STRUCTURAL CHARACTERIZATION AND LIGAND SPECIFIC PROTEIN INTERACTIONS OF ANDROGEN RECEPTOR

By: Stacy Baker

The Ohio State University
School of Allied Medical Professions
Medical Technology

Honors Thesis
2007-2008

Honors Committee:

James T. Dalton, Ph.D.
Research Co-Advisor
The Ohio State University
Professor of Pharmaceutics
Department of Pharmacy

Sally V. Rudmann, Ph.D., MT (ASCP) SBB
Research Co-Advisor
The Ohio State University
Professor and Director
Division of Medical Technology

Tammy Bannerman, Ph.D.
The Ohio State University
Faculty, Medical Technology Division

Terri Bruckner
Clinical Instructor
School of Allied Medical Professions

TABLE OF CONTENTS

ABSTRACT

Background: Androgen receptor (AR) is a protein in the human body that binds various steroidal androgens such as testosterone and causes specific anabolic and androgenic activities. Current methods of androgen therapy are limited in use because of their inability to target anabolic versus androgenic activities. Selective androgen receptor modulators (SARMs) are nonsteroidal AR ligands that hold clinical promise in that they have the ability to selectively target anabolic versus androgenic activities, because they have the ability to act as agonists and antagonists in various tissues. Despite all that is known about SARM pharmacology, there is limited information about their structure, stability, and protein-protein interactions.

Study Design and Methods: The question arises of whether or not two known SARMs (007 and 014) differ in their biochemical properties when bound to AR. To explore the question of which complex is the most stable, AR-ligand complexes (i.e., AR-007 and AR-014) were formed with the SARMs and they were exposed to heat to determine if the two had different degradation rates. To determine differences in three-dimensional conformation, the complexes were degraded by typsin to explore if they yielded different patterns of degradation. Finally, to determine whether or not the two complexes have different protein-protein interactions, heat shock protein 70 (hsp70) was measured via a Western blot technique to determine if the two complexes have different levels of association with hsp70.

Results: AR-007 degraded more quickly than AR-014 when exposed to heat. AR-007 and AR-014 were broken down by trypsin in a similar fashion. AR-007 and AR-014 seem to have a tighter and more coiled conformation than AR-DHT (dihydrotestosterone), which was used as a positive control, because AR-DHT was broken down more readily by trypsin than the AR-SARM complexes. The AR-007 complex also had a stronger association with hsp70 than the AR-014 complex.

Conclusion: The faster degradation of AR-007 when exposed to heat and its stronger association with hsp70 indicates that it is the least stable out of the two SARM complexes with AR. We conclude that SARM-induced conformational changes in the AR contribute to their differing pharmacology from steroidal androgens. These conformational changes are ligand-specific and affect the stability of the AR and its interactions with other intracellular proteins.

ACKNOWLEDGEMENTS

Dr. James Dalton – Thank you for giving me the opportunity to work with you in your laboratory. I appreciate all of the help and insight that you have given me during my research experience. You have opened my eyes to the world of research, and a possible future career! I have learned a great deal while working in your laboratory, and I am truly appreciative for this experience.

Natalie Goldberger – There is no way that I could have done this without you. You have been a mentor to me throughout this whole process. I cannot tell you how helpful you have been to me over the past year. Thank you for spending so much of your time helping me and guiding me through this process. I truly appreciate everything you have done for me!

Dr. Sally Rudmann – Thank you for getting me motivated and excited about doing research. You are a large part of the reason why I decided to begin doing research in the first place. You have been incredibly helpful to me by guiding me through the process, making sure I met all of the deadlines, and making sure I kept myself on track. I am very grateful for all of the extra time you have put into being my research advisor.

Dr. Margaret Teaford – Thank you for guiding me through my research project. You have been very helpful in keeping me on track to complete my research project, and I appreciate all of the help you have offered me.

Dr. Tammy Bannerman – I would like to thank you not only for serving on my research committee but for serving as my academic advisor. I could not have asked for a better advisor, and I appreciate your help in guiding me through my academic career including my research project.

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Androgen Receptor Functions

Androgen receptor (AR) is a protein that functions as an intracellular hormone receptor. It specifically binds androgens, such as testosterone, which leads to the regulation of various bodily functions. AR is expressed the strongest in certain types of tissues in the body including the prostate and epididymis in males, the adrenal gland, skeletal muscle, the liver, skin, and in the central nervous system (1). There are two basic types of bodily functions that are regulated by AR: androgenic activities, which include the development of male secondary characteristics (voice, hair, skin, etc.) as well as spermatogenesis, and anabolic activities, which include regulation of the size and strength of muscle and bone as well as regulation of the hematocrit (2).

When testosterone is secreted by the body, it binds to AR and guides the expression of proteins associated with both androgenic and anabolic activities. Testosterone is considered a non-selective AR ligand because it stimulates androgenic and anabolic activities to an identical extent with similar potency.

AR is a approximately 110 kDa in size. The gene that codes for AR is located on the X-chromosome at the location of Xq11.2-12 (3). Figure 1 shows

the structure of AR with its various regions. The $-NH_2$ terminal of AR is responsible for transcriptional activation of both full-length AR and constitutively active AR, which is full-length AR minus its ligand-binding domain (3). Toward the center of the molecule is the DNA-binding domain, which allows AR to bind to portions of DNA in target tissues and turn on or turn off the transcription of proteins that regulate anabolic and androgenic activities in the body. The ligand-binding domain (LBD) of AR, which is located in the –COOH terminal end, is where a ligand such as testosterone would bind to either activate or deactivate AR, causing it either to bind to DNA in the nucleus of the tissues it acts upon, leading to mRNA transcription and protein translation, or just the opposite.

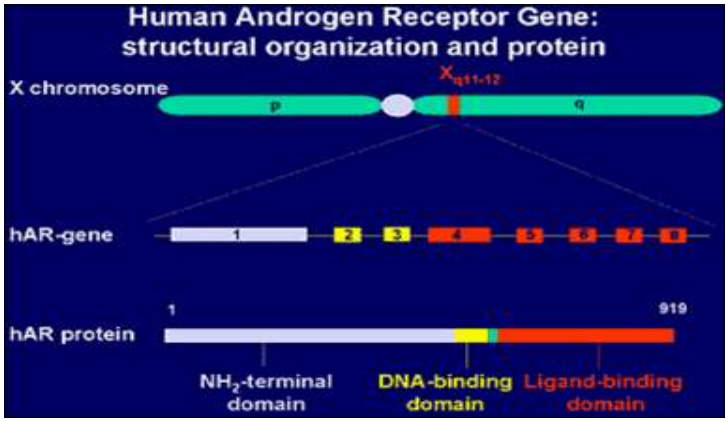


Figure 1: Molecular structure of human androgen receptor. Includes the location of AR on the X-chromosome, the exon breakdown, and the various domains of the molecule. Taken from (10).

1.2 Ligands of Androgen Receptor

The LBD of AR plays an important role in regulation. When there is an agonist such as testosterone bound to the LBD then AR can do its job: binding

to DNA and causing an increase in androgenic and anabolic activities. When there is an antagonist bound to the LBD of AR (or when the AR is unbound), then the androgenic and anabolic activities of the body are turned off (2).

As was previously stated, testosterone is a major endogenous molecule that binds to AR at the LBD. However, there are many different ligands besides testosterone that have been studied which can regulate the function of AR. One important molecule that is synthesized in the prostate and other organs is dihydrotestosterone (DHT). DHT is a more potent metabolite of testosterone that functions as a regulator of male characteristics, a function similar to that of testosterone, but plays a larger role in the maturation and enlargement of the prostate (4). DHT is considered an agonist, which is a ligand that turns on transcription and increases androgenic and anabolic activities in the body.

Bicalutamide is another ligand that binds to the LBD of AR. Bicalutamide is not synthesized by the body, but has been developed as a drug to suppress the action of AR. It is referred to as an antagonist, which turns off transcription and decreases androgenic and anabolic activity in the body. Bicalutamide is used in the treatment of prostate cancer.

Although there are many other ligands that have been discovered, a particularly interesting class that has been developed are called the selective androgen receptor modulators (SARMs) which have the ability to act as both agonists and antagonists, possessing tissue selectivity.

1.3 Selective Androgen Receptor Modulators

SARMs are a new class of synthetic non-steroidal molecules that mimic some but not all of the pharmacologic effects of testosterone. Our laboratory discovered two SARMs, known as 007 and 014, that demonstrate tissue selective androgenic and anabolic activity. Figure 2 shows the chemical structures of these SARMs. Not only do SARMs demonstrate a high level of tissue selectivity when bound to the LBD of AR, but they show a high potency (5). Although the pharmacologic effects of SARMs have been studied already, minimal information is known about their interactions with AR, their molecular mechanisms of activity, and their effects on AR stability. This is a major research question that has arisen in the field of pharmacology and the study of AR. Better understanding of the way that these SARMs work would allow for discovery of SARMs with improved activity, hopefully leading to the treatment of diseases involving the AR.

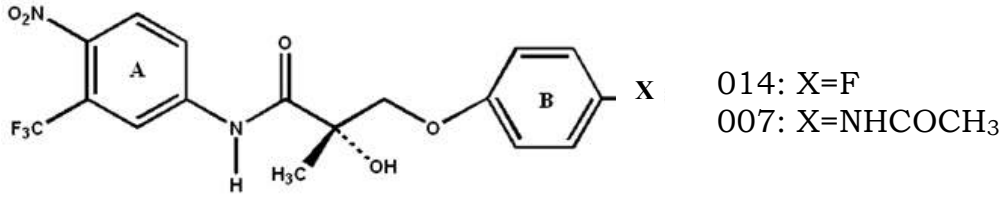


Figure 2: Structures of SARMs 007 and 014. Taken from (2).

