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Identification And Characterization Of Fkbp52-Specific Inhibitors For The Treatment Of Prostate Cancer

Johanny Tonos De Leon
University of Texas at El Paso, johanny.tonos@gmail.com

IDENTIFICATION AND CHARACTERIZATION OF FKBP52-SPECIFIC INHIBITORS FOR THE TREATMENT OF PROSTATE CANCER

JOHANNY TONOS DE LEON

Department of Biological Sciences

APPROVED:

Marc B. Cox, Ph.D., Chair

Kristine Garza, Ph.D.

Robert Kirken, Ph.D.

Siddhartha Das, Ph.D.

Mahesh Narayan, Ph.D.

Patricia Witherspoon, Ph.D.
Dean of the Graduate School

DEDICATION

I would like to thank my parents, María and Javier, my sister, Christina, and my aunt, María for their continued encouragement, love and support. My mom's outgoing confidence and good cheer, my sister's strength and kindness, my aunt's hopefulness and my dad's ability to find strength within himself to inspire me to become a stronger person. I thank God for having them in my life (ustedes son lo más importante para mí y los amo con todo mi corazón). I would also like to thank Jeffrey Kugelman for being my best friend, companion and soul mate. Your love and sense of humor has kept me going through difficult times (gracias por ser mi otra mitad, te amo muy más). To the Cox lab members Diondra, Cheryl, Yenni, Nai, Jackie, Marissa, Heather, Paul and Dr. Sivils, thanks for your friendship, patience and sharing your love for science. To Raquel and Walker, thanks for being like family to me, you guys are awesome!

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IDENTIFICATION AND CHARACTERIZATION OF FKBP52-SPECIFIC INHIBITORS FOR THE TREATMENT OF PROSTATE CANCER

By

JOHANNY TONOS DE LEON

DISSERTATION

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ABSTRACT

Steroid hormone receptors require the ordered assembly of various chaperone and cochaperone proteins in order to reach a functional state. The final stage in the receptor maturation process requires the formation of a mutimeric complex consisting of Hsp90 dimer, p23, and one of several large immunophilins. Studies conducted previously demonstrated that the large immunophilin FKBP52 acts to potentiate glucocorticoid, androgen, and progesterone receptor signaling pathways. The aim of these studies was to identify and characterize FKBP52-specific inhibitors that would not only serve as tools for the pharmacological analysis of FKBP52-receptor interactions, but may also lead to novel drugs with significant therapeutic potential. A modified receptor-mediated reporter assay in yeast used to screen a natural compound library for FKBP52 inhibitors led to the identification of multiple hits. Structure activity relationship studies using the yeast-based assays led to the characterization of fifteen additional inhibitors, some of which display dramatically increased potency. Surface plasmon resonance studies confirmed that these inhibitors disrupt FKBP52 function through interaction with the AR hormone binding domain. In addition, scintillation proximity binding and fluorescence polarization assays demonstrated that the compounds bind to a previously unrecognized regulatory surface domain on the AR LBD termed BF3. Interestingly, mutations within the BF3 surface lead to increased dependence on FKBP52 for normal function. ELISA and Western immunoblotting analysis in prostate cancer cells demonstrated that the compounds inhibit Prostate-Specific Antigen and FKBP51 expression. In addition, androgen-dependent cell proliferation was prevented after treatment with the inhibitory

molecules. In summary we have identified molecules that inhibit FKBP52 regulation of AR function and represent an exciting new approach for the treatment of prostate cancer. In addition, these studies provide new insight into FKBP52-AR structural and functional interactions.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

GLOSSARY

SHR – steroid hormone receptor

HSP - heat shock protein

FKBP52 – 52 kDa FK506-binding protein

GR – glucocorticoid receptor

AR – androgen receptor

PR – progesterone receptor

HBD – hormone binding domain

LBD – ligand binding domain

BF3 – binding function 3

AF2 – activation function 2

HRPC – hormone refractory prostate cancer

NCI – National Cancer Institute

PSA – prostate specific antigen

FKBP51 – 51 kDa FK506-binding protein

ARE – androgen response element

MEFs 52KO – mouse embryonic fibroblast cells that lack FKBP52

DHT – dihydrotestosterone

Pc – prostate cancer

EPc – early stage prostate cancer

LPc – late stage prostate cancer

MJC – Marc and Johanny's compounds

AR-P723S – mutant androgen receptor (a proline was substituted by a serine at the position 723)

URA – uracil

LEU – leucine

TRP – tryptophan

OD – optical density

DMSO – dimethyl sulfoxide

IC – inhibitory concentration

RLU – relative light units

CO_2 – carbon dioxide

MEM – minimum essential medium

RPMI – Roswell Park Memorial Institute medium

DMEM – Dulbecco's Modified Eagle's medium

LNCAP – androgen-sensitive human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis of a 50-year-old Caucasian male

VCAP – "vertebral-cancer of the prostate", cell line derived from a vertebral metastatic lesion of a patient with hormone refractory disease

LAPC4 – "Los Angeles prostate cancer from patient 4" cell line established from a human prostate cancer xenograft

22RV1 – prostate cancer cell line derived from the androgen-dependent xenograft CWR22R and it is hyperdiploid with two clonal sublines

MDA-kb2 – breast cancer cell line that stably expresses a luciferase reporter gene responsive to the androgen receptor

