferase reporter gene luc2P in response to activation of several nuclear receptors such as glucocorticoid receptor and androgen receptor.

In accordance with optimization results, each transfection was performed using 3.75µl of Lipofectamine Reagent, 0.5µg of *Renilla Luciferase* DNA, 0.5µg of *Firefly* DNA and 0.5µg of AR mutant or wild-type AR DNA, and 3µl P3000 TM Reagent. Transfections were performed following the manufacturer's protocol. Transfected cells were incubated for 24 hours at 37°C in

a humidified 5% CO_2 incubator. Following incubation, media was removed and replaced with 2ml of DMEM supplemented with 10% charcoal-stripped FBS per well. 0.02mM DDT compounds dilutions were then added to each well, followed by the addition of the DHT dilutions shown in table 2. Cells were incubated for 24 hours, and Dual Luciferase experiments were performed the following day.

Cell Sample	DHT Concentration (uM)
1	1
2	.1
3	.01
4	.001
5	.0001
6	.00001

Table 2: Concentration of DHT for individual wells

3.7 Dual-Luciferase Assay

After 24 hours of incubation, HEK 293 cells were washed with 1mL of PBS, and 250uL of Passive Lysis Buffer (PBS) was added to detach the cells. Cells were then scraped and transferred into microcentrifuge tubes. To enhance the lysis results, two cycles of freezing in dry ice, ethanol and thawing in a 37°C water bath were performed. Reporter assays were then performed using the Promega Dual-Luciferase Assay Kit and protocol. 1X Passive Lysis Buffer, LAR II (Luciferase Assay Substrate in Luciferase Assay Buffer II) and Stop & Glo reagents were prepared before each trial. 100uL of LAR II was added on microcentrifuge tubes. 20uL of

PLB cell lysate was then transferred and briefly mixed. Samples were immediately placed on the Luminometer to obtain the first absorbance reading of 10seconds (firefly luciferase activity). After all readings were completed, 100uL of Stop & Glo reagent was added and briefly mixed in each sample to obtain the *Renilla* Luciferase activity. Data was then analyzed to obtain the ratio of absorbances and was converted to a percentage for plotting. Dose-response curves were created and IC_{50} values were determined using Sigma Plot (Systat Software).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Inhibition of AR Activity by DDT and Related Compounds

Inhibition of dihydrotestosterone (DHT) binding to AR: Various concentrations of DDT or related compounds were tested using a filter binding assay. Binding of ^{3}H-DHT to the AR ligand binding domain was measured by the amount of radioactivity bound to the filter and the data was plotted in a dose-response curve (Figure 10). Results obtained showed that the DDT and related compounds inhibited DHT from binding to AR with IC_{50} values ranging from 2 to 10μM, under these conditions (Table 3).

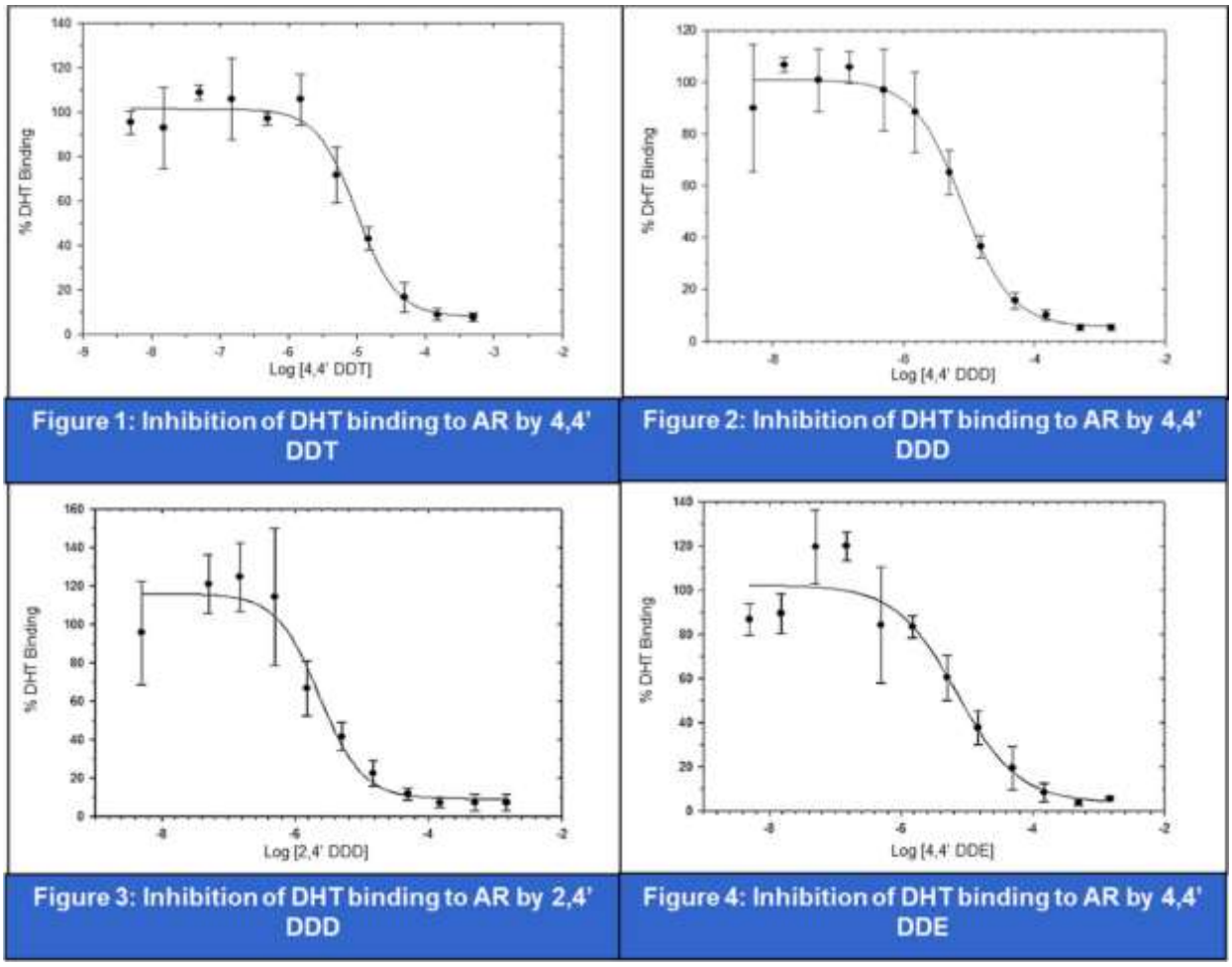

Figure 10: Inhibition of DHT binding to AR by DDT and related compounds.

A second set of experiments measured the ability of DDT and related compounds to cause the release of DHT from AR. In these experiments, the radioactive steroid was first bound to the AR LBD and then the DDT or related compound was added second. The data was again plotted as a dose-response curve (Figure 11). Under these conditions, DDT and related compounds caused the release of DHT from the AR, with IC_{50} values ranging from 54 to 82μM (Table 3).