1.4 Androgen Therapy

SARMs hold much clinical promise when it comes to patients who need androgen therapy. Androgen therapy is used for a wide variety of diseases and

ailments that occur in both men and women where androgens either function insufficiently or are not produced in an adequate amount. Some of these conditions include hypogonadism, delayed puberty, anemia, primary osteoporosis, endometriosis, and various muscular diseases, to name a few (6).

There are a variety of issues when it comes to the administration of testosterone to a patient who has an inadequate amount. First of all, it is worrisome to administer to women, due to the fact that they may develop undesirable male characteristics if given large enough doses (1). A downfall of testosterone therapy in men is that, as previously mentioned, it may enlarge the prostate and worsen benign prostatic hyperplasia, a common disease in older men. As androgens may also stimulate the growth of prostate cancer, treatment of men with undetected prostate cancer is another concern. Acne is a common side effect in men and women due to the effects of testosterone on the skin. Due to the fact that testosterone is not able to target specific tissues, it simply affects all tissues of the body, which leads to these side effects during treatment.

Bicalutamide has proven to be a successful drug for the treatment of prostate cancer because of its antagonistic activity. However, along with shrinking the prostate, it also causes muscle-wasting and other undesirable effects due to the fact that it does not selectively target the prostate, but it acts on all tissue receptors of AR (7).

The advantage that SARMs have to offer is tissue selectivity. When they are bound to the LBD of AR, they are able to mimic the beneficial effects of

testosterone, such as muscle growth, but avoid unwanted effects, such as prostate growth (2). This is true due to the fact that SARMs have weak antagonist activity in the prostate and agonist activities in the muscle and bone. This leads to the conclusion that SARMs can effectively be used to treat muscle-wasting conditions, hypogonadism, and age-related frailty without causing concern for prostate enlargement or stimulation of undetected prostate cancer.

Although SARMs offer new alternatives for the use of androgen therapy, we understand very little about how they work. Do SARMs interact differently with the AR than steroidal androgens? Does the AR act or work in unique ways when bound to a SARM? Are there differences in stability or protein interactions between AR bound to 007 and AR bound to 014?

There are many different ways to approach the question of the stability of AR-ligand complexes. Typically, less stable proteins degrade faster when exposed to heat. The biochemistry of AR indicates that it changes its three-dimensional conformation when bound to a ligand (6). If there are differences in the way that AR coils and folds when it is bound to each of these SARMs, it leads to stability information about these complexes. If they coil into a tighter three-dimensional structure, then they are less available for protease cleavage, and indeed more stable. Also, looking at protein-protein interactions could be a useful tool. If the AR-ligand complexes associate with more chaperone proteins when transported into the cell, then that suggests that it is a less

stable or less active complex due to the roles that chaperone proteins often play in protecting intracellular proteins from the harsh cellular environment.

Some important research questions to address: are there differences in stability between AR-007 and AR-014? Are there different protein-protein interactions between the two complexes that could lead to the assumption that they have different stabilities and mechanisms of action? Are there structural differences between AR-007 and AR-014 that could allow one complex to be more stable than the other? Finding the answers to these questions could aid in the understanding of the mechanism of action of SARMs, and eventual development of even better drugs.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Androgen Receptor Expression

Although androgen receptor (AR) is expressed in a variety of human tissues, it is difficult to acquire enough of it to study in a laboratory. There are two common techniques to overcome this hurdle. The first is to use an immortalized cell line that expresses high concentrations of the AR. The LnCap human cell line is an androgen-sensitive human prostate adenocarcinoma cell line (8). Cells derived from the prostate, and especially prostate cancer cells, express a lot of the AR due to their dependence on androgens (i.e., testosterone and dihydrotestosterone) for growth. These cells grow and divide rapidly in the laboratory and provide a replenishable source of AR.

The second technique is use of the baculovirus *Autographa californica* expression system in Sf9 insect cells. *A. californica* is pathogenic to many species of insect cells, and has the ability to form intracellular structures called polyhedrons, which have the ability to induce site-specific mutations on the insect genome via recombination of co-transfected cells (9). One of the proteins that this system has been identified as being able to express is AR. By

transfecting Sf9 insect cells with *A. californica,* laboratories are able to obtain a large enough sample of functionally active AR to study. Another benefit of this system is that Sf9 insect cell derived AR has similar immunological and functional properties as its mammalian AR counterparts (10).

2.2 Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) are synthetic compounds that have the ability to act as full agonists, mixed agonists/antagonists, or pure antagonists in tissues expressing estrogen receptors (7). This means that they have the ability to mimic the effects of estrogen in some tissues while blocking it in others. SERMs have been developed to the point where they are used successfully in the clinical setting. Selective androgen receptor modulators (SARMs), like SERMs, have the potential to act in the same manner, only with tissues in which androgens play a role. By studying the mechanisms of action and the clinical benefits of SERMs, more ideas can be formulated about SARMs, which are not yet at the point to where they can be used clinically.

SERMs act on estrogen receptor (ER) in tissues, while SARMs act on androgen receptor (AR) in tissues. ER can be found in a variety of tissues such as breast tissue, and in the cardiovascular, skeletal, and central nervous systems. Estrogens also play an important role in the reproductive system of females.

It is important to regulate the use of estrogens as treatment options because, although they have many positive effects, they have major negative effects as well. Estrogens can effectively inhibit bone resorption, preventing osteoporosis, benefit the cardiovascular system preventing risk of coronary heart disease, and some studies even suggest they may have the ability to delay the onset of Alzheimer's disease (11). However, a major negative effect of estrogens is that there is still concern for the stimulation of breast cancer, in which the risk increases for women who are on estrogen therapy. Much of these same concerns are applicable to the use of androgens for therapy options as well because, although they have positive effects in one area of the body, such as the muscles and bones, they may have detrimental effects on another, such as the prostate.

SERMs offer a solution to these issues associated with the use of estrogen therapy. They have the ability to act on the pituitary gland, leading to an increase in follicle-stimulating hormone, and the uterus in an agonist or antagonist fashion, while acting on breast tissue in an antagonist fashion, decreasing breast cancer risk (12). Given all of the positive outcomes and therapeutic issues that SERMs have solved, the medical world is optimistic about the use of SARMs as a replacement for androgen therapy, and hope that they can yield similar beneficial results in the respective tissues that they act upon.

By studying the mechanism of action of SERMs, it is possible that the mechanism of action of SARMs can be deciphered, if it acts in a similar

fashion. SERMs function through chaperone proteins that serve as co-activators and co-repressors in various cell types in which ER acts. SERMs also function through the conformational change of ER when a SERM binds to it, which determines how strongly the SERM-ER complex recruits these chaperone co-activators and co-repressors (12). When there is a high ratio of co-activators to co-repressors, the action of ER on its target tissue increases, whereas if there is a low ratio, ER will not have a strong effect on its target tissues.

2.3 Benefits of Androgen Therapy for Women

Even though many think of androgens, like testosterone, as male hormones, they have some important benefits for women also. Androgens are produced in the ovaries, adrenal glands, and fat cells in women, and they are important for sexual drive in women. Even though the use of androgen therapy for enhancement of the sexual drive in women has not yet been approved by the Food and Drug Administration, it has been approved for conditions such as Turner's Syndrome, premature menopause, AIDS-related and age-related wasting conditions, and even improvement of sexual function (13).

Turner's Syndrome (TS) is a condition in which a woman has only one X chromosome, instead of the two they should normally have. This in turn causes underdeveloped female characteristics and a lower level of verbal intellectual ability, spatial cognition, working memory, organization, and

planning (14). Androgens such oxandrolone have proven to be a beneficial treatment option in improving the cognitive shortcomings of women with TS (14). However, oxandrolone has a 10-100 times lower affinity toward AR than testosterone or dihydrotestosterone and is not selective, meaning that its long-term effects are unknown but likely similar to the endogenous steroidal androgens (15). The use of SARMs could overcome these disadvantages because they have a stronger affinity toward AR, and have the potential for selective and hopefully better, long-lasting results.

Premature menopause is another condition that can be treated using androgen therapy. Women affected by premature menopause typically have low libido, fatigue, and diminished well-being (16). Testosterone replacement therapy has proven to be beneficial in improving these undesirable effects. Due to the fact that there is an age-related decline in the production of testosterone by both the adrenal gland and the ovaries, this decline can be attributed as having an effect on the onset of menopause, playing a factor in causing these symptoms (17).

Another benefit of androgen therapy in women is in the way that it builds muscle mass and bone. This is beneficial for both aging women and for AIDS patients, both of whom typically have lower muscle mass and bone density caused by their wasting conditions. The use of androgens in these types of patients has proven to be beneficial, because androgens have the ability to strengthen and build muscle and bone (18).

Androgen replacement therapy in women is an important treatment option for women with a vast variety of conditions. Common side effects include skin conditions (such as greasy hair and skin, itchy scalp, and hair loss), and also a decrease in HDL-cholesterol and total cholesterol (15). This is where the use of SARMs as an alternative to androgen replacement therapy could benefit women. They have the potential to have the same positive, anabolic effects in women while avoiding the negative side effects (1).