FBS – fetal bovine serum

PBS – phosphate buffered saline

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrzolium bromide

SDS-HCL – sodium dodecyl sulfate in Hydrochloric Acids solution

DNA – deoxyribonucleic acid

MPER – mammalian protein extraction reagent

EDTA – ethylenediaminetetraacetic acid

ESI-QTOF-MS – electrospray ionization-time-of-flight mass spectrometry

PDR5 – pleitropic drug resistance 5 gene

Ab – antibody

SDS – sodium dodecyl sulfate

CMF-PBS – calcium magnesium free phosphate buffered solution

BSA – bovine serum albumin

DAPI – 4’,6’-diamino-2-phenylindole fluorescent stain

[^{3}H] – tritium

SRC2 – nuclear receptor co-activator 2

SRC3 – nuclear receptor co-activator 3

ELISA – enzyme-linked immunosorbent assay

CHAPTER 1

INTRODUCTION

I. Androgen Receptor Function

Nuclear receptors are proteins responsible for sensing the presence of hormones and other molecules. In response to hormone, these receptors regulate the expression of specific genes in order to control cell development, homeostasis, and metabolism [1]. The androgen receptor is a nuclear receptor [2] that is activated by the binding of the androgenic hormone testosterone and its derivatives [3]. Androgen-regulated genes are critical for the development and maintenance of the male sexual phenotype [4, 5]. Figure 1.1 shows a model of nuclear steroid hormone receptor function were the ligand binds the receptor, chaperone proteins disassociate, and the activated receptor dimer enters the nucleus and interacts with DNA to mediate changes in gene expression [6]. The androgen receptor dimer binds to a specific DNA sequence known as a hormone response element. Androgen receptors interact with additional proteins in the nucleus resulting in “up” or “down-regulation” of specific gene transcription [6]. Up-regulation or activation of transcription results in the increased synthesis of messenger RNA, which in turn is translated by ribosomes to produce specific proteins.

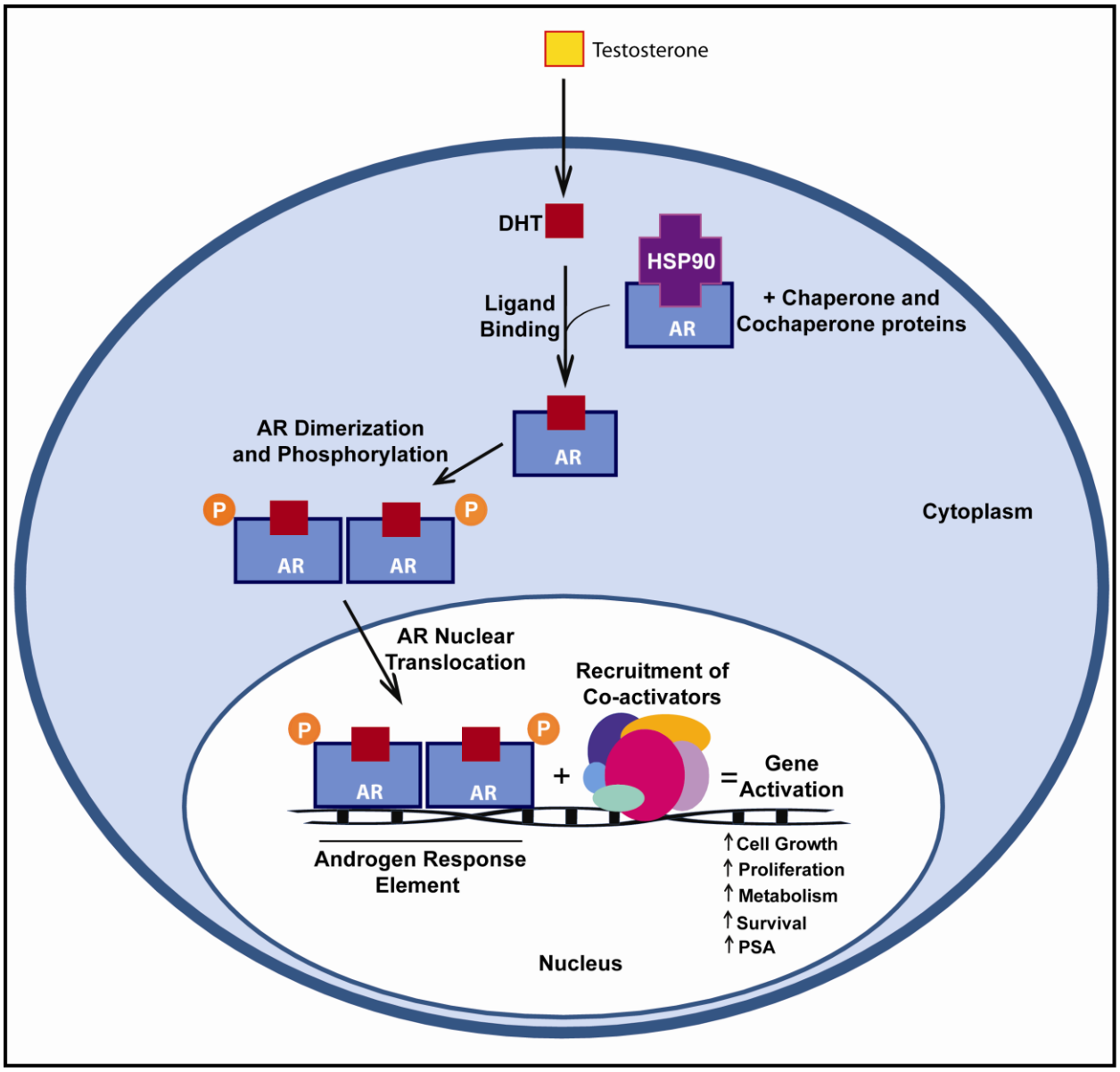

Figure 1.1. Androgen Receptor Action.