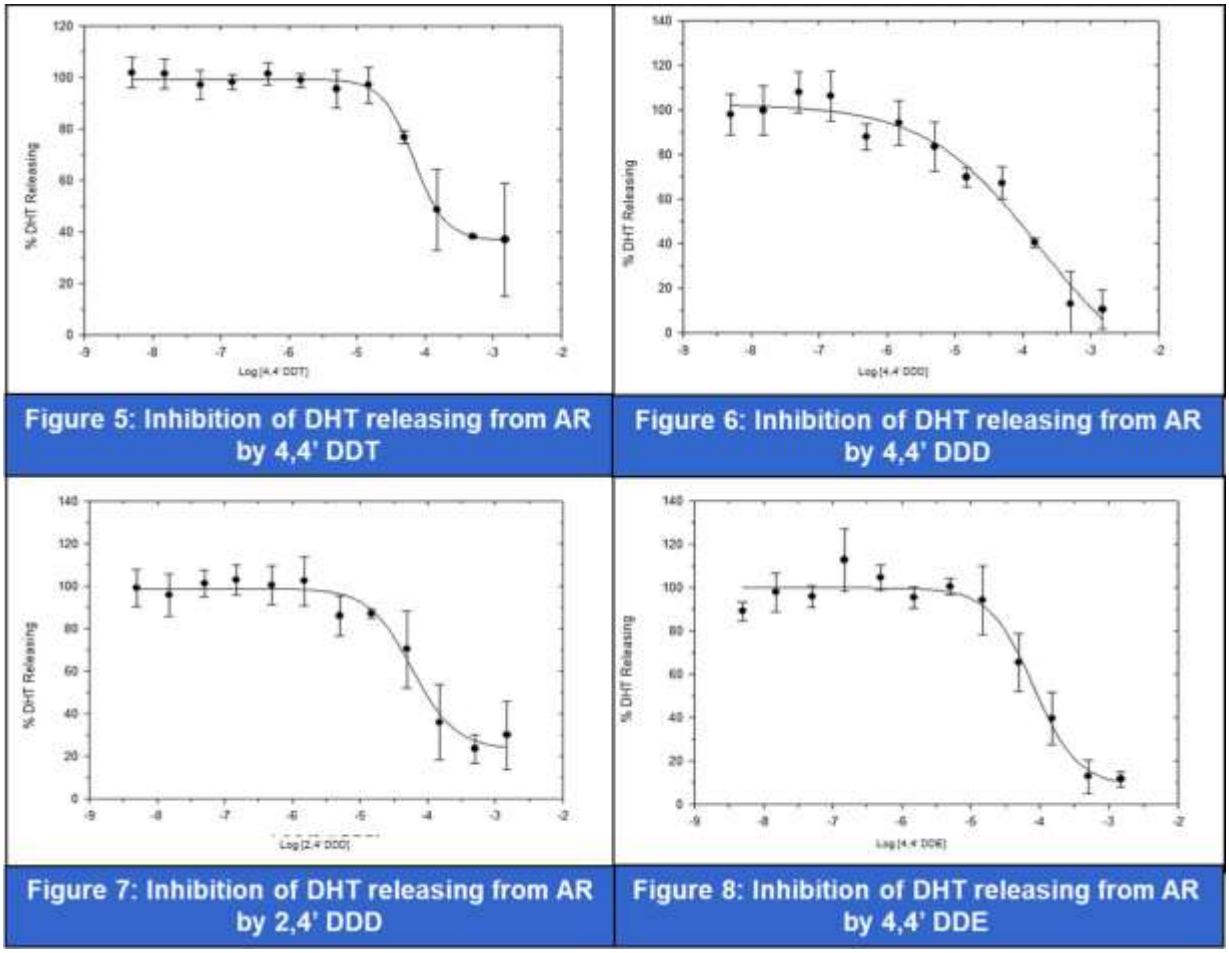

Figure 11: Inhibition of DHT release from AR by DDT and related compounds.

Table 3: Effect of DDT Compounds on DHT Binding to and Releasing from AR		
Compound	**Binding IC_{50} (µM)**	**Releasing IC_{50} (µM)**
4,4' DDT	**10**	**68**
2,4' DDD	**2**	**59**
4,4' DDD	**8**	**54**
4,4' DDE	**8**	**82**

4.2 Androgen Receptor Molecular Model

Molecular modeling studies on AR (2PIT) (Figure 12) showed us what amino acids are present in the BF3 site and could potentially be in contact with DDT bound in the site.

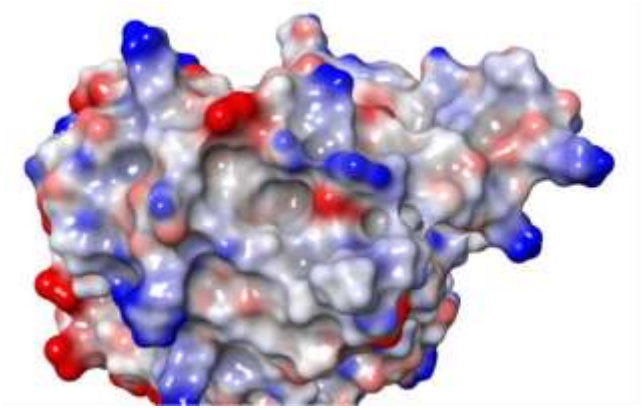

Figure 12: 2PIT-Wild Type AR

Amino acid Phe673 is found near the junction of Helix 1, identified as a hydrophobic cleft on AR. Asn727, Gly724, and Pro723 are found in the H3-H5 loop. Amino acids Phe826, Leu830, Tyr834, and Glu837 are found in Helix 9, which runs from one end of the site to the other, as shown in Figure 13. F673, G724, and L830 were chosen for mutagenesis.

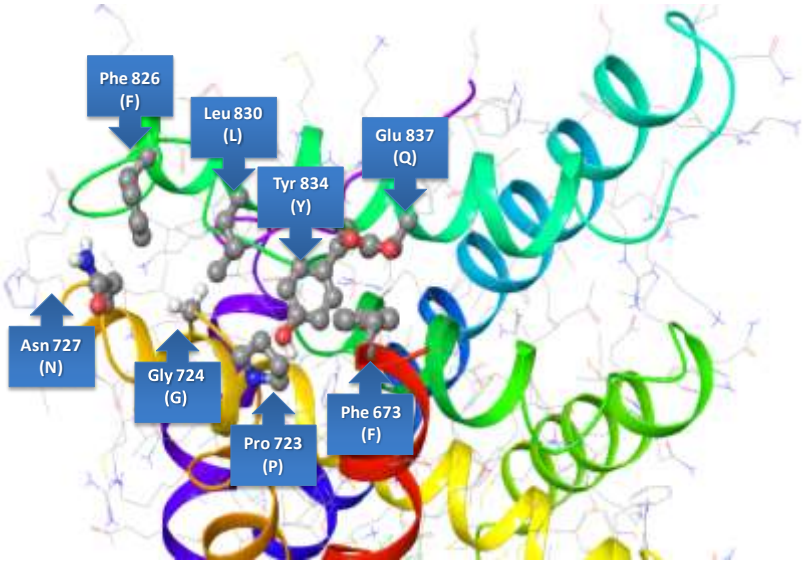

Figure 13: 2PIT-Wild Type AR with Amino Acids lining the BF3 Site

Mutations that showed the greatest structural change in the BF3 site were selected to test the selectiveness of our compound for the BF3 site. These structural changes are shown in Figure 14.

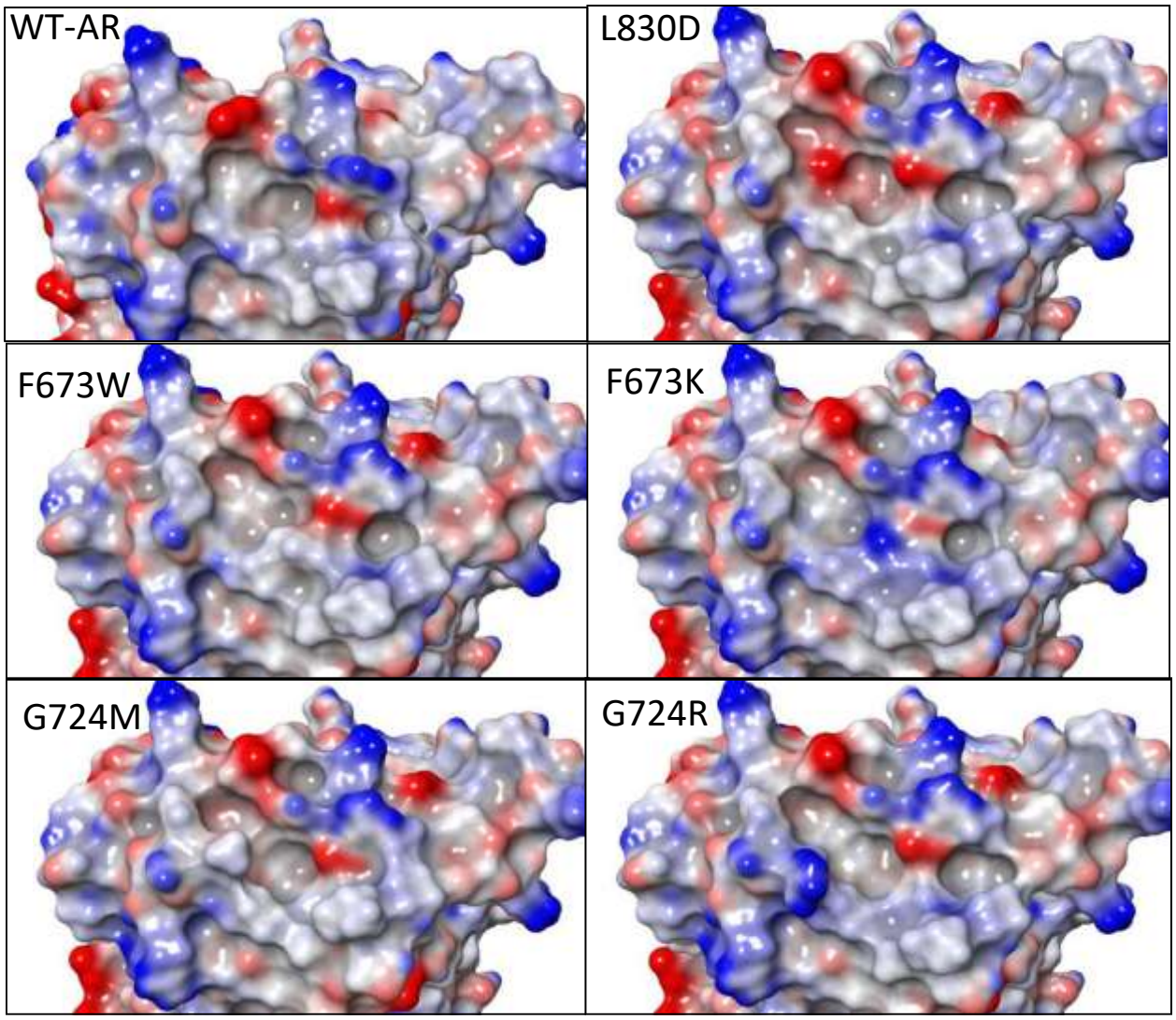

Figure 14: Surface rendering of the AR BF3 site that show the wild type alongside the individual mutations.