2.4 Biochemistry of SARMs

SARMs have the ability to not only bind to AR with a very high affinity, but they have the ability to cause it to act as either an agonist, turning on transcription, or an antagonist, turning it off (2). The mechanism as to how this occurs is very complicated, and the biochemical interactions must be observed as to how this phenomenon takes place.

The SARMs 014 and 007 bind to AR with a very high affinity. The reason for this is their ability to interact with hydrophobic and hydrogen bond partners in the LBD of the AR. The structures of the SARMs needed to be R-isomers at the alpha carbon when incorporating a thioether of sulfonyl linkage, have an electrophilic para-substituent or hydrogen bond partner on the B-aromatic ring, have a nitro group in the para-substituent in the A-aromatic ring, and have a tri-fluoromethyl at the meta position of the A-ring (see figure 2) (19). This structure can have modifications made on other areas of the molecule, providing great flexibility in chemical design and synthesis.

How is it that SARMs cause AR to act as an agonist and an antagonist? The answer likely lies in the fact that they have the ability to cause conformational changes in AR (20). AR has 11 alpha-helices in its structure (3). The helices are numbered up to 12; however, the number 11 helix is missing. When steroid agonists such as testosterone or dihydrotestosterone bind to AR at the ligand-binding domain (LBD), helix 12 closes over the binding pocket, which makes an area of the AR available for coactivator interaction. On the other hand, when antagonists bind to the LBD, helix 12 does not fully close and the coactivator interaction site is not formed (19). It is possible that the tissue selectivity of SARMs could depend on the unique way that they cause the structure of AR to change, causing the recruitment of coactivators or corepressors (20).

2.5 Chaperone Proteins of AR

AR also interacts with various intracellular proteins while it is being transported to the nucleus after ligand binding. Since testosterone is a steroidal hormone, it has the ability to cross the cell membrane and enter the cytoplasm of cells. Prior to testosterone or any other ligand being bound, AR forms complexes with specific chaperone proteins. Two of these proteins are heat shock protein 70 (hsp70) and heat shock protein 90 (hsp90). They aid in the movement of AR through the cytoplasm and to the nucleus along microtubular tracks (21).

Given the complexity of the nucleus, these heat shock proteins must be able to transport AR to the exact location in the genome at which it needs to act (22). There is a signal transduction pathway within the nucleus that allows a hormone receptor, such as AR, to bind to its intended genomic location. The exact mechanism for AR is not known; however, a similar hormone receptor, glucocorticoid receptor (GR) has a pathway that is thought to be similar to that of AR, as shown in figure 3. With GR, the microtubules interact with a molecule called Dynein, which is bound by its intermediate chains to an immunophilin, which then reacts simultaneously with hsp70 and hsp90 (which is bound to molecule p23), both of which interact with the GR-ligand complex (23). This complicated scheme is what allows GR to move through the cytoplasm and to its site of action. Researchers hope that by studying this mechanism, they can come up with a similar mechanism and identify similar chaperone proteins for the transport of AR through the cytoplasm and to the nucleus.

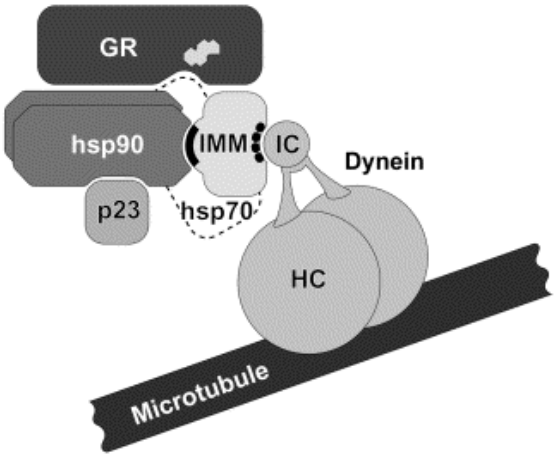


Figure 3: GR movement along microtubules with associated chaperone proteins. Taken from (23).

CHAPTER 3

MATERIALS AND METHODS

3.1 Methodology

There are three goals that this study intends to achieve. The first goal is to determine whether or not the stability of AR is affected by the ligand bound, whether it is dihydrotestosterone (DHT) or a Selective Androgen Receptor Modulator (014 or 007). It is important to explore this research question because if there are differences in stability between these ligands, then it can be deduced that the conformational differences that affect protein-protein interactions and DNA interactions are likely to exist. It might also help to explain observed differences in the pharmacologic effects of different ligands in different tissues.

Another goal is to determine whether or not the level of AR-chaperone protein interaction is dependent on the ligand bound. A higher level of protein-protein interactions will lead to the conclusion that one complex is more stable than other. A higher level of protein-protein interactions may indicate that that complex is less stable than one that has less protein-protein interactions, because proteins typically use chaperone proteins as a stability factor for their journey through the cytoplasm and into the nucleus of cells. Also, are there

ligand-specific protein-protein interactions for AR? Different protein-protein interactions will indicate that these AR-ligand complexes have different mechanisms of traveling through the cytoplasm and into the nucleus. This will indicate that the two SARMs may cause AR to behave in slightly different ways.

Finally, we examined the three-dimensional structure of AR change when bound to each of the three ligands. Different conformational changes could indicate that each of the ligands could cause AR to function in different ways. It could also lead to conclusions about the stability of each of these AR-ligand complexes. A tighter, more coiled three-dimensional structure would likely be more stable than a looser one, because it is less likely to be degraded by proteases and other degradation factors.

3.2 Design, Data, and Instrumentation

3.2.1 Cell Preparation

In order to isolate a sample of AR that could be used to investigate the research questions, it needed to be isolated from certain types of cells in a maximal amount. The cells that were used for all experiments were Sf9 insect cells, because they have the ability to express AR in a large quantity. The cells were grown in 90 mL of *Grace's Insect Cell Media*, supplemented with 10% FBS, to a concentration of 2.0×10^6 cells/mL. After the cells reached the desired density, they were then divided into two Erlenmeyer flasks. After that, 9 mL of high-titer AR baculovirus stock was added to each flask. The cells were then infected with the baculovirus *Autographa californica* so that they would

strongly express AR (10). The AR construct contained a hexahistidine (His_6) tag to simplify purification.

The Sf9 cells with amplified AR were cultured and treated with various ligands. The cells that were used for the following experiments were divided into five different samples: cells treated with Bicalutamide (an antagonist), cells treated with DHT (an agonist), cells treated with SARM 014, cells treated with SARM 007, and cells treated with no ligand at all (negative control). The ligand treatment conditions (also known as the standard conditions) consisted of the addition of 1uM of ligand to the cultured cells after 24 hours of AR baculovirus infection, and then again 48 hours after baculovirus infection. The cells were isolated via centrifuge, and stored at -80 degrees Celsius until further use.

3.2.2 Protein Purification

The first step of the protein purification was cell lysis. The purpose of lysing the cells was to extract the AR protein from the Sf9 insect cells so that it could be further purified. First, each 15 mL sample was treated again with 1 ul/mL of their prospective ligands (Bicalutamide, DHT, 014, 007, or no ligand), to give a final concentration of ligand of 1 uM, after being re-suspended in 100 mL of *Protein Lysis/Wash Buffer* containing Hepes and a protease inhibitor, to prevent the AR protein from being digested prematurely. The suspended cells were subjected to four freeze-thaw cycles in dry ice/100% ethanol to physically lyse the cells by breaking down the cellular membrane, which caused the

contents in the interior of the cells to be released. The cell lysate was then centrifuged at 18,000 RPM for 40 minutes at 4 degrees Celsius. The supernatant, which was the portion containing AR along with other cell proteins, was collected and stored at -80 degrees Celsius for later use.

The next step of the protein purification was TALON® Metal Affinity Chromatography. The TALON® metal affinity resin was equilibrated with 10 mL of *Hepes Protein Lysis/Wash Buffer* containing the prospective ligands for each sample and a protease inhibitor, and then centrifuged to pellet the resin. The cell lysate from the ultracentrifuge was then divided into two halves, which were incubated with the pre-equilibrated resin for one hour each at 4 degrees Celsius by gentle agitation. This allowed for the AR protein to stick to the resin, thus extracting it from the cell lysate, and theoretically away from the other proteins that were in the sample. The resin was centrifuged and washed with 10 mL *Hepes Protein Lysis/Wash Buffer*, again containing the prospective ligands, five times for each sample. The resin was transferred to a column, and the absorbed proteins were eluted with *Elution Buffer* containing 25 mM imidazole and 50 mM imidazole. A total of 9-10 elution portions were collected for each sample. The protein portions were stored at -80 degrees Celsius until further use.

The next step in the protein purification assay was to confirm the level of purity using SDS Polyacrylamide Gel Electrophoresis. The 9-10 elution portions of proteins from the five different samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). This process separated the

various proteins in the elution portions based on their electrophoretic mobility. The gels were stained with a *Coomassie* stain, and photos were taken of each gel. The purpose of this procedure was to verify the purity of the AR protein in each of the elution portions.