Figure 1.1. Androgen Receptor Action. Circulating testosterone enters prostate cells and is converted into dihydrotestosterone (DHT). When activated by the binding of the hormone ligand, the androgen receptor (AR) dissociates from chaperone proteins and becomes phosphorylated. AR dimerizes, translocate into the nucleus and binds directly to DNA at the androgen-response elements (ARE) in the promoter regions of the target genes. Activation or repression of the target genes control biological responses such as cell growth, proliferation, homeostasis, metabolism and prostate-specific antigen secretion (PSA).

II. Model of Chaperone-Mediated Steroid Hormone Receptor Assembly

Steroid hormone receptors are ligand-dependent transcription factors. In the absence of hormone the receptors exist in the cytoplasm and/or the nucleus complexed with heat shock protein 90 (Hsp90) and other cochaperones. Upon hormone binding the receptor-Hsp90 complex dissociates and the receptors dimerize in the nucleus where they bind to hormone response elements enhancing the expression of a wide variety of genes. The interests in our laboratory focus on chaperone proteins that bind to and regulate steroid hormone receptors. At least ten chaperone and cochaperone proteins are required for normal receptor function, including Hsp70 and Hsp90. All of these factors offer the potential opportunity for therapeutic intervention. We are interested in understanding the manner in which these factors influence receptor folding, hormone binding, nuclear translocation, dimerization, and DNA binding. Work conducted previously demonstrated that the maturation of the steroid hormone receptors to the mature hormone binding confirmation is a highly ordered, dynamic process that involves at least ten chaperone and cochaperone components, all of which present an opportunity for therapeutic intervention. The steroid receptor folding pathway depicted in Figure 1.2 has been thoroughly reviewed [7, 8] and is briefly described here.

Much of the knowledge of steroid hormone receptor folding has been gleaned from *in vitro* receptor assembly assays for progesterone receptor (PR) [9-14] and glucocorticoid receptor (GR) [15], but the process is likely to be similar for other steroid receptors given that their associated chaperone complexes are nearly identical.

Unfortunately, these assays reveal nothing about early nascent chain folding. Thus, our knowledge of the steroid hormone receptor folding process is restricted to folding events subsequent to nascent chain folding. However, it is thought that the receptors associate with Hsp70 and its cochaperone, Hsp40, early in the folding process, possibly even as the receptor is being translated on the ribosome [16]. The Hsp70 complex is not only thought to aid in the early folding process, but is a prerequisite for receptor association with the Hsp90 complex. After association with the receptor, the Hsp70 complex recruits the Hsp interacting protein (Hip), Hsp organizing protein (Hop), and Hsp90. Hop acts as a scaffolding protein to bridge the Hsp70 and Hsp90 complexes by way of its multiple tetratricopeptide repeat domains. Following Hsp90 association a rearrangement occurs in which the interaction with Hsp70 is lost and the p23 cochaperone and one of several immunophilin proteins are recruited to the receptor-Hsp90 complex. It is this form of the complex that the receptor is capable of high affinity hormone binding. In the case of PR the mature conformation of the receptor lasts only several minutes [17], which correlates with the slow ATPase cycle of Hsp90 [18-22]. After several minutes the complex dissociates and the receptor is recycled back into the chaperoning pathway. Thus, the receptor-folding pathway is a dynamic, ordered process that involves many transient interactions. In the absence of Hsp90 the receptors are unstable and degraded rapidly through the proteosomal degradation pathway.

Given that chaperone interactions with the receptors are restricted to the ligand binding domains (LBD), it is generally thought that the chaperones assist the LBD in achieving and maintaining the high affinity hormone binding conformation through the classic “folding and holding” chaperone role. However, it is also clear that some of the

chaperone components have a direct regulatory role in the receptor signaling pathways. The Hsp90 complex effectively silences steroid hormone receptor function until hormone binding occurs by blocking access to the receptor nuclear localization signal, receptor dimerization, and the association of transcriptional coactivators [reviewed in 23]. Additionally, evidence suggests that the large FK506 binding protein (FKBP) immunophilin FKBP52 can directly influence receptor hormone binding [24-26] and consequently its function.