4.3 Mutations Selected Based on Conformational Changes

Mutation F673W made the BF3 site more rounded and smaller in size, preventing the compounds from fitting in the BF3 site. Mutation F673K added a positive charge with the amino

group from the lysine(K) side chain and decreased the size of the cavity. Mutation G724M has created a more narrowed BF3 site due to the methionine creating a wall-like structure on one side of the binding site. Mutation G724R gives the BF3 site a positive charge due to the nitrogen atoms present in the arginine sidechain, and the binding site appears deeper, which could reduce the affinity between the BF3 site and the compounds. Finally, mutation L830D makes the BF3 site more open, but with a negative charge at the center, which could lead to hydrophilic interactions with the inhibitor compounds.

4.3.1 Site Directed Mutagenesis Results

AR DNA sequencing results were conducted and prepared by Functional Biosciences, Inc. Each mutation was confirmed by DNA sequencing. The entire nucleotide and amino acid sequence of the wild type AR is shown in Appendix A. However, highlighted point of mutations can be seen in Figure 15. Plasmids with confirmed mutations were chosen and a large-scale DNA prep was made from each one, which was used to transfect the HEK 293 cells for expression in vivo.

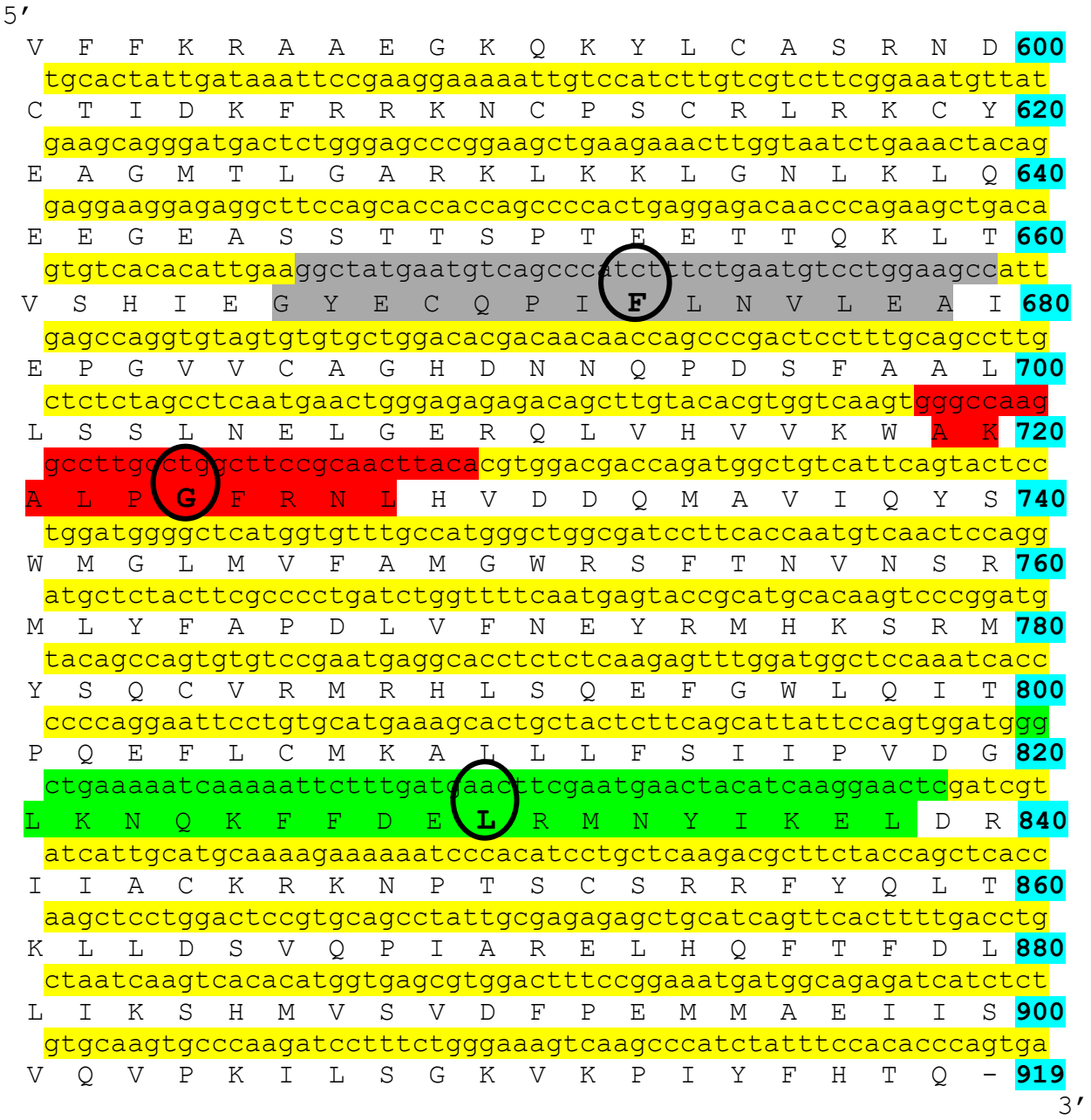

Figure 15: Nucleotide and Amino Acid Sequence of Wild-Type AR (WT-AR). Mutation targets are circled with primers selected highlighted as follows: F673 grey, G724 red, and L830 green.

4.4 Dual Luciferase Activity Results

To test the affinity of the DDT compounds for the BF3 site, dual luciferase activity assays was performed on extracts of HEK293 cells transfected with wild type AR or mutated AR along with the *Renilla* luciferase TK and Firefly vectors.

4.4.1 Cytotoxicity of DDT compounds in HEK 293 Cells Results

A cytotoxicity test was performed to ensure a non-toxic concentration of our DDT compounds was used in these experiments. The DDT was found to be highly toxic at a concentration of 1mM DDT (Figure 16). However, to ensure our results were not affected by the toxicity of DDT or related compounds to the HEK 293 cells, 10 μM DDT was selected as the optimum DDT compound concentration to perform the effects of AR activity.