The yield of the various elution portions of proteins was determined via quantification using the Bio-Rad Protein® assay. The *Bradford* assay was used, which is a colorimetric assay based on the color change of Coomassie Brilliant Blue® dye, which measures total protein concentration of a specific sample. The "standard assay" conditions were used because the concentration of the protein was expected to be between 20 and 140 ug/mL. If the protein concentrations were above 140 ug/mL, dilutions of the elution portions were made. Absorbance levels were read at a wavelength of 595 nm. Standards of known concentrations of bovine serum albumin (BSA) were used to match the wavelength readings to the actual protein concentrations. Three different dilutions of BSA in water were used to make the standards: 62 ug/mL, 125 ug/mL, and 250 ug/mL.

3.2.3 Stability Experiments

The next portion of the experiment included an exploration of conformational information and stability of AR when it is bound to different ligands. This portion of the experiment attempted to answer the question of whether or not the stability of androgen receptor is affected by the ligand bound. The stability of the various AR-ligand complexes and the affinity to

which each ligand binds to AR was explored to determine whether or not different ligands create different conformational changes.

In these experiments, the various AR-ligand complexes were subjected to heat for various amounts of time. Considering that heat normally has the effect of denaturing proteins, if the AR-ligand complexes remained intact for a long period of time, it suggests that they are more stable than those that degraded.

One elution portion of four of the five samples of purified AR was selected for further analysis: AR-no ligand (second fraction, 50 mM imidazole), AR-DHT (fifth fraction, 25 mM imidazole), AR-014 (first fraction, 50 mM imidazole), and AR-007 (first fraction and second fraction, 50 mM imidazole). The specific fractions were chosen because according to the SDS-PAGE gels, they were the purest of all of the protein fractions, containing virtually only AR and no other proteins. The first stability experiment entailed establishing a negative control by subjecting a small portion of the AR-no ligand protein to 37 degree Celsius heat for four different time periods: 0 min, 15 min, 30 min, and 60 min.

The second stability experiment involved subjecting 1.05 ug of the AR-007, AR-014, and AR-DHT complexes to 37 degree Celsius heat for 0 hours, 6 hours, 9 hours, and 12 hours. The third stability experiment involved a repetition of the second experiment, but using 1.15 ug of the samples and subjecting them to 37 degree Celsius heat for 0 hours, 12 hours, 24 hours, and 36 hours. The samples from all three experiments were subjected to SDS-

PAGE analysis to determine the percentage of each sample that had degraded over time.

After the gels were imaged, a program called Image J® was used to quantify the AR protein bands for each sample on each gel. These values are a reflection of peak intensities of the AR band as it compares to the rest of the proteins in the same lane. Theoretically, these values should reflect the relative amounts of AR remaining in each sample.

3.2.4 Protein-Protein Interaction Experiments

To examine whether or not protein-protein interactions are different amongst the various AR-ligand complexes, another experiment was conducted. A protein that is hypothesized to be associated with AR is heat shock protein 70 (hsp70). The goal of this experiment was to determine if hsp70 was more strongly associated with AR-DHT, AR-007, or AR-014. If it associated more strongly with one over the other, then it could be concluded that that complex is less stable or at least conformationally different.

The AR protein for this experiment was isolated from the cell lysates of the Sf9 insect cells, treated with baculovirus *Autographa californica* and with DHT, 007, and 014 at the standard conditions. The three cell lysates were subjected to immunoprecipitation, which is a technique that uses an antibody specific to the desired protein (AR) to precipitate out that protein.

The immunoprecipitation technique involved pre-clearing 2.0 mg of lysate from each sample by adding 1.0 ug of a control IgG and 50 uL of protein-

A agarose after one wash with a binding buffer. After the wash, each sample was brought to a total volume of 500 uL by adding binding buffer. The samples were incubated for one hour at 4 degrees Celsius. Each sample was centrifuged at 2500 rpm for 30 seconds at 4 degrees Celsius. The supernatant was transferred to a microcentrifuge tube, combined with 2 ug of the primary AR antibody (labeling the –COOH terminal of the protein), and incubated for 24 hours at 4 degrees Celsius with rocking. Then, 50 uL of protein-A agarose was added after one wash with the binding buffer. The samples were then incubated at 4 degrees Celsius for two hours. The immunoprecipitates were collected via centrifugation, washed three times with PBS, resuspended in Electrophoresis Sample Buffer®, and boiled. At this point, the samples were stored at -20 degrees Celsius until later use.

A Western blot method was used to detect the AR protein in each sample, by using gel electrophoresis to separate proteins by shape and size. The samples were labeled by a rabbit anti-AR antibody at the –COOH terminal, and then a secondary anti-rabbit antibody. Results were obtained by developing the membranes on a film. After evidence suggested that AR was indeed present in equal quantities in all samples, the Western blot was repeated, only this time samples were labeled by an anti-hsp70 antibody. Results were examined to determine if different amounts of hsp70 were associated with AR-DHT, AR-007, and AR-014.

3.2.5 Partial Proteolysis

In order to evaluate the stability and conformation of AR-007, AR-014, and AR-DHT, a partial proteolysis experiment was performed. Pure protein samples of AR (the same samples that were used in the stability experiments) were used for this experiment. The three AR samples (1 ug) treated with DHT, 007, and 014 were each combined with 0.02 ug of trypsin, an enzyme that digests proteins. Each sample was divided into four different sub-samples: one that was not treated with trypsin, one that was treated for 10 minutes, one that was treated for 20 minutes, and one that was treated for 30 minutes. Results were examined using Western blotting: once using an antibody labeling the $-NH_2$ terminal and once using an antibody labeling the –COOH terminal of the protein. The films were examined to determine if different protein products were seen when each of the AR-ligand complexes was degraded. This information would serve to evaluate whether or not the three proteins had different conformations and stabilities once exposed to the protein-destructive trypsin.

CHAPTER 4

RESULTS AND CONCLUSION

4.1 Results

4.1.1 Stability Experiments

Three pure protein fractions were used for the stability experiments. The purest protein fractions were used because the idea was to use a sample that contained virtually only AR with no other proteins. By using only AR, it could be reasonably certain that interfering proteins would not skew the data in any way or make the data invalid. The pure protein fractions that were used for all of the following experiments are shown in figure 4 (AR-014), figure 5 (AR-007), and figure 6 (AR-DHT).

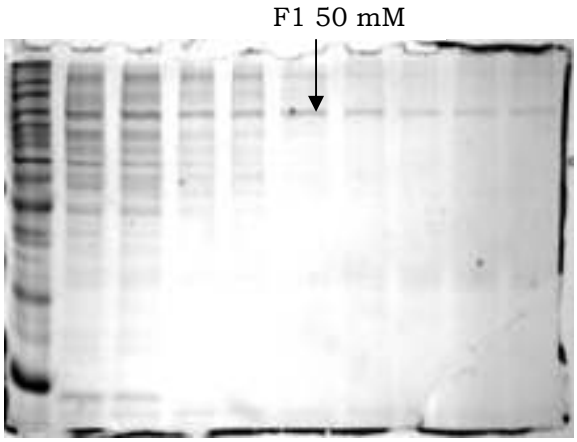


Figure 4: Pure AR-014 Samples

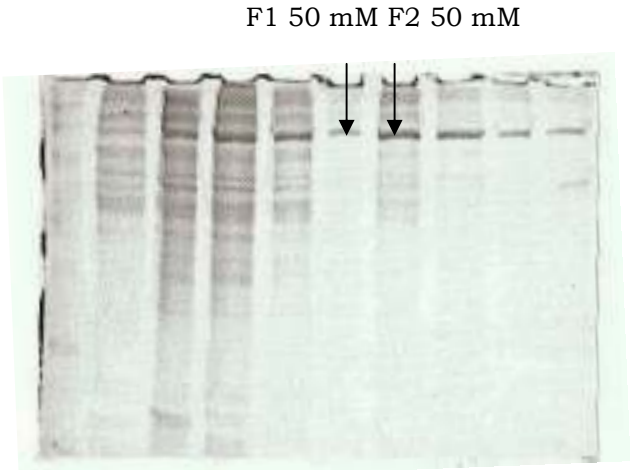


Figure 5: Pure AR-007 Samples

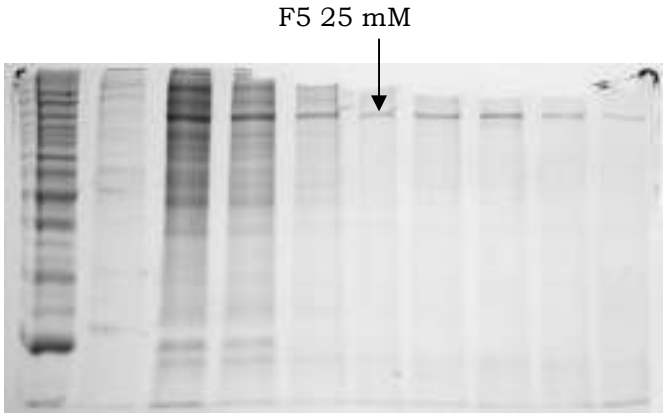


Figure 6:
Pure AR-DHT Samples

Another sample was used as a negative control. This sample consisted of only AR, and it did not have a ligand to make a complex with. As can be seen in Figure 7, AR when it is not complexed with a ligand is not very stable. It was difficult to obtain a sample that had enough pure AR in it to carry out the experiment with no ligand present.