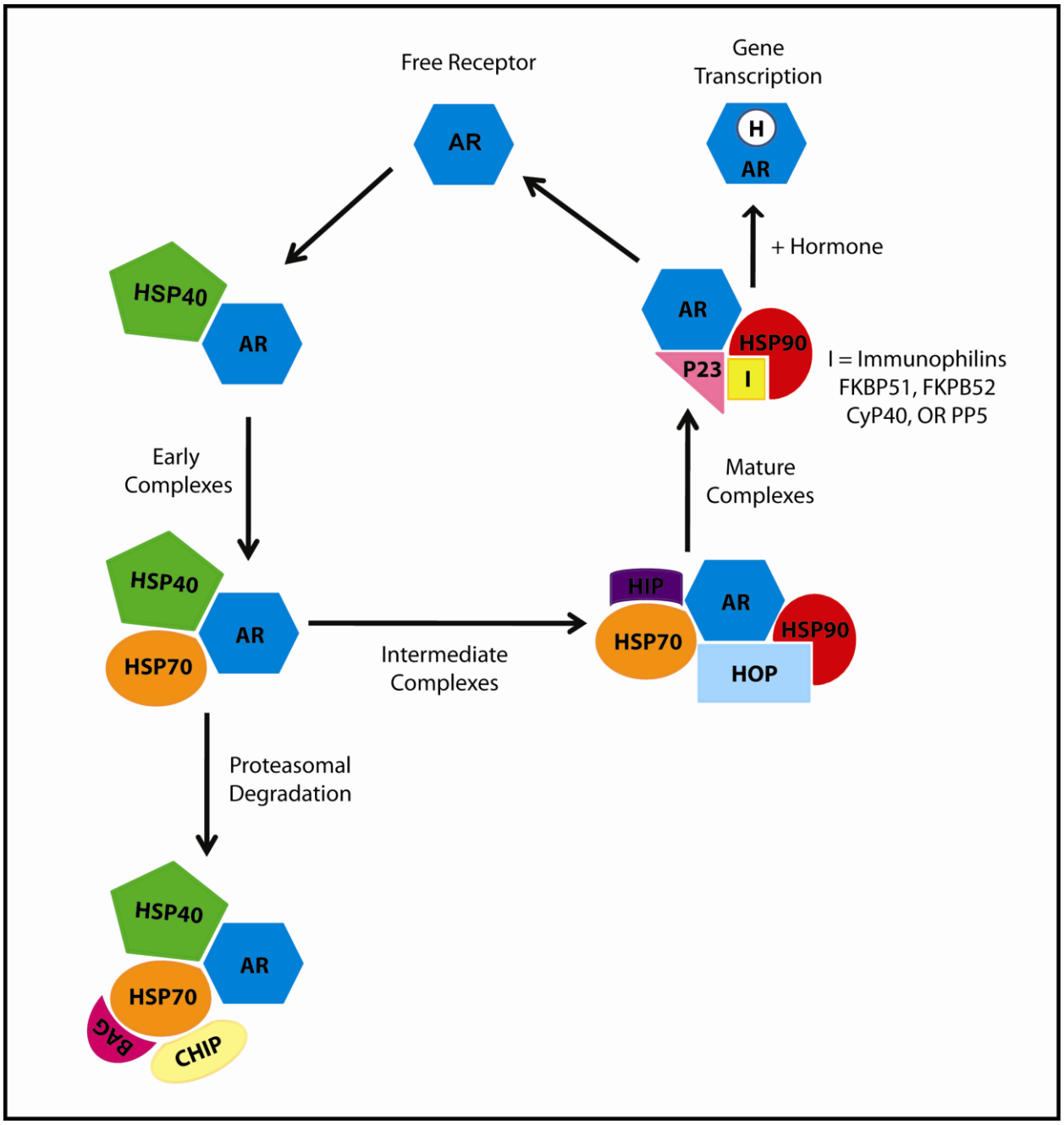

Figure 1.2. Chaperone-Mediated Receptor Folding Pathway.

Figure 1.2. Chaperone-Mediated Receptor Folding Pathway. The SHR maturation process involves the interaction with chaperone and co-chaperone proteins and an immunophilin protein in the last step. After this dynamic process the receptor is in a mature hormone-binding conformation.

III. Steroid Hormone Receptor-Associated Immunophilins

The immunophilins comprise several protein families including the FK506 binding proteins (e.g. FKBP51 and FKBP52), cyclosporine A binding proteins (e.g. Cyp40), and a protein phosphatase (e.g. PP5). Four immunophilins have been identified to associate with steroid receptor-Hsp90 complexes, all of which are listed in the above examples [10, 27-32]. The Hsp90-binding immunophilins contain a single TPR domain (Tetratricopeptide repeat), which comprises the Hsp90-binding domain. In addition, the FKBPs and Cyp40 contain a peptidyl-prolyl isomerase (PPIase) domain and PP5 contains a protein phosphatase domain. Although all four of these TPR-containing proteins compete for binding the Hsp90-steroid receptor complexes, only the association of FKBP51 and FKBP52 with AR, GR or PR complexes appear to represent relevant functional interactions [reviewed in 5].

Over expression of FKBP52 in the budding yeast *S. cerevisiae* and in various mammlian cell culture systems potentiates AR, GR and PR-mediated expression of a reporter gene up to 20-fold, however it has no effect on estrogen receptor (ER) and mineralicorticoid receptor (MR) function [25, 26, 33]. Using FKBP52 mutants unable to bind Hsp90 (K354A), display PPIase activity (FD67DV), or immunosuppressive ligands that bind to and inhibit the PPIase enzymatic activity, Dr. Smith lab demonstrated that FKBP52 potentiation of receptor function requires a functional PPIase domain and interaction with Hsp90 (Fig. 4B). Studies conducted by the Smith lab and others suggest that FKBP52 potentiation occurs through enhancement of receptor hormone binding affinity [24, 26]. Domain swapping experiments between the various steroid receptors

demonstrated that FKBP52 potentiation localized to the receptor ligand binding domain [25]. Thus, a plausible hypothesis is that the FKBP52 PPIase binds to and catalyzes the isomerization of a relevant proline substrate in the receptor ligand binding domain, thereby increasing the affinity for hormone. However, it is not the PPIase activity that is required for potentiation, but the PPIase (also termed FK1) domain. The current prevailing hypothesis is that the FKBP52 PPIase catalytic pocket is part of an interaction surface and the interaction is disrupted by the immunosuppressive ligands, which protrude out from the catalytic pocket. We do not know the identity of the interaction partner, but evidence suggests that it is the steroid receptor ligand binding domain.

The physiological significance of the FKBP52 effects observed in cellular studies has been confirmed in a mouse gene knockout model. Both male and female FKBP52 knockout (52KO) mice are infertile [26, 33]. The 52KO male mice display specific reproductive phenotypes directly linked to defects in androgen receptor function. The observed phenotypes are consistent with partial androgen receptor insensitivity including ambiguous external genitalia, the presence of nipples that persist through puberty, and dysgenesis of the prostate and seminal vesicles. The 52KO female mice display defects in implantation and decidualization that are directly related to defects in progesterone receptor function. Although the 52KO mice do not display any obvious phenotypes related to defects in GR function, we have observed a 3-fold higher level of serum corticosterone in both the 52KO males and females as compared to their wild type litter mates [34]. Thus, the 52KO mice are likely compensating for reduced GR function.