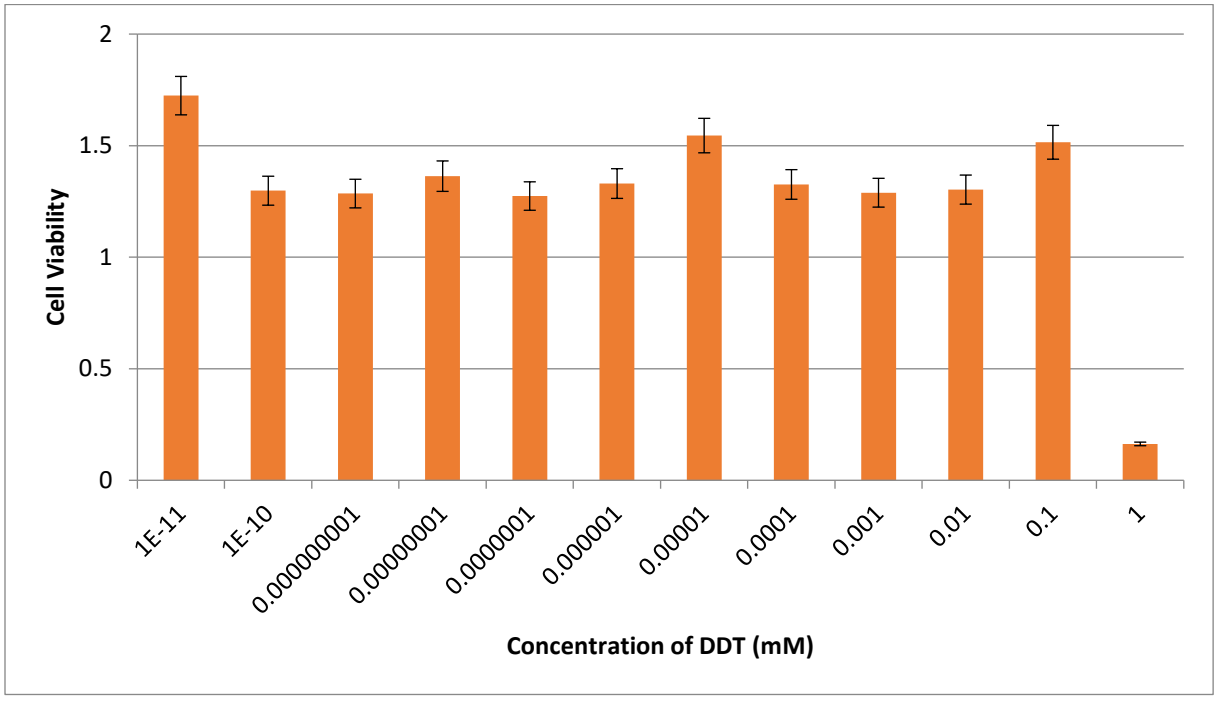

Figure 16 : Cytotoxicity of DDT on HEK 293 Cells

4.4.2 Optimal Concentration of DNA

HEK 293 cells were transfected with the *Renilla* Luciferase reporter, Firefly reporter and WT AR DNA amounts as shown (Figure17). Results showed that the optimum amount of each DNA to use for our protocol was 0.5ng AR, 0.5ng Firefly, and 0.5ng *Renilla*.

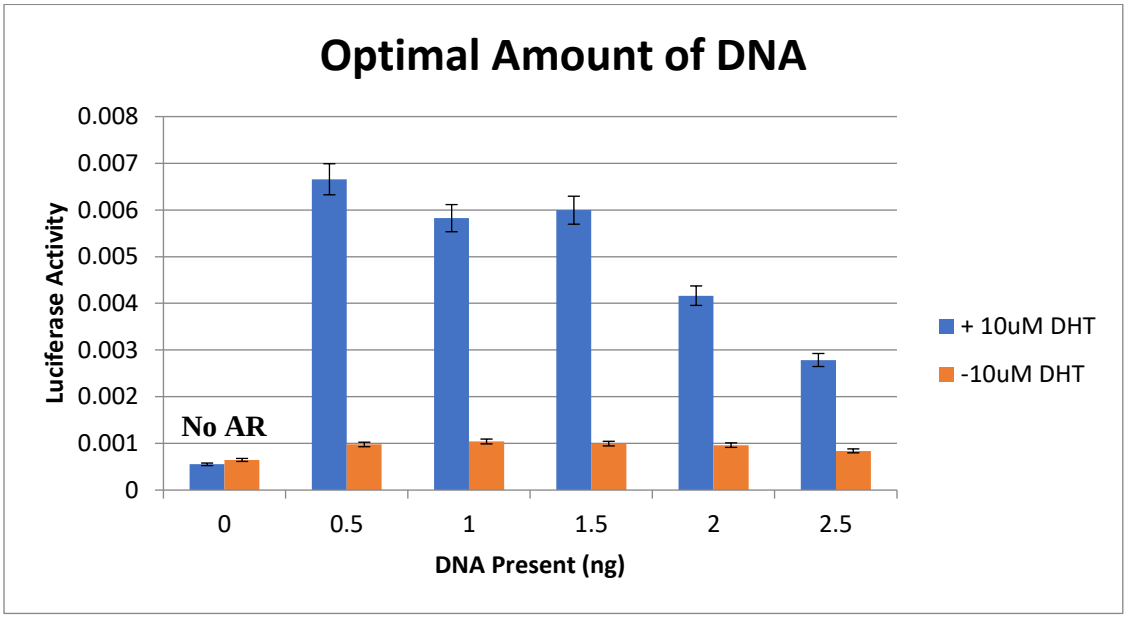

Figure 17: Optimal Amount of DNA for Transfection

The first two samples in Figure 16 were transfected only with the *Renilla Luciferase* and Firefly vectors, showing a dependency on AR in the assay. The optimal amount of DNA used to transfect was 0.5ng for all three vectors. The Blue bars show the activation of the AR by the addition of DHT, versus the orange bars showing no activation of AR when DHT is not present. Therefore, the activity of AR is measured in these assays by the amount luciferase activity (Figure 17).

4.4.3 Effects of varying DDT levels on AR activity.

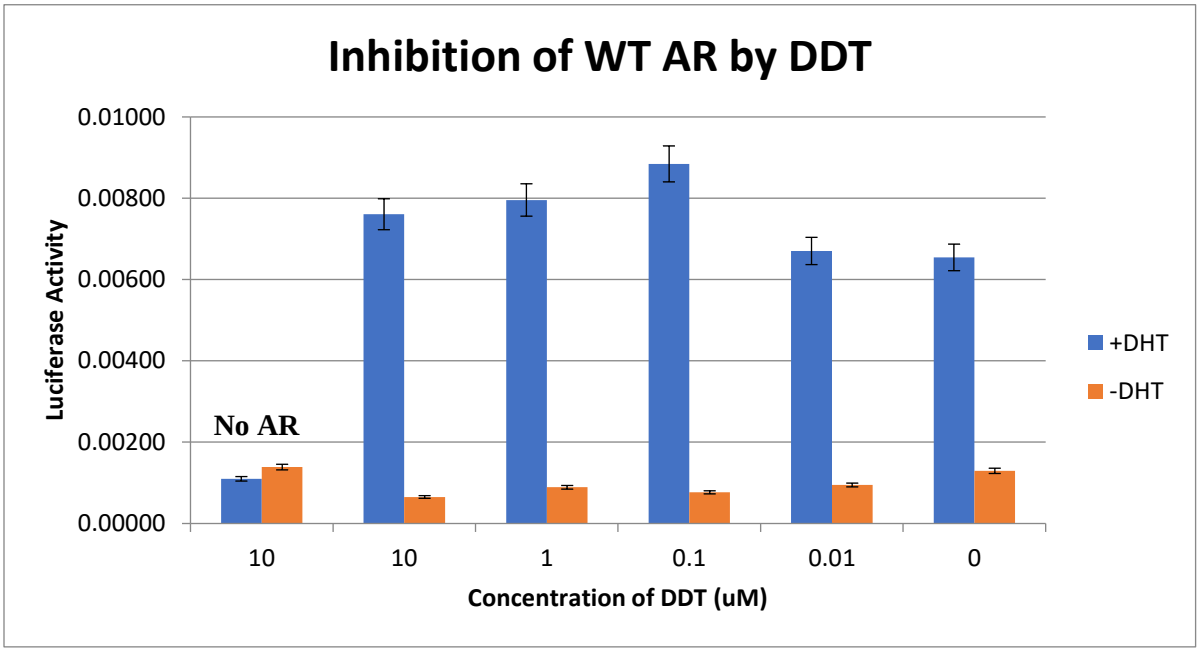

Figure 18: Inhibition of Wild Type AR by DDT. The two bars on the left show no activity when AR DNA is absent.

Varying levels of DDT were tested for an effect on AR activity. The purpose was to find a DDT concentration range for determining an IC_{50} value for inhibition. However, no inhibition of AR activity was observed for concentrations up to 10 μM (Figure 18). Several variations were tested, including different concentrations of DDT, different timed periods of transfection of AR DNA, and different concentrations of DNA. None of those trials showed any significant inhibition of AR activity. This was consistent with previous published observations, where not enough inhibition was observed using varying concentrations of DDT to get a full dose-response curve in HepG2 cells.[Maness] However, it was found that varying levels of DHT in the presence of a constant concentration of the test compound gives a reliable measure of the potency of the inhibitor. The more potent the inhibitor, the higher the DHT concentration necessary to overcome the inhibition, and the greater the shift in the EC_{50} for DHT.[Maness] Therefore, varying

the concentration of DHT was tried either in the presence or absence of 10 μM DDT (Figure 19).