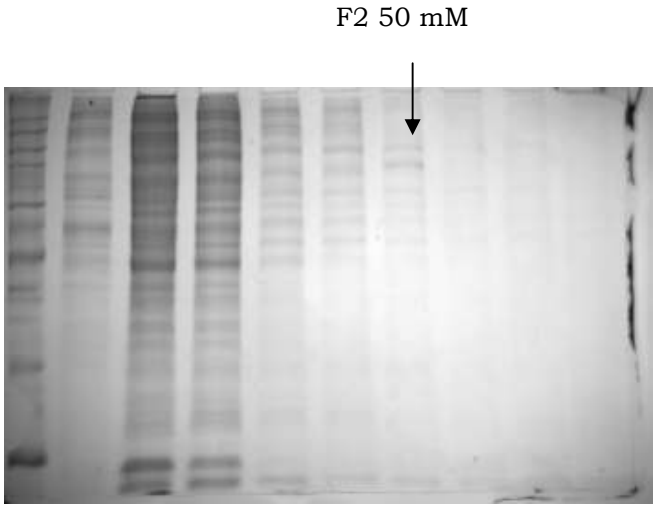


Figure 7: Pure AR-No Ligand Samples

It was important to ensure that each sample was made up of a pure sample of AR, complexed to one of three ligands, because it could then be said that the sample was essentially made up of all AR-ligand complexes, and minimal background protein. This made the experiment more accurate and meaningful.

It was also important to determine the concentrations of the protein samples that were used in the experiments, to ensure that equal amounts of each sample were being used. The concentrations were determined using the Bio-Rad Protein® assay, as described above. The concentrations can be found in Figure 8.

FIGURE 8: TABLE OF PROTEIN CONCENTRATIONS USED FOR STABILITY EXPERIMENTS

Protein Fraction	AR-014 (F1 50 mM)	AR-007 (F1 50 mM)	AR-007 (F2 50 mM)	AR-DHT (F5 25 mM)	AR-No Ligand (F3 50 mM)
Concen-tration	0.077 ug/uL	0.142 ug/uL	0.074 ug/uL	0.119 ug/uL	0.067 ug/uL

The negative control experiment involved exposing 4.28 ug of the AR-no ligand sample to 37 degrees Celsius for 0 minutes, 15 minutes, 30 minutes, and 60 minutes. The samples were subjected to SDS-PAGE analysis and results in figure 9 were obtained. The results suggest that AR degrades readily when it is not complexed with a ligand. Thus, ligands increase the stability of AR in general.

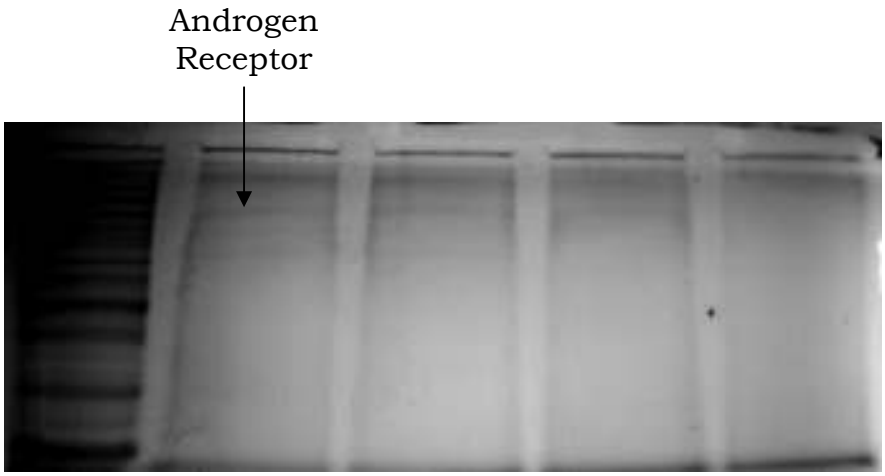


Figure 9:
Stability Experiment Results with
AR-No Ligand

The next question that was addressed during this experiment was which ligands cause AR to be the most stable, or are there even any differences in stability between them? Figure 10 describes the conditions of the first stability experiment. Figures 11 and 12 describe the results of the experiment via SDS-PAGE analysis.

Figure 10: Stability Experiment 1 Conditions

Fraction	Time Period At 37 degrees	Concentration (ug/ul)	Volume (ul)	Amount of AR added (ug)
AR-014 F1 50 mM	**0 hr, 6 hr, 9 hr, 12 hr**	**0.077**	**13.6**	**1.05**
AR-007 F2 50 mM	**0 hr, 6 hr, 9 hr, 12 hr**	**0.074**	**14.2**	**1.05**
DHT F5 25 mM	**0 hr, 6 hr, 9 hr, 12 hr**	**0.119**	**8.82**	**1.05**

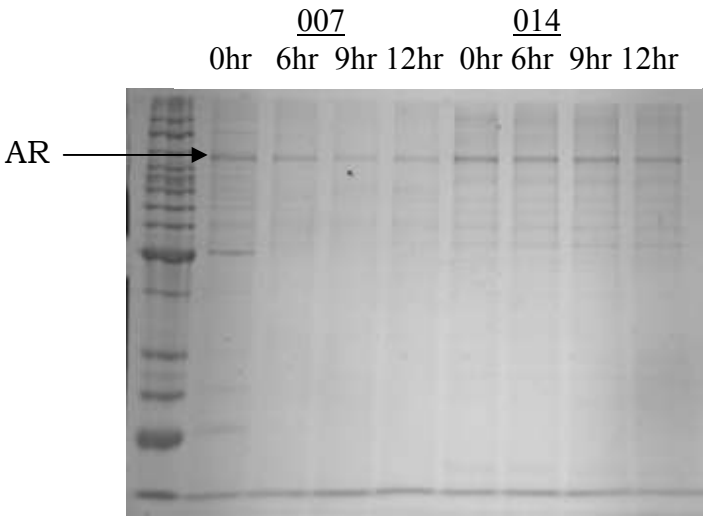


Figure 11: Stability Experiment 1 Results with AR-014 and AR-007

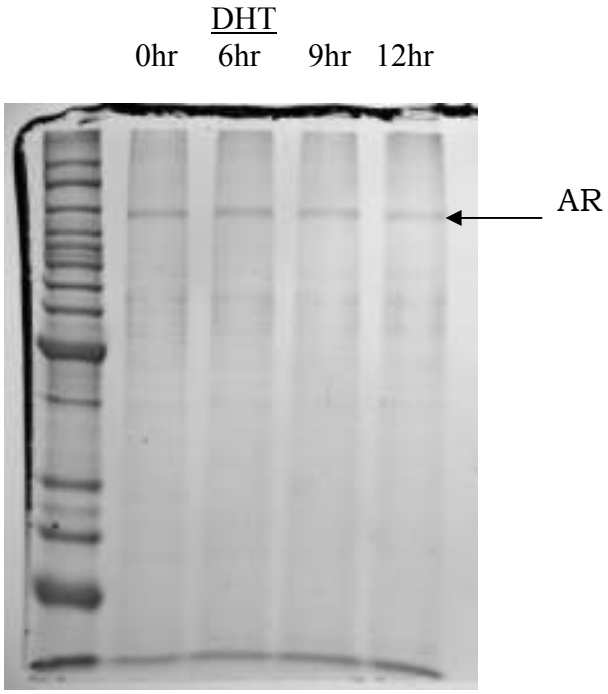
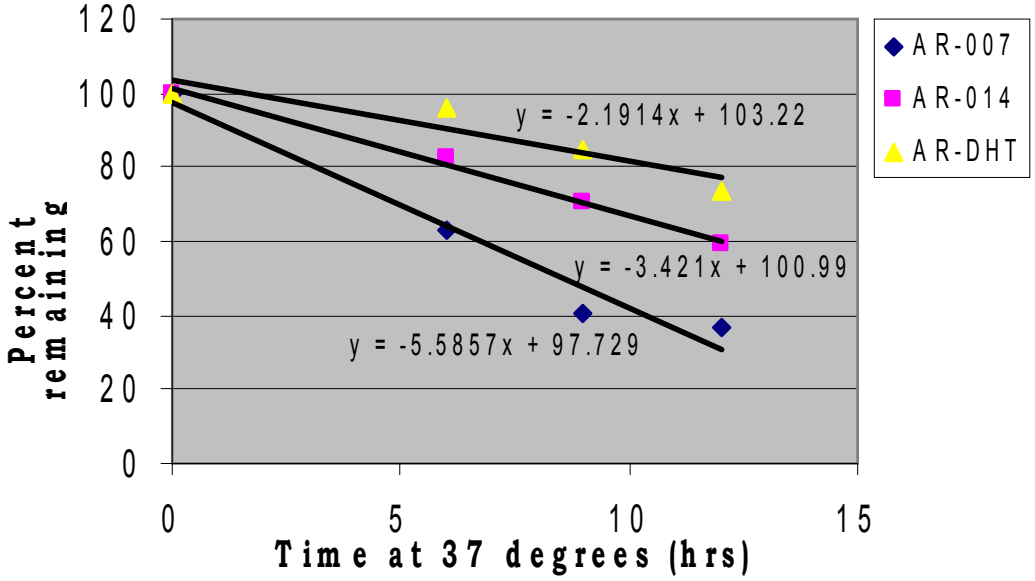


Figure 12: Stability Experiment 1 Results with AR-DHT

The results of the SDS-PAGE gels were confirmed via quantification. This was done via a program called Image J® which uses relative band intensities as a measurement tool. When the relative band intensities were graphed as a function of percent of the sample remaining vs. time at 37 degrees Celsius. The results can be observed in figure 13. The graph suggests that AR-007 degrades at a faster rate than AR-014 and AR-DHT.