High resolution crystal structures have been solved for full-length FKBP51 [35] and for overlapping fragments of FKBP52 [36, 37]. FKBP52 shares ~70% sequence similarity and is structurally similar to FKBP51. Figure 1.3 indicates FKBP51 and FKBP52 structural comparisons and domain organizations. In contrast to FKBP52, FKBP51 overexpression in both yeast and mammalian cell assays has no effect on receptor function alone. However, co-expression of FKBP51 and FKBP52 demonstrated that FKBP51 acts to antagonize FKBP52-mediated potentiation of receptor function [25]. It is likely that FKBP51 is doing more than simply competing with FKBP52 binding with Hsp90 as the other steroid receptor-associated immunophilins do not antagonize FKBP52 potentiation. Thus, we believe that FKBP51-mediated antangonism of FKBP52 potentiation is functionally relevant for steroid hormone receptor regulation. In a physiological setting, FKBP51 expression is induced by glucocorticoids, progesterone and androgens [38-44]. Thus, the hormone-induced expression of FKBP51 may represent a negative feedback loop. Although we have not observed any obvious phenotypes in the FKBP51 knockout mice (51KO), this negative feedback loop may be important under specific physiological conditions. In addition, the loss of both FKBP51 and FKBP52 in mice results in an embryonic lethal phenotype [34]. Thus, FKBP51 and FKBP52 clearly have some redundant developmental functions. Finally, some of the strongest physiological evidence for a relevant role of FKBP51 in steroid receptor regulation comes from studies in New World primates. Cortisol resistance in New World primates has been attributed to FKBP51 [45, 46]. In conjunction with collaborators from Smith lab that demonstrated squirrel monkey FKBP51 displays a higher basal level of

expression and a more robust inhibition of GR ligand pontency as compared to human FKBP51 [47].

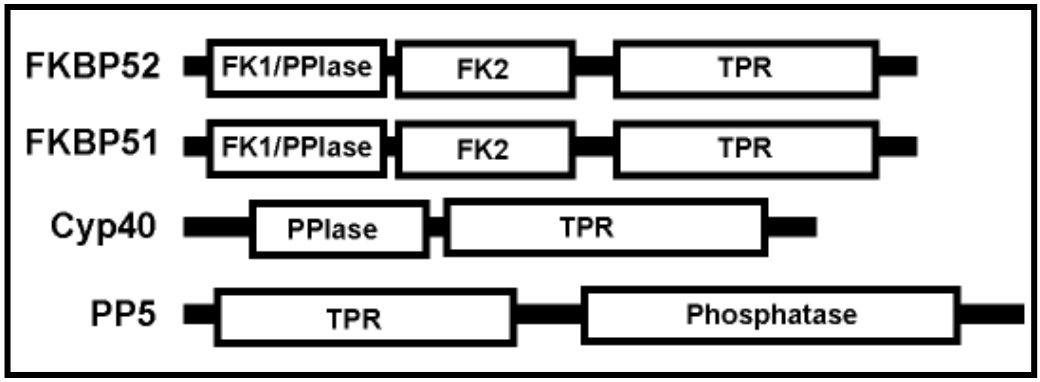

Figure 1.3. Receptor-Associated Immunophilins.

Figure 1.3. Receptor-Associated Immunophilins. A common feature shared by Immunophilin proteins is the presence of the TPR motif and additional functional domains. The TPR domain helps in protein-protein interactions and is responsible for Hsp90 binding. Two members of the FKBP family which possess Hsp90 binding abilities are FKBP51 and FKBP52. In FKBP51, the TPR domain projects at an angle from the rest of the protein, but in FKBP52 the TPR domain is more extended in comparison with the rest of the protein. FKBP51, FKBP52 and Cyp40 have the ability to bind immusuppressive drugs by their PPIase domains. Cyclophilins (CyP) bind the drug cyclosporine-A while the FKBP's bind the immune suppressant FK506. In both FKBP51 and FKBP52, the FK1 domain has functional PPIase activity, while the FK2 domain is a PPIase like domain with unknown function. FK1 regions are conserved between these proteins, with the exception of a few amino acids at the edge of the PPIase domain. The FK loop is the hinge region that connects FK1 to FK2. FKBP51 exhibits ionic bonding between the FK2 and TPR domains, whereas FKBP52 lacks these bonding abilities and is therefore more flexible in nature. FKBP51 and FKBP52 are 60% identical and approximately 70% similar in amino acid conservation.

IV. Prostate Cancer

According to the National Cancer Institute (NCI) prostate cancer is the major leading cause of death in males in the United Sates. There have been 217,730 new cases of prostate cancer and 32,050 deaths from prostate cancer in 2010. In addition, data from previous years indicate that 16.22% of males born today (1 in 6 males) in the United States will be diagnosed with prostate cancer at some point during their lifetime. These lifetimes risk statistics are available at the NCI website (http://seer.cancer.gov/statfacts/html/prost.html). Prostate cancer (PCa) occurs when cells within the prostate grow uncontrollably, creating small tumors [44, 48]. Prostate cancer growth is dependent on androgens and the androgen receptor function of regulating gene expression [48-50].