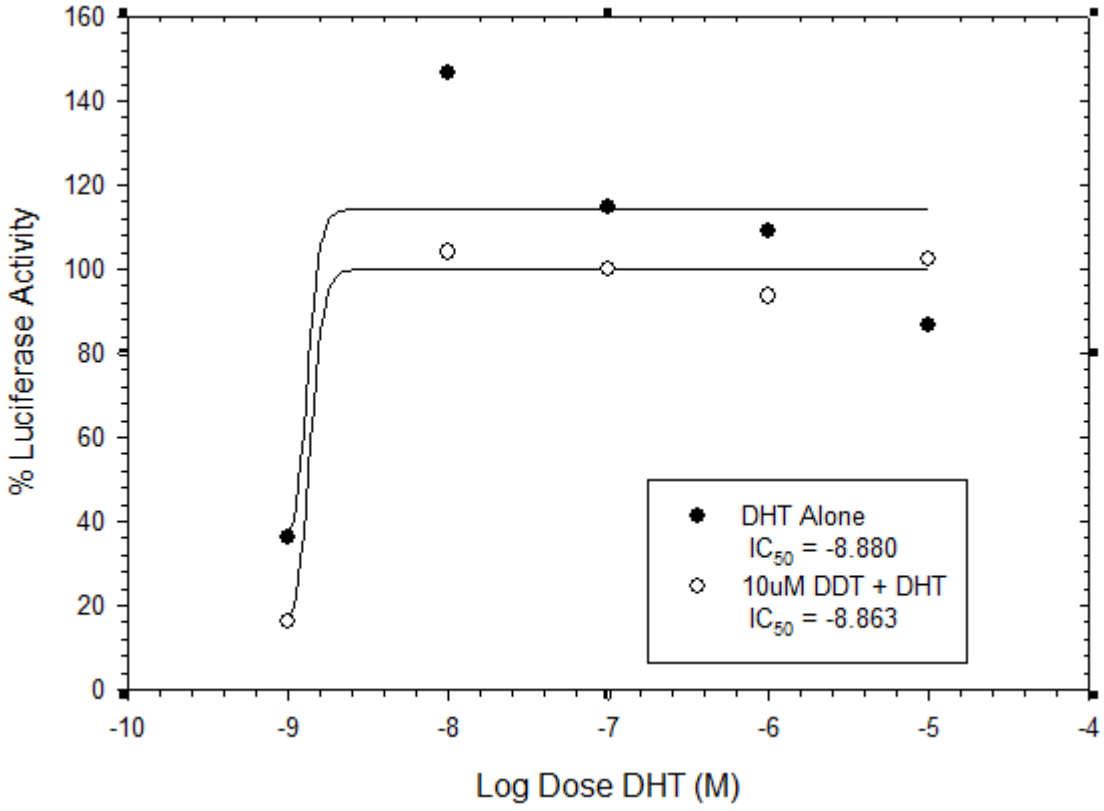

Figure 19: Inhibitory effect of DDT on AR activation by DHT.

When the concentration of DHT was varied in the presence or absence of 10 μM DDT, a transition was observed from low concentrations of DHT that did not show AR activity (luciferase activity) to high concentrations of DHT that did express AR activity (Figure 18). However, no effect of the presence or absence of DDT was observed on the concentration of DHT needed for AR activation. Other research on the inhibition of AR activity has shown that DDE is a more potent inhibitor of AR activity in vivo, compared with DDT.[Kelce] Previous results (Section 4.1 above) showed that DDE inhibited DHT binding with an IC_{50} value of 8 μM, and induced the release of DHT with an IC_{50} value of 82 μM (Table 3). Therefore, the luciferase experiment was repeated using DDE to inhibit AR activity (Figure 20).

4.5 Measurement of DDE Inhibition of AR Activity

Varying concentrations of DHT were tested either in the presence or absence of 10 μM DDE (Figure 20). Again, a transition was observed from low concentrations of DHT that did not show AR activity to high concentrations of DHT that did. This time, however, the presence of 10 μM DDE shifted the dose-response curve for DHT higher by approximately 8-fold, compared with the absence of DHT, from a log EC_{50} of -8.872 to a log EC_{50} of -8.006 (Figure 20). We now had an assay for the effect of DDE on AR activity in vivo.

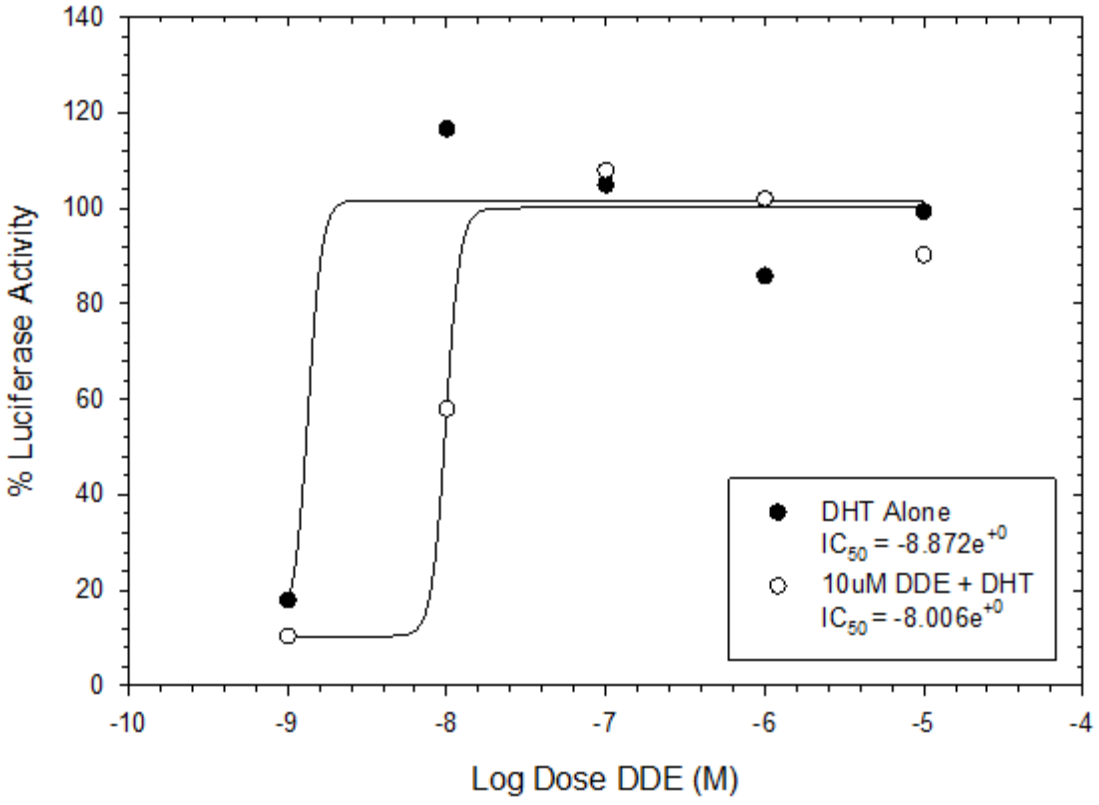

Figure 20: Effect of DDE on Activation of WT AR by DHT concentrations ranging from 1×10^{-5} to 1×10^{-9} M

4.5.1 Effect of DDE Inhibition on DHT Activation of Wild Type AR

To locate the dose-response curves more in the center of the range of DHT concentrations tested, we shifted the concentration range from between 1×10^{-5} and 1×10^{-9} to between 1×10^{-6} and 1×10^{-11} M. Now the two dose-response curves were centered within the concentration range tested, this time showing an 8.4-fold increase in the EC_{50} value for AR activation in the presence of DDE (Figure 21).

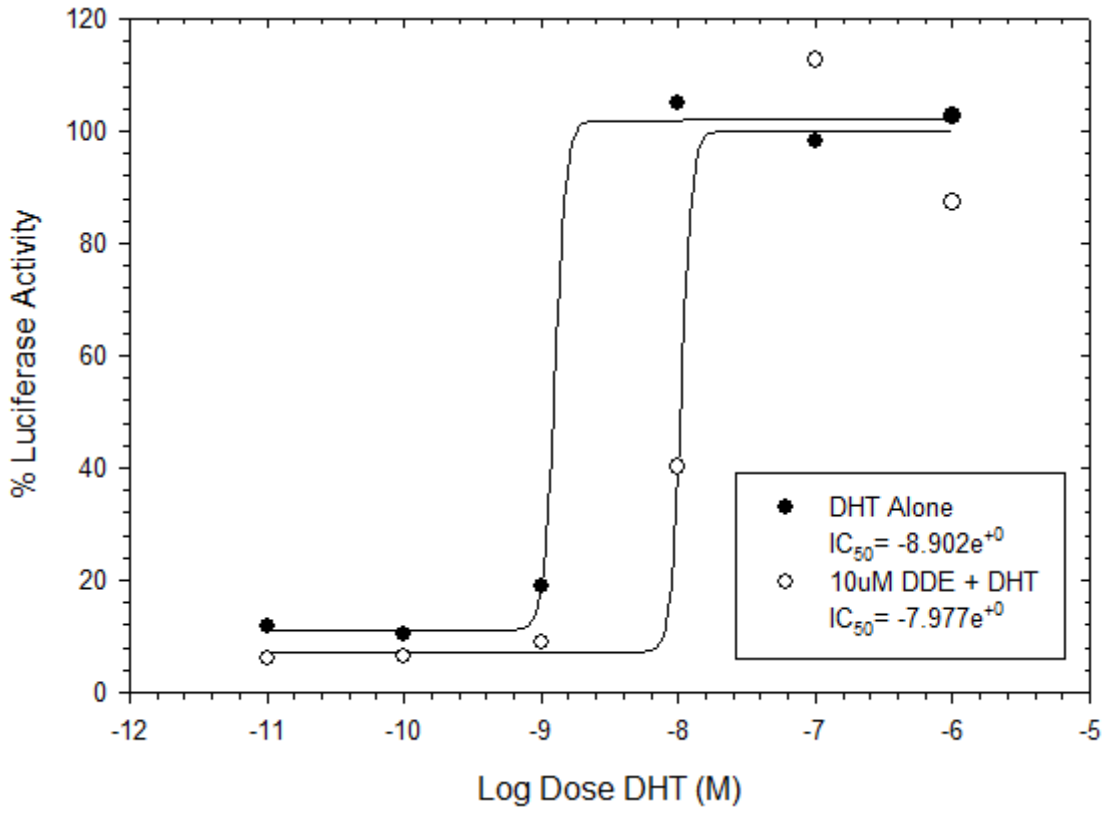

Figure 21: Effect of DDE on Activation of WT AR by DHT concentrations ranging from 1×10^{-6} to 1×10^{-11} M