Figure 13: Stability Experiment 1 Graph



In order to confirm these results, the stability experiment was repeated with similar conditions. However, this time, the AR-ligand complexes were exposed to 37 degree Celsius heat for longer periods of time. A slightly larger amount of AR was added also. Figure 14 describes the conditions used in this experiment. Figures 15 and 16 describe the results obtained for stability experiment 2.

Figure 14: Stability Experiment 2 Conditions

Fraction	Time Period At 37 degrees	Concentration (ug/ul)	Volume (ul)	Amount of AR added (ug)
AR-014 F1 50 mM	0 hr, 12 hr, 24 hr, 36 hr	0.077	15.0	1.15
AR-007 F1 50 mM	0 hr, 12 hr, 24 hr, 36 hr	0.142	8.1	1.15
DHT F5 25 mM	0 hr, 12 hr, 24 hr, 36 hr	0.119	9.7	1.15

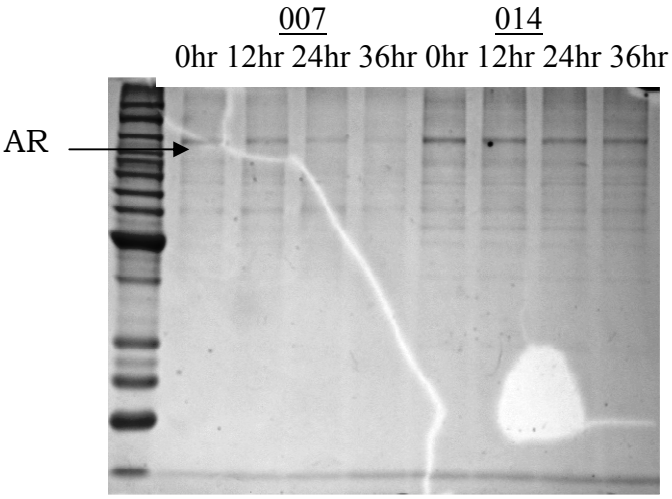


Figure 15: Stability Experiment 2 Results with AR-014 and AR-007

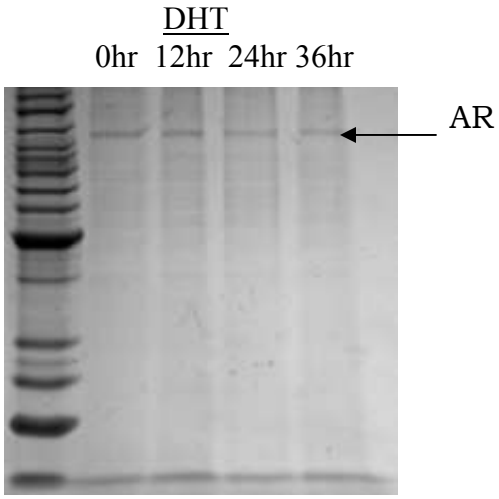
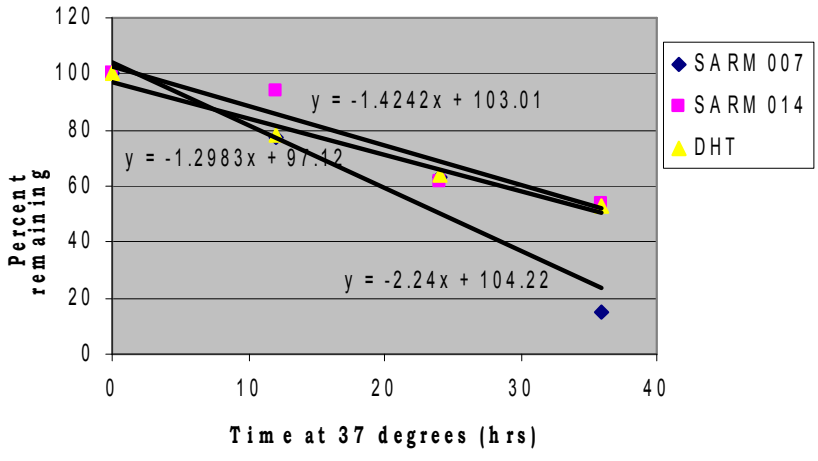


Figure 16: Stability Experiment 2 Results with AR-DHT

These results seem to be very similar those obtained from the first stability experiment. Figure 17 shows these results after quantification using Image J®. In both studies, AR-007 was the least stable of the receptor complexes, while AR-DHT and AR-014 showed better and approximately equal stability to heat.

Figure 17: Stability Experiment 2 Graph



4.1.2 Protein-Protein Interaction Experiments

To examine whether or not the various AR-ligand complexes displayed different protein-protein interactions, immunoprecipitatates, which contained AR and any proteins closely associated with it, were analyzed via Western blotting. The first set of data that was collected involved the antibody labeling of AR in each of the samples. The purpose of this was to indicate that AR was indeed present in all immunoprecipitation samples in equal amounts. A negative control was used to indicate that there was a minimal amount of non-specific antibody binding in the samples. For the negative control, no primary antibody was added to the Western blot membrane. In this instance, 2.0 mg of AR-007 was used as the negative control, but it theoretically did not matter which sample was chosen. Positive controls of 5% of the cell lysates were used to confirm that AR was present initially, before immunoprecipitation, in all samples used. All of the immunoprecipitation products had 2.0 mg of cell lysate, yielding approximately the same amount of product for each sample. The results in figure 18 were obtained for the western blot labeled with anti-AR.

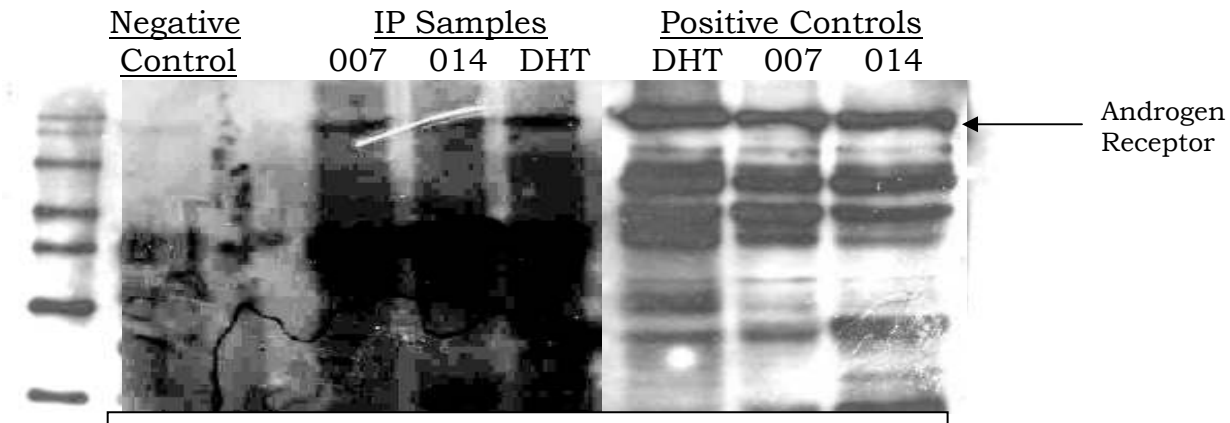


Figure 18: Western Blot with antibody labeling of Androgen Receptor

It can be confirmed from these results that AR was indeed present in all samples, seen at about 110 kDa. The negative control shows that a negligible amount of non-specific antibody binding contributes to the band intensities. The next step in the experiment was to determine whether or not heat shock protein 70 (hsp70) associated with the AR-ligand complexes. Since hsp70 is a protein thought to be associated with AR, it should be present in all samples. The results in figure 19 are those that were obtained when the Western blot membrane was probed with an anti-hsp70 antibody. A large amount of hsp70 was found in all samples, but appeared to differ based on the ligand bound to the AR.

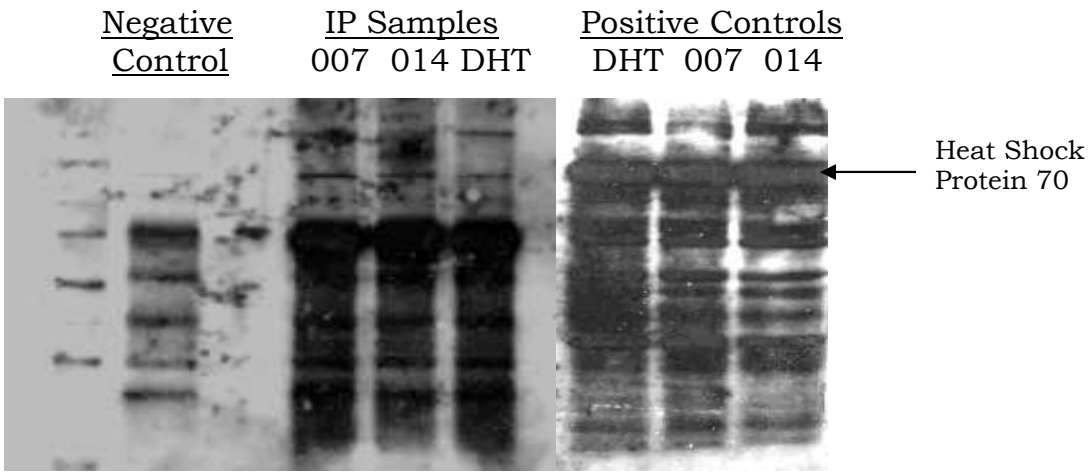


Figure 19: Western Blot with antibody labeling of Heat Shock Protein 70

To confirm these results, the bands were quantified using Image J® and compared with one another in terms of the ratio of hsp70:AR. Considering about the same amount of AR was present in all samples, the higher the ratio of hsp70:AR then the more AR-hsp70 association was present. The results can be seen in figure 20. The graph confirms the results from the Western blot films in that it suggested that AR-007 associated more strongly with hsp70 than the other two AR-ligand complexes.