Several mechanisms of action for prostate cancer development have been reported [51-53] (figure 1.4). The first mechanism requires the presence of androgens to activate AR, followed by receptor translocation to the nucleus. The second mechanism results from amplification of AR that is activated in the absence of hormone or mutations in the receptor that make it capable of activation by either cytokines or growth factors. This later model suggest prostate cancer cell growth and tumor formation independent of the presence of hormones. Subsequent up-regulation of genes that promote cell growth and proliferation would result in tumor formation in the prostate. In the later mechanism, AR action is bypassed and an overexpression of Bcl-2 leads to the activation of genes that cause the formation of tumors within the prostate [52].

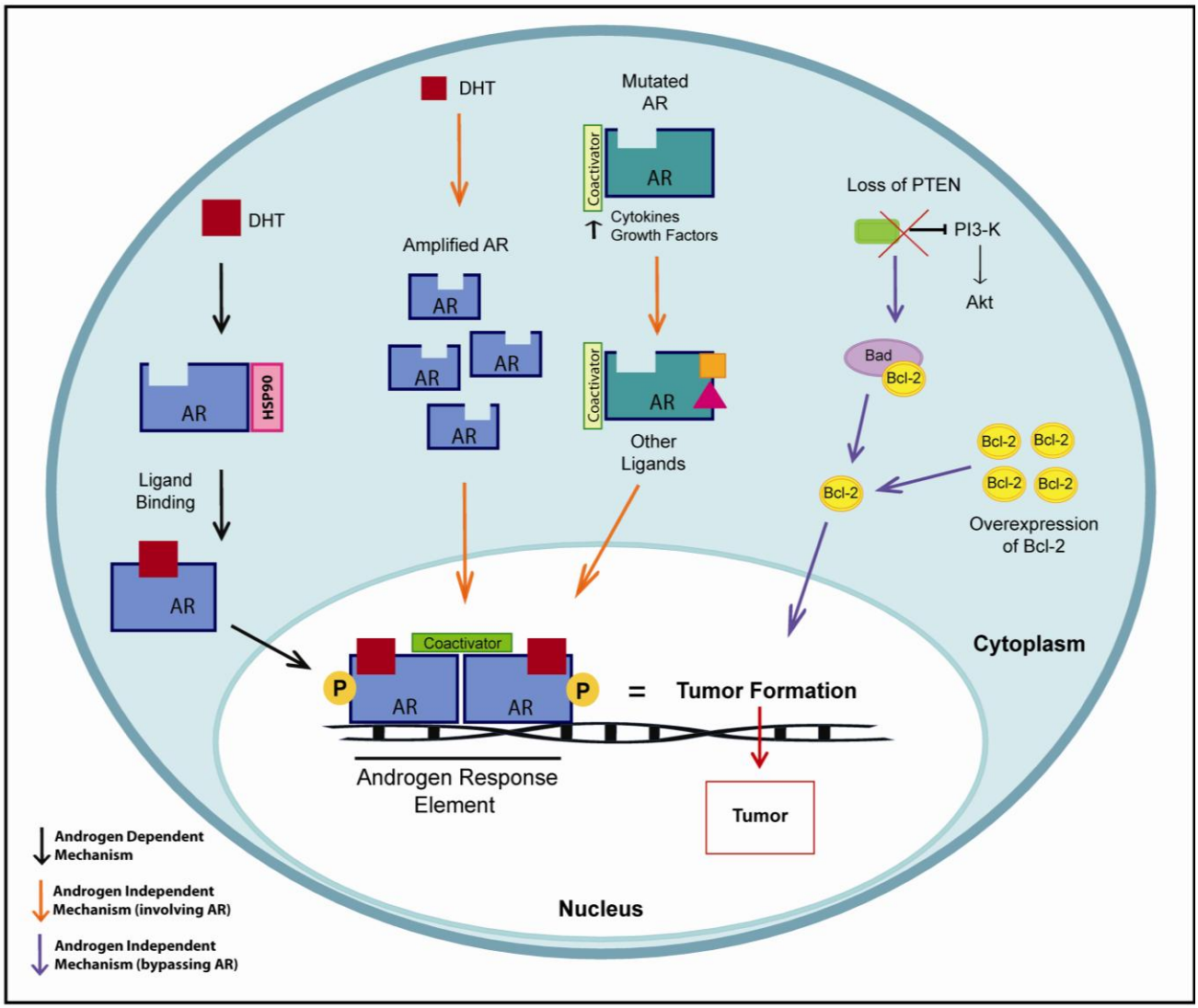

Figure 1.4. Prostate Cancer Development Mechanisms.

Figure 1.4. Prostate Cancer Development Mechanisms. The androgen-dependent mechanism requires the presence of androgenic hormone to activate the Androgen Receptor (AR) which leads to up regulation of genes that promote cell growth and subsequent cell proliferation and tumor formation. In the androgen-independent mechanism there is amplification of AR that is activated by additional molecules that are not typically androgenic hormones. This second mechanism describes prostate cancer cell growth and tumor formation independent of the presence of hormones.

The prostate is an androgen-dependent tissue that requires adequate AR activity for its development and maintenance. Dihydrotestosterone (DHT) is the primary ligand for AR in prostate tissue and it is synthesized from testosterone by reductase enzymes. As previously mentioned, during the AR maturation process the receptor interacts with cochaperone proteins, such as FKBP52, in order to induce high affinity androgen binding and to promote AR translocation to the nucleus [49]. The effect of AR in the transcriptional machinery of the cell is crucial for prostate cancer development since androgens promote cell growth, proliferation and survival [54-56] and are a major stimulator of tumor progression [57]. Therefore, under abnormal conditions, AR hyperactivity can contribute to the development of prostate cancer [58].