This validated the technique for the measurement of DDE inhibition of AR activation, allowing the analysis of the effect of the BF3 point mutations on the inhibition by DDE of AR activation.

4.5.2 Effect of DDE on DHT Activation of Mutated F673W AR

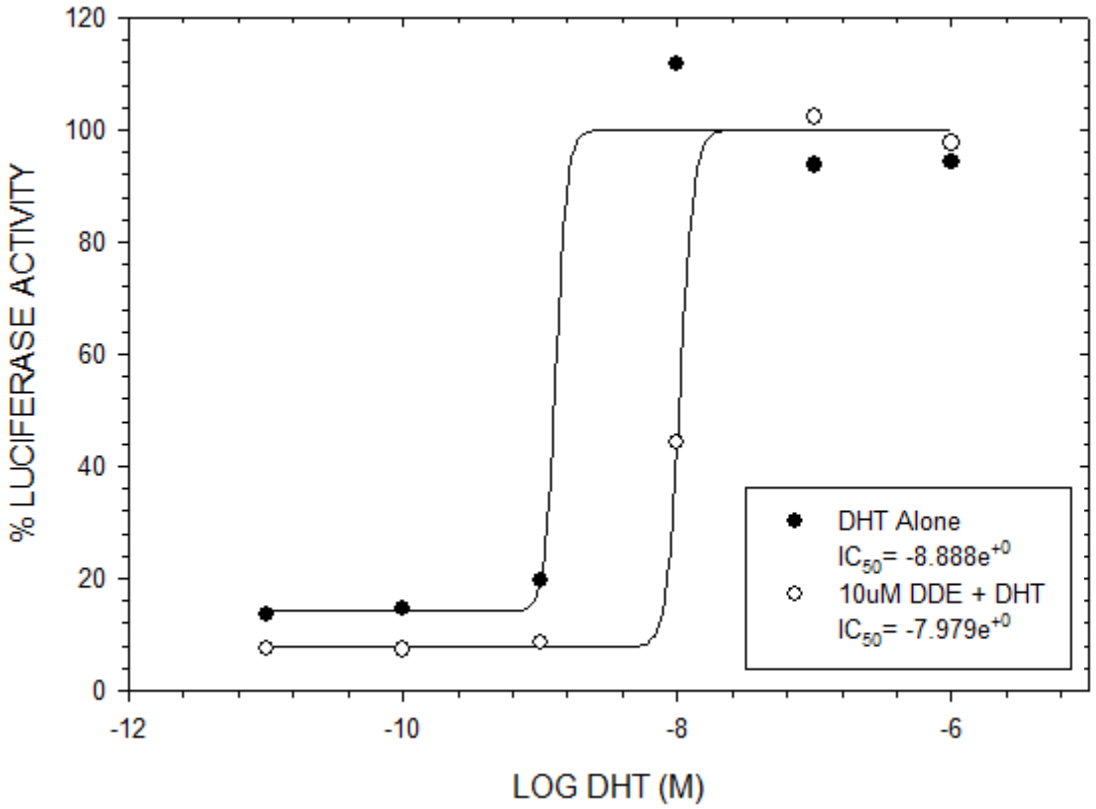

Figure 22: Effect of DDE on DHT Activation with Mutated F673W AR

Using the AR F673W mutation, described previously as making the BF3 site more rounded and smaller in size, the activation by DHT was shown to be reduced by about 8-fold by DDE (Figure 22, Table 4).

4.5.3 Effect of DDE on DHT Activation of Mutated F673K AR

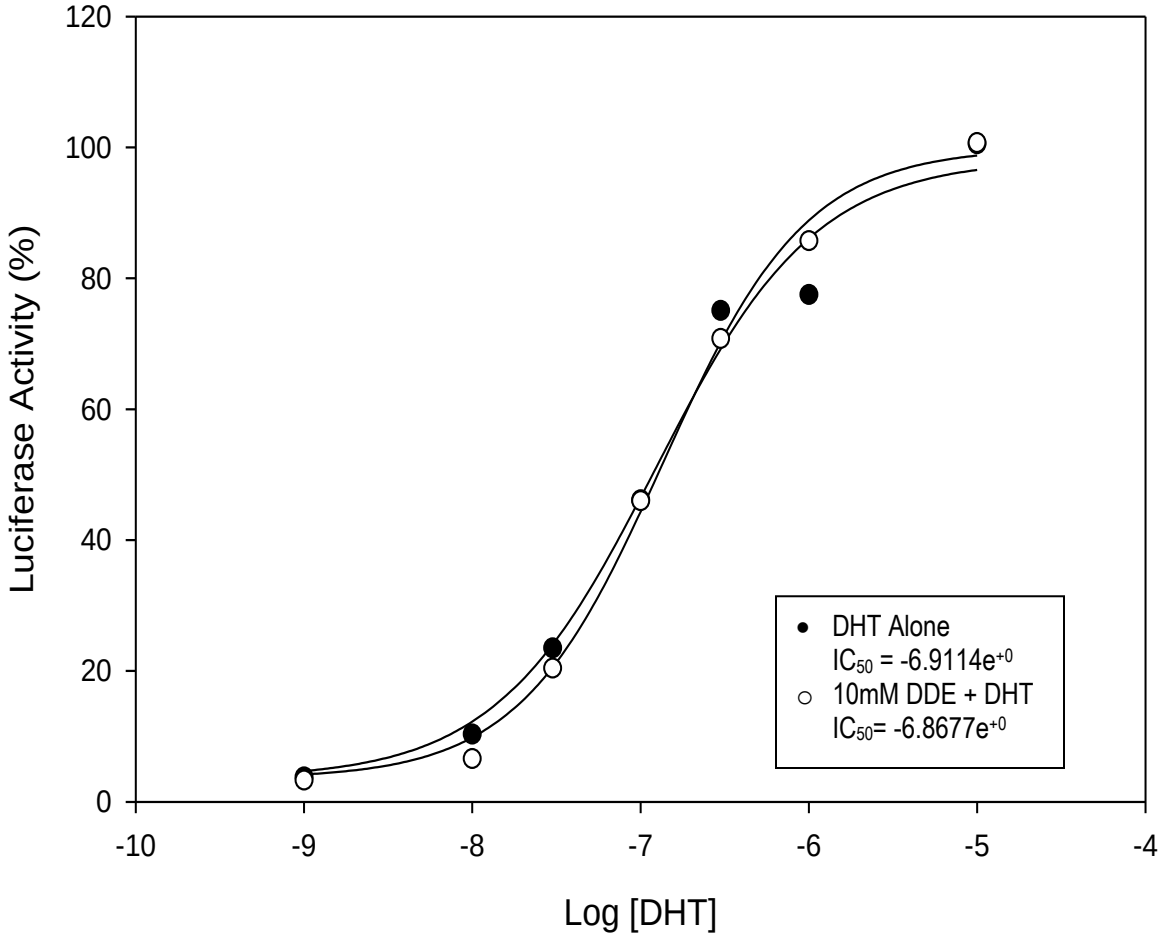

Figure 23: Effect of DDE on DHT Activation with Mutated F673K AR

Using the AR F673K mutation shows that the positive charge introduced with the Lysine(K) amino group and the tighter binding site reduces the inhibition by DDE of AR activation. The inhibition by DDE is reduced to 3.7-fold when this mutation is present (Figure 23, Table 4).