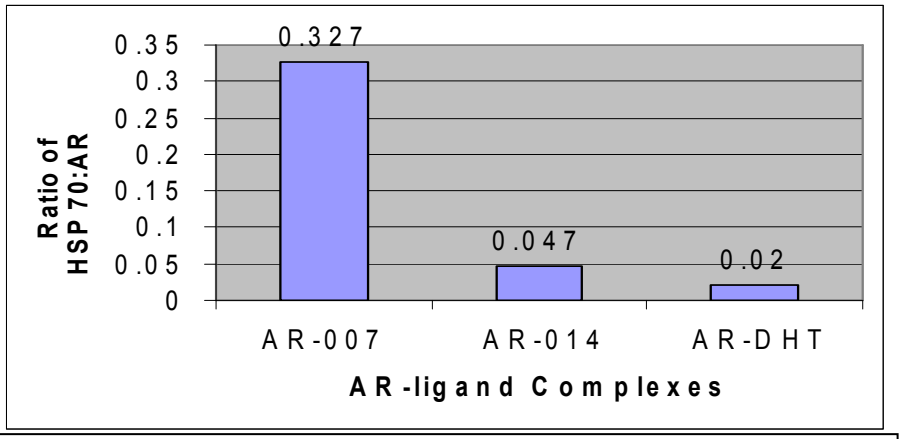


Figure 20: Protein-Protein Interactions Graph

4.1.3 Partial Proteolysis

In order to examine whether AR-DHT, AR-007, and AR-014 have different three-dimensional orientations, a partial proteolysis experiment was conducted. The idea was to determine if trypsin, an enzyme which degrades proteins, clips and cuts up these three AR-ligand complexes in different ways. If it does, then it suggests that these three complexes indeed have different three-dimensional conformations. Pure AR protein samples were used for this experiment. The three samples, AR-014, AR-007, and AR-DHT were degraded

with trypsin for a total of 0 minutes, 10 minutes, 20 minutes, and 30 minutes. The patterns of degradation were observed using Western blot analysis. There were two sets of data in this experiment. The first set of data came from antibody labeling at the –COOH terminal, and the second came from antibody labeling at the $–NH_2$ terminal. The purpose of labeling both ends was to get a better picture of how exactly the proteins were being degraded. The results of the –COOH labeled terminal are shown in figure 21 and figure 22 and the results of the $–NH_2$ labeled terminal are shown in figure 23 and figure 24.

Figures 21-24 suggest that at the C-terminal, AR-007 and AR-014 have tigher, more coiled conformations than AR-DHT because they avoid degradation by trypsin better for longer periods of time. The darker and more prominent band at the top of the gel, which represents the largest protein fraction, shows that the sample contains a larger portion of protein that has not been degraded by trypsin. Similar results at the N-terminal support this theory.

Figure 21: Partial proteolysis results labeled at the C-terminal

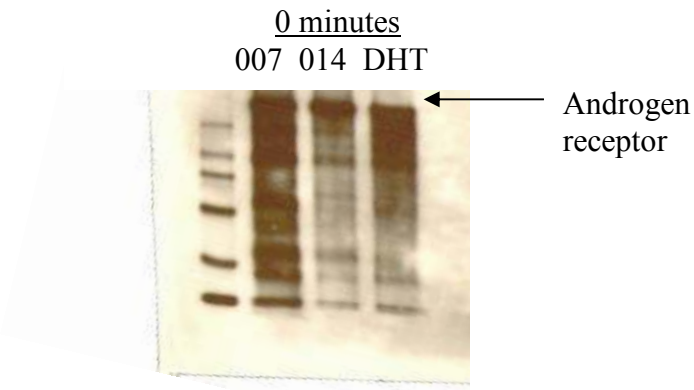


Figure 22: Partial proteolysis results labeled at the C-terminal

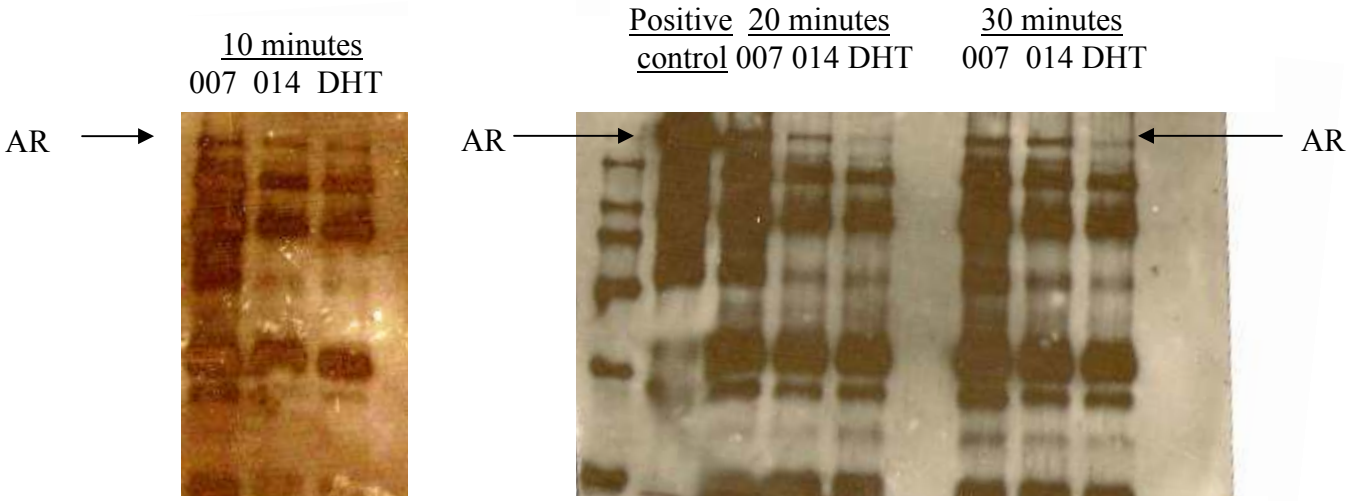


Figure 23: Partial proteolysis results labeled at the N-terminal

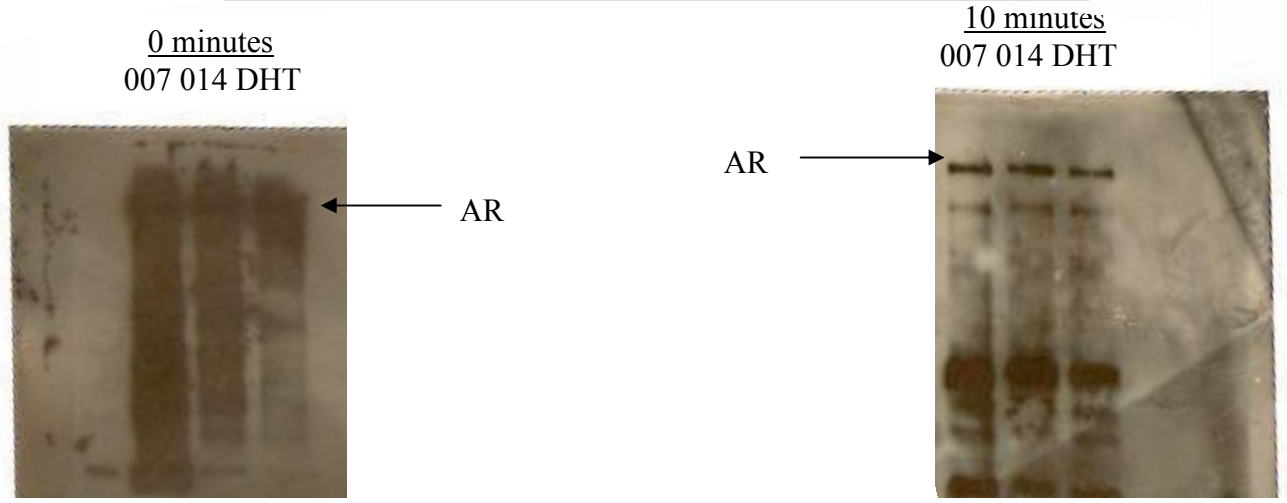
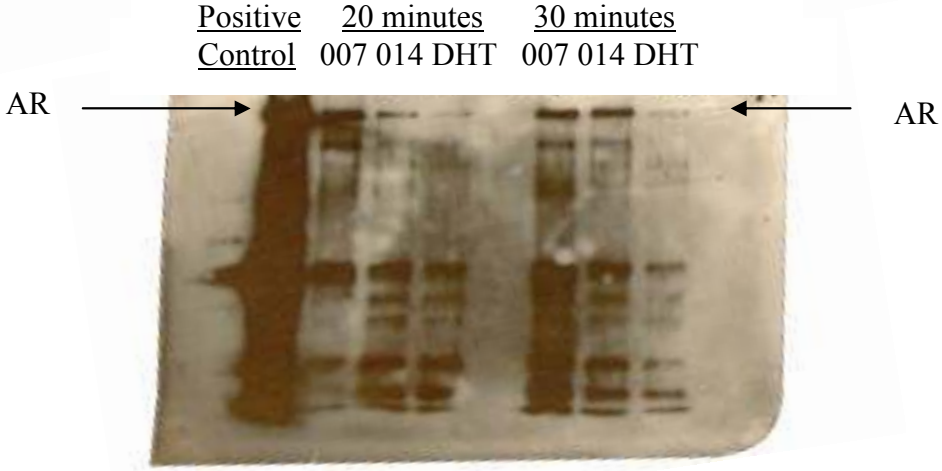


Figure 24: Partial proteolysis results labeled at the N-terminal



4.2 Discussion

The results from the stability experiments showed an interesting pattern. For the negative control, where AR was not complexed with a ligand, there was hardly any sign of a band on the SDS-PAGE gel indicating that there was AR in the sample. There was a slight band at time point 0 minutes, but it diminished quite quickly as time went on. It appears that all of the AR was degraded after only 30 minutes. This leads to the conclusion that AR is broken down readily when it is not complexed to a ligand. Therefore, ligands in general cause AR to be more stable than when it is by itself (i.e., unbound).