During early stage prostate cancer (EPc), the cancer progress is totally dependent on androgen accessibility. This requirement is the basis for the frequently used androgen ablation therapies that repress AR function by means of blocking the action of androgens by decreasing their levels in circulation or by competing for receptor binding. As a result cell growth and proliferation is prevented, AR-target genes' expression is reduced and tumor progression is repressed, making this therapy a principal option to be used for the treatment of EPc [48, 55, 59, 60]. However, androgen-resistant prostate cancer can reoccur and maintain a dependency on AR activity for growth and proliferation. This state is referred to as androgen independent, ablation resistant, or late stage PCa [61, 62]. Large scale gene-profiling studies have shown that late stage prostate cancer cells and tumors continue to express AR and secrete prostate specific antigen (PSA) like early stage prostate cancer cells [48, 49, 61]. These results indicate that AR remains important for androgen-independent

prostate cancer development and AR-regulated gene expression contribute to late stage PCa cell growth and progression [33, 44, 50, 63-65]. This phenomenon can be attributed to mutations in AR that make it reactive in the absence of DHT and cause oncogenic transformation of the prostate, impaired function of AR regulators, or changes in the signal transduction pathways that regulate AR activity [49]. At this point of prostate cancer progression the receptor might have the ability to be activated by molecules other than its primary ligand [48], thus new strategies to target AR activity are needed.

Co-regulators of the androgen receptor are beginning to be acknowledged as possible candidates for the treatment of these hormone-related and hormone-resistance diseases. The immunophilin protein FKBP52 is expressed in prostate tissue from prostatic hyperplasia patients [66] and interacts with the androgen receptor in prostate cancer cells [67] suggesting that it has a vital role in AR-mediated PCa cell growth and gene transcription. MALDI-TOF MS/MS studies of prostate needle biopsies [61] confirmed that FKBP52 is up-regulated in prostate cancer cell lines as compared to normal prostate cells, [67, 68] suggesting that this immunophilin is involved in AR activity in these tissues. As mentioned previously, FKBP52 has a positive physiological role in up-regulating AR activity, not only by interacting with AR-chaperone complexes, but also by potentiating receptor function [33, 55, 69] and enhancing AR-dependent transcription [33, 70]. In addition, studies of FKBP52 knockout mice have shown that this protein is essential for AR activity in the prostate [33, 71]. Therefore it has been postulated that AR positive regulators can be selectively targeted to achieve inhibition of AR-induced cell proliferation of PCa cells.

An alternative treatment for late stage PCa might be the inhibition of AR activation mechanisms, such as altering the interactions with chaperone proteins during the AR maturation process. AR mutations commonly found in late stage prostate cancer patients are positioned within our putative FKBP52 interaction surface on the androgen receptor. Previous studies from Dr. Cox's laboratory have shown that these mutations result in a mutant AR that displays increased dependence on FKBP52 for normal function [34]. Over-expression of FKBP52 positively affects the hormone-independent activity and basal signaling of mutated AR (L710H) in yeast models and stimulates hormone-independent signaling of AR (L710H) in PCa cells [72] suggesting that FKBP52 can bind AR and have chaperone function in prostate cancer cells. Therefore, targeting proteins involved in AR activation pathways that physically interact with AR, such as FKBP52, can inhibit androgen-induced cell growth and gene transcription in PCa cells by preventing hormone binding to the receptor and/or receptor translocation to the nucleus. Based on these observations we assume that in early and late stage PCa, AR is still dependent on molecular chaperones for its functional properties, FKBP52 cochaperone can increase AR activity and FKBP52 inhibitors will prevent AR regulation of PCa growth and progression.

V. Dissertation Goal

The maturation of steroid receptors to the final hormone binding conformation is a process that involves several chaperone proteins, all of which present an opportunity for therapeutic intervention into prostate cancer. HSP90 binds to immunophilin proteins (FKBP52) for subsequent interactions with steroid receptors, kinases, and other cellular signaling factors that play important physiological and potentially pathological roles in mammals. The inhibition of FKBP52 represents an attractive therapeutic option for the treatment of diseases that are dependent upon a functional hormone signaling pathway. Gaining a better understanding of the mechanism by which FKBP52 regulates receptor function and of the molecular determinants of FKBP52 specificity, will support downstream efforts in the therapeutic targeting of Hsp90 client proteins with a high degree of specificity. FKBP52-specific drugs could potentially have an advantage over the Hsp90 inhibitors that are currently being tested in clinical trials for the treatment of various cancers and endocrine diseases since FKBP52 is more selective for Hsp90 client proteins. Thus, FKBP52 inhibitors should be less toxic as compared to Hsp90 inhibitors.

The ability of steroid receptors to activate pathways defined as key regulators of cell growth is likely to play an important role in the development of resistance to endocrine therapies [73]. The goal of these studies is to develop novel small molecule inhibitors of FKBP52 that can be used to treat hormone-dependent diseases such as prostate cancer, benign prostatic hypertrophy, diabetes and obesity. In addition theses small

molecules could potentially be used as male contraceptives and research reagents for the study of androgen receptor interactions and their function.