4.5.4 Effect of DDE on DHT Activation of Mutated G673M AR

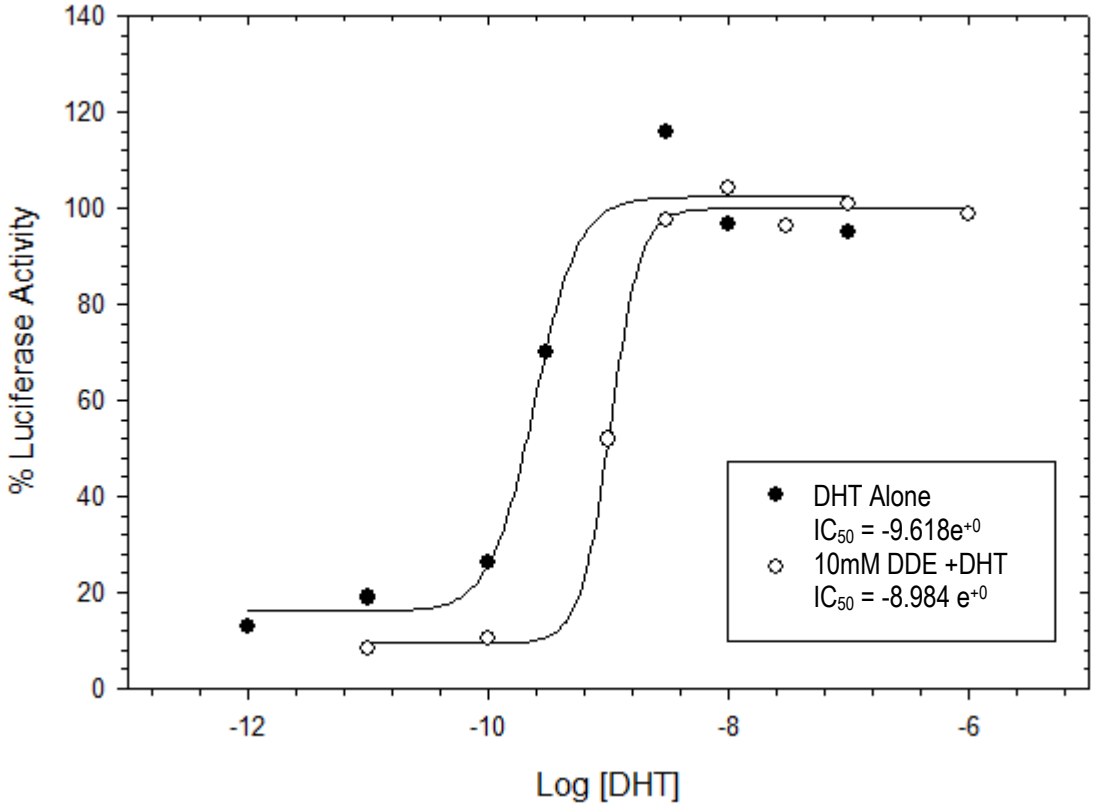

Figure 24**:** *Effect of DDE on DHT Activation with Mutated G724M AR*

The AR G724M mutation yields a narrowed BF3 site due to replacing the glycine with the bulky side chain of methionine. Results indicate this reduces the inhibition of AR activation by DDE to 4.3 fold (Figure 24, Table 4).

4.5.5 Effect of DDE on DHT Activation of Mutated G673R AR

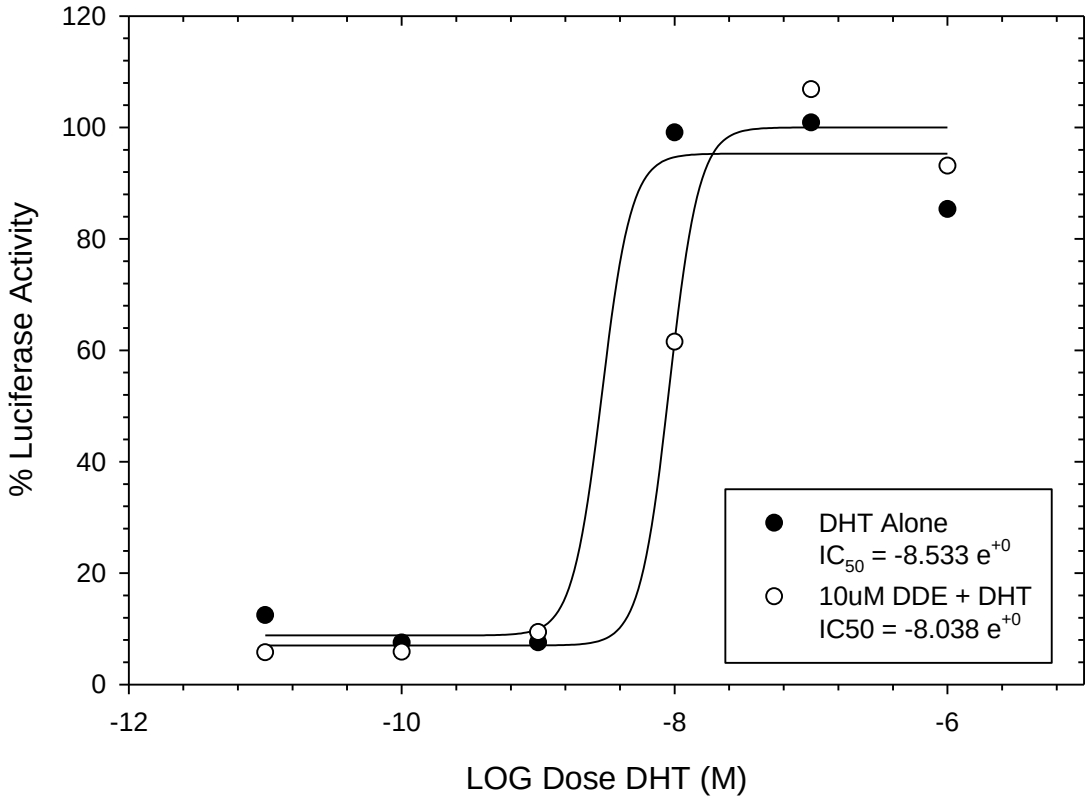

Figure 25: Effect of DDE on DHT Activation with Mutated G724R AR

DDE inhibition using the AR G724R mutation demonstrates that the bulky positive guanidinium group present in arginine inhibits the interaction of DDE with AR and drops the DDE inhibition of AR activation by 3.1-fold. (Figure 25, Table 4).

4.5.6 Effect of DDE on DHT Activation of Mutated L830D AR

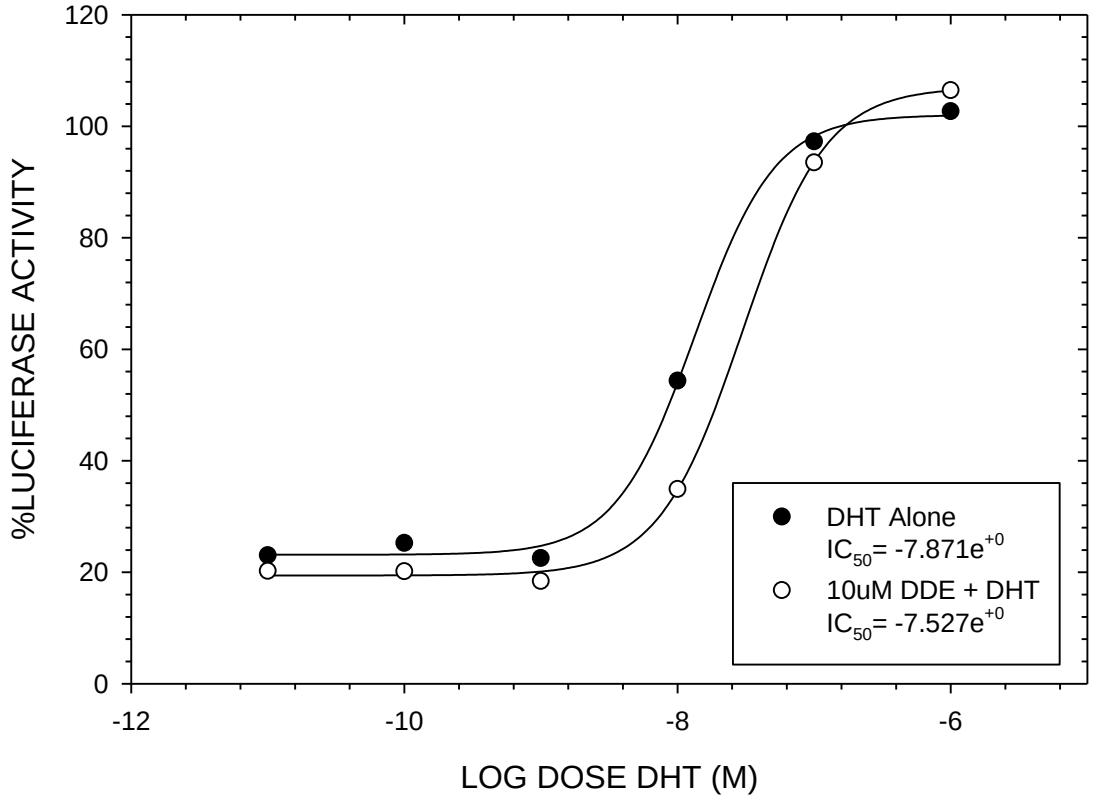

Figure 26: Effect of DDE on DHT Activation with Mutated L830D AR

Results of using the AR F673K mutation, which yields a more open BF3 site and a negative charge at the center of the site, also show reduced inhibition by DDE. A reduction of inhibition to 2.2-fold is observed when DDE is present (Figure 26, Table 4).