In the first stability experiment, the main observation was that, as time progressed, the AR-DHT and AR-014 complexes seemed to be more stable. They appeared to have less degradation during the 37 degree Celsius heating period, which was shown by darker bands on the SDS-PAGE gel and subsequent image analysis. There were more of the AR-DHT and AR-014 complexes left at 12 hours than the AR-007 complex, which appeared to have a less intense band and therefore a smaller relative concentration at 12 hours.

Similar results were obtained in the follow-up stability experiment with the longer time points. Again, AR-007 seemed to degrade at a quicker rate than AR-014 and AR-DHT. Therefore, it can be concluded that the AR-014 and AR-DHT complexes are more stable than the AR-007 complex because they were able to withstand the heat exposure for a longer period of time, while AR-

007 degraded and was not able to withstand the heat exposure. The graphs in figure 13 and figure 17 confirm these results.

In the protein-protein interactions experiment, where each AR-ligand complex was examined to see their amounts of association with heat shock protein 70 (hsp70), another interesting result was obtained. It appeared that the band intensities of hsp70 were weakest for the AR-014 and AR-DHT samples than for the AR-007 complex. These results indicate that hsp70 associates more closely with AR-007 than with AR-014 and AR-DHT, which was concluded as a result of the darker band of hsp70 with AR-007. The quantitative data image analysis confirmed that AR-007 associated more strongly with hsp70 than the other two AR-ligand complexes.

The data in the first two experiments correlate well with one other. The results from the stability experiments showed that AR-007 is less stable than AR-014 and AR-DHT because it breaks down readily when exposed to heat. The results from the protein-protein interactions experiment also indicate that AR-007 is a less stable complex because it had the strongest association with hsp70. Typically, less stable molecules associate with more chaperone proteins when they travel through the cellular environment. More proteins provide better protection for the molecule so that it can avoid degradation.

The purpose of the partial proteolysis experiment was to obtain more information about the three-dimensional structures of AR-007, AR-014, and AR-DHT. According to the results in figure 21, AR-DHT is broken down into the smallest fractions at the –COOH terminal. This can be concluded because

the largest band, or the top band, is the faintest. AR-007 and AR-014 seem to have approximately the same intensity at the top band at all time points, which indicates that a larger portion of the intact AR is present. This suggests that they have a tighter, and more coiled conformation than AR-DHT because they are not being easily digested by the trypsin. However, this possible conclusion conflicts existing data regarding the known conformational changes that occur with DHT and SARMs. A further experiment could be performed to determine why AR-007 and AR-014 are more resistant to trypsin proteolysis. This may lead to information about their functions and how they can act as both agonists and antagonists.

Looking at the results from the $-NH_3$ terminal in figure 22, at 10 minutes all of the bands are slightly fainter, but they are in approximately equal intensities. This suggests that all of the AR complexes have a more coiled conformation at the $-NH_3$ terminal as compared to the –COOH terminal. Looking at the 20 minute time point, AR-007 has a heavier band at the top than AR-014 and AR-DHT. However, at the 30 minute time point, AR-007 and AR-014 have approximately the same band intensities at the top band. In both the 20 and 30 minute time points, AR-DHT appears to have been degraded more so than the AR-007 and AR-014 samples. Again, this leads to the conclusion that AR-007 and AR-014 are more stable and appear to have a more coiled conformation in general than AR-DHT.

These results suggest that SARMs induce unique AR conformations that affect its stability, interactions with other intracellular proteins, and perhaps

even pharmacologic action and tissue selectivity. The studies reported herein are preliminary in nature, but suggest that detailed characterization of the biophysics of AR will eventually shed light on the molecular mechanisms of these exciting new drugs.

REFERENCES

1. Gao, W. and Dalton, J.T. *Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs).* **2007**, 241-248.

2. Gao, W.; Bohl, C.E.; Dalton, J.T. *Chemistry and Structural Biology of Androgen Receptor.* **2005**, 3352-3368.

3. Brinkmann, A.O. *Androgen Receptor: Functional Domain Structure*. **2006**.

4. Fang, H., Tong, W., Branham, W.S., Moland, C.L., Dial, S.L., Hong, H., Xie, Q., Perkins, R., Owens, W., and Sheehan, D.M. *Study 202 of Natural, Synthetic, and Environmental Chemicals for Binding to the Androgen Receptor.* 2003, 1338-1358.

5. Gao, W. *Pharmacology of Selective Androgen Receptor Modulators (SARMs).* The Ohio State University, 2004.

6. Yin, D.; Gao W.; Kearbey J.D.; Xu, H.; Chung, K.; He, Y.; Marhefka, C.A.; Veverka, K.A.; Miller, D.D.; Dalton, J.T. *Pharmacodynamics of Selective Androgen Receptor Modulators.* **2002**, 1334-1340.

7. Allan, G.F. and Sui Z. *Therapeutic androgen receptor ligands.* **2003**, e009.

8. Bonaccorsi, L., Carloni, V., Muratori, M., Salvadori, A., Giannini, A., Carini, M., Serio, M., Forti, G., and Baldi, E. *Androgen Receptor Expression in Prostate Carcinoma Cells Suppresses a6b4 Integrin-Mediated Invasive Phenotype*.* **2000**, 3172-3182.

9. Smith, G.E., Summers, M.D. , and Fraser, M.J. *Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations Within the Polyhedrin Gene.* **1983**, 46: 584-593.

10. Xie, Y.; Sui, Y.; Shan, L.; Palvimo, J.J.; Phillips, D.M.; Janne, O.A. *Expression of Androgen Receptor in Insect Cells.* **1992**, 4939-4948.

11. Dutertre, M. and Smith, C.L. Mole*cular Mechanisms of Selective Estrogen Receptor Modulator (SERM) Action.* Baylor College of Medicine, **2000**, 431-437.

12. Windahl, S.H.; Galien, R.; Chiusaroli R.; Clement-Lacroix P.; Morvan, F.; Lepescheux L.; Nique, F.; Horne W.C.; Resche-Rigon M.;

Baron, R. *Bone protection by estrens occurs through non-tissue-selective activation of the androgen receptor.* **2006**, 2500-2509.

13. Basson R. "The female sexual response: a different model." J Sex Marital Ther. **2000**, 26(1): 51-65.

14. Ross, J.L., Roeltgen, D., Stefanatos, G.A., Feuillan, P., Dushner, H., Bondy, C., and Culter, G.B. Jr. *Androgen-Responsive Aspects of Cognition in Girls with Turner Syndrome.* **2003**, 88(1): 292-296.

15. Arlt, Wiebke. *Androgen Therapy in Women.* **2006**, 154(1): 1-11.

16. Davis, Susan. *Androgen Replacement in Women: A Commentary.* **1999**, 84(6), 1886-1891.

17. Mushayandebvu, T., Castracane, D.V., Gimpel, T., Adel, T., Santoro, N. **1996** *Evidence for diminished midcycle ovarian androgen production in older reproductive aged women.* Fertil Steril. 65: 721–723.

18. Davis, S.R., McCloud, P.I., Strauss, B.J.G., Burger, H.G. *Testosterone enhances estradiol's effects on postmenopausal bone density and sexuality.* Maturitas. **1995**, 21: 227–236.

19. Brown, Terry. *Nonsteroidal Selective Androgen Receptor Modulators (SARMs): Designer Androgens with Flexible Structures Provide Clinical Promise.* J. Endocrinology. **2004**, 49(12): 5417-5419.

20. Kuil, C.W., Berrevoets, C.A., Mulder, E. *Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization—studies on the mechanism of antiandrogen action.* J Biol Chem. **2005**, 270: 27569–27576.

21. Kimmins, S., MacRae, T.H. *Maturation of Steroid Receptors: An Example of Functional Cooperation Among Molecular Chaperones and their Associated Proteins.* Cell Stress and Chaperones. **2000**, 5(2): 76-86.

22. DeFranco, D.B. *Role of molecular chaperones in subnuclear trafficking of glucocorticoid receptors.* **2000**, 1241-1249.

23. Pratt, W.B.; Galigniana, M.D.; Harrell, J.M.; DeFranco, D.B. *Role of hsp90 and the hsp90-binding immunophilins in signaling protein movement.* **2004**, 857-872.