4.5.7 Quantitation of Inhibitory Effects using DDE with WT or Mutated AR

Results show an 8.4-fold higher EC_{50} value for wild type AR in the presence of DDE, indicating an interaction between DDE and a site on AR. Lower EC_{50} values are observed for inhibition of four of the mutant AR proteins by DDE (Table 4). This supports our hypothesis that the BF3 site serves as the site for interaction for DDE, and likely also for the site of interaction

for many other hydrophobic compounds with a diphenyl structure. Occupation of the BF3 site by a diphenyl compound allosterically regulates AR conformational structure and activity. Therefore, BF3 may be a promising candidate for an AR regulatory site which can be taken advantage of for the prevention of adrenal cancer.

Table 4: Inhibition Values for WT-AR and AR Mutations

DNA	Log EC_{50} -DDE	EC_{50}	Log EC_{50} +DDE	EC_{50}	Ratio
WT AR	-8.902	1.25E-09	-7.977	1.05E-08	8.4
F673K	-6.9114	1.23E-07	-6.8677	1.36E-07	1.1
F673W	-8.888	1.29E-09	-7.979	1.05E-08	8.1
G724R	-8.533	2.93E-09	-8.038	9.16E-09	3.1
G724M	-9.618	2.41E-10	-8.984	1.04E-09	4.3
L830D	-7.871	1.35E-08	-7.527	2.97E-08	2.2

CHAPTER V

CONCLUSION

The BF3 site on the androgen receptor surface can serve as a regulatory site for androgen receptor activity. The diphenyl compound DDE showed inhibition of wild type AR activity, but showed reduced inhibition using AR proteins mutated in the BF3 site. This reduction in inhibition observed with the mutant BF3 sites confirms our hypothesis that DDT and related compounds interact with AR and allosterically regulate its activity via the BF3 site. This suggests a novel understanding for the mechanism of action of DDT as well as other endocrine disrupting chemicals with a diphenyl structure. In addition, it indicates the potential value of the BF3 site as useful target for the development of anticancer medications.

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APPENDIX A

APENDIX A

NUCLEOTIDE AND AMINO ACID SEQUENCE OF WILD TYPE AR

Complete nucleotide and Amino Acid Sequence of Wild-Type AR (WT-AR). Mutation targets are circled with primers selected highlighted as follows: F673 grey, G724 red, and L830 green.

5′

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atggaagtgcagttagggctgggaagggtctaccctcggccgccgtccaagacctaccga
M  E  V  Q  L  G  L  G  R  V  Y  P  R  P  P  S  K  T  Y  R  20
ggagctttccagaatctgttccagagcgtgcgcgaagtgatccagaacccgggccccagg
G  A  F  Q  N  L  F  Q  S  V  R  E  V  I  Q  N  P  G  P  R  40
cacccagaggccgcgagcgcagcacctcccggcgccagtttgctgctgctgcagcagcag
H  P  E  A  A  S  A  A  P  P  G  A  S  L  L  L  L  Q  Q  Q  60
cagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcaagagact
Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  E  T  80
agccccaggcagcagcagcagcagggtgaggatggttctccccaagcccatcgtaga
S  P  R  Q  Q  Q  Q  Q  Q  G  E  D  G  S  P  Q  A  H  R  R  100
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G  P  T  G  Y  L  V  L  D  E  E  Q  Q  P  S  Q  P  Q  S  A  120
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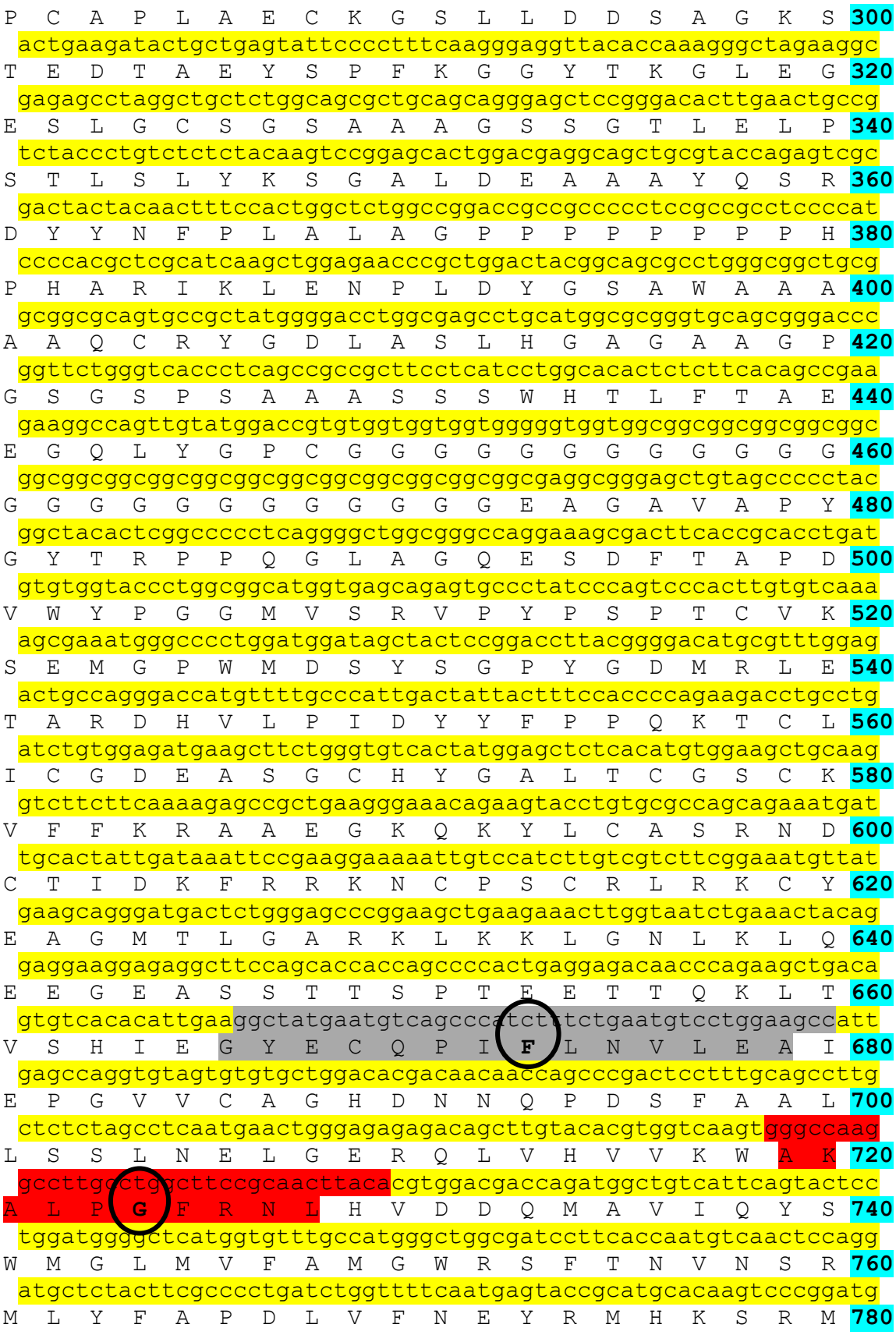
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3’

BIOGRAPHICAL SKETCH

Tahyra M. Resto Santos. She was born in Fajardo, Puerto Rico in 1993. She obtained her high school diploma from Sharyland High School in 2011. She first attended South Texas College and obtained her associates in chemistry in 2013. She then transferred to The University of Texas-Pan American to obtain her Bachelor's in Chemistry in 2015. As an undergraduate, she began working for Dr. Frank Dean research in the Department of Chemistry in UTPA. She was then accepted in UTRGV to pursue a master's degree of science in chemistry program in 2015. As a master student, she continued working as a research assistant for Dr. Dean's laboratory. Also, she began working as a teacher assistance for General Chemistry Laboratory courses. Finally, she completed her master in chemistry and received her M.S. degree in May of 2019. Her permanent address is tahyraresto@yahoo.com.