CHAPTER 2

IDENTIFICATION OF MOLECULES THAT SPECIFICALLY INHIBIT FKBP52-ENHANCED RECEPTOR FUNCTION

I. Rationale

Previous studies have established FKBP52 as an important positive regulator of steroid hormone receptor function. Biochemical and cellular studies suggest that FKBP52 directly contacts the receptor hormone binding domain within the receptor-Hsp90 complex, which ultimately results in increased hormone binding to the receptor. The studies detailed herein were in part conducted in order to better understand FKBP52-receptor interactions. In addition, the steroid hormone receptor-specific phenotypes observed in the fkbp52-deficient mice have firmly established FKBP52 as an attractive therapeutic target for the treatment of hormone-dependent diseases. Thus, a small natural compound library screen in a yeast system was performed in order to identify FKBP52-specific inhibitors that could serve as research tools for the study of FKBP52-receptor interactions and as therapeutic candidates for the treatment of disease. Previous experiments performed by Dr. Neckers' laboratory at the National Cancer Institute identified two initial compounds (H7 and H8) as possible FKBP52-specific inhibitors. Using these original hits, we have acquired molecules that represent slight structural modifications and then assessed them for their ability to prevent FKBP52 potentiation of receptor activity.

II. Materials and Methods

Yeast Strains and Assays. *β*-galactosidase reporter assays developed previously described [25, 74-76] were used as a quantitative indicator of hormone receptor activity and for the yeast-based medium-throughput compound library screens. The basic reporter strains used for wild type AR, indicated AR point mutant, and GR assays were based on a W303a genetic background (MATa *leu2-112ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2)* and all contained a *URA3*-marked steroid receptor-mediated β-galactosidase reporter plasmid (pUCΔs-26X, kind gift from Brian Freeman, University of Illinois, Urbana-Champaign, IL). The parent plasmids for all other yeast expression vectors were described previously [77]. The AR point mutants were generated directly in the yeast expression vector by site-directed mutagenesis using the QuickChange kit (Stratagene) according to the manufacturer's instructions. For hormone-responsive reporter assays, the indicated strains were co-transformed with the following three plasmids: a hormone-inducible β-galactosidase reporter plasmid, a *LEU2*-marked high-copy number plasmid constitutively expressing the indicated steroid hormone receptor from a glyceraldehyde phosphate dehydrogenase (GPD) promoter, and a *TRP1*-marked high-copy number plasmid with or without human FKBP52 where indicated. Transformants were selected and maintained on synthetic complete dextrose medium lacking uracil, leucine, and tryptophan. All hormones were obtained from Sigma (St. Louis, MO) and were stored as 10 mM stock solutions in ethanol. Hormone dilutions were generated so that the ethanol vehicle never exceeded 1% total volume in the yeast cultures. The hormone concentrations were optimized by performing dose-response curves. Hormone-induced reporter activity was measured from yeast extracts

as described previously [25] with a single two-hour time point measurement. All assays were performed minimally in triplicate and data presented from at least three independent experiments that produced consistent results.

Compound Library Screening. The yeast-based *β*-galactosidase reporter assays used to screen the library of compounds were performed as previously described [78] with modifications to a 96-well plate format (Figure 2.1). The strain and plasmids used for the library screens were the same as those described above except the parent yeast strain was deleted for the pleiotropic drug resistance 5 (*PDR5*) gene in order to avoid the potential transport of candidate compounds out of the yeast. Two reporter strains were used in the library screen. The control strain contained a wild type human AR expression plasmid and an empty *TRP1*-marked plasmid, and the tester strain contained an AR-P723S expression plasmid and a *TRP1*-marked human FKBP52 expression plasmid. The assay protocol was designed to identify compounds that specifically reduce signaling from the tester strain and not the control strain (FKBP52-specific inhibition). The use of the wild type AR control strain controlled for specificity and general toxicity including effects on growth, transcription, translation and protein stability. The AR-P723S point mutant was previously described as being hypersensitive to FKBP52 potentiation [33]. The use of this mutant in these assays increased the sensitivity of detection as signaling at the hormone doses used in the tester strain depends entirely on the presence of FKBP52. Preliminary dose response curves were performed and the DHT concentrations used in the library screening assays were based on the EC_{50} values determined in both the control and tester strains. The Diversity Set

Library available through the Developmental Therapeutics Program of the National Cancer Institute was chosen for the screen. This library contains approximately 2000 identified compounds that were selected to have representative diverse chemical structures derived from a collection of almost 140,000 compounds. For the screening assays the cultures were grown in synthetic complete medium lacking uracil, leucine, and tryptophan at 30.0 ˚C in a shaking water bath and the optical density at 600 nm ($O.D._{600}$) was monitored until the cultures were in exponential phase growth ($O.D._{600}$ = 0.08 to 0.10). Cultures were then aliquoted into a 96-well plate at 100 µl per well and compounds added. The compounds were stored in dimethyl sulfoxide (DMSO), while yeast can tolerate up to 5% DMSO without significant effects on the assay results. Thus, care was taken not to exceed the 5% DMSO limit. All library compounds were assayed in the control and tester strains at a single concentration of 50 µM. Hormone was added 1 hr after compound addition and at 2 hrs 100 ul of Tropix Gal-Screen reagent (Applied Biosystems, Foster City, CA) was added to each well. The plates were incubated for an additional1 hr and 30 min at room temperature and light emission measured on a microplate luminometer (Luminoskan Ascent, Thermo Labsystems). Two compounds were identified (H7 and H8) to inhibit FKBP52-enhanced receptor function but did not affect AR function alone. These compounds were further analyzed for effects in wild type AR and GR reporter assays as described above. An additional 28 compounds that represented slight structural modifications of H7 were design by Johanny Tonos De Leon under the supervision of Dr. Marc Cox, purchased from the Sigma Rare Chemicals Library (Sigma-Aldrich, St. Louis, MO) and also assayed for their effects on FKBP52-regulated AR-P723S, AR and GR function. Chem Draw

software was used to design the structural modifications of the additional chemical compounds in order to determine the active motifs of the inhibitory molecules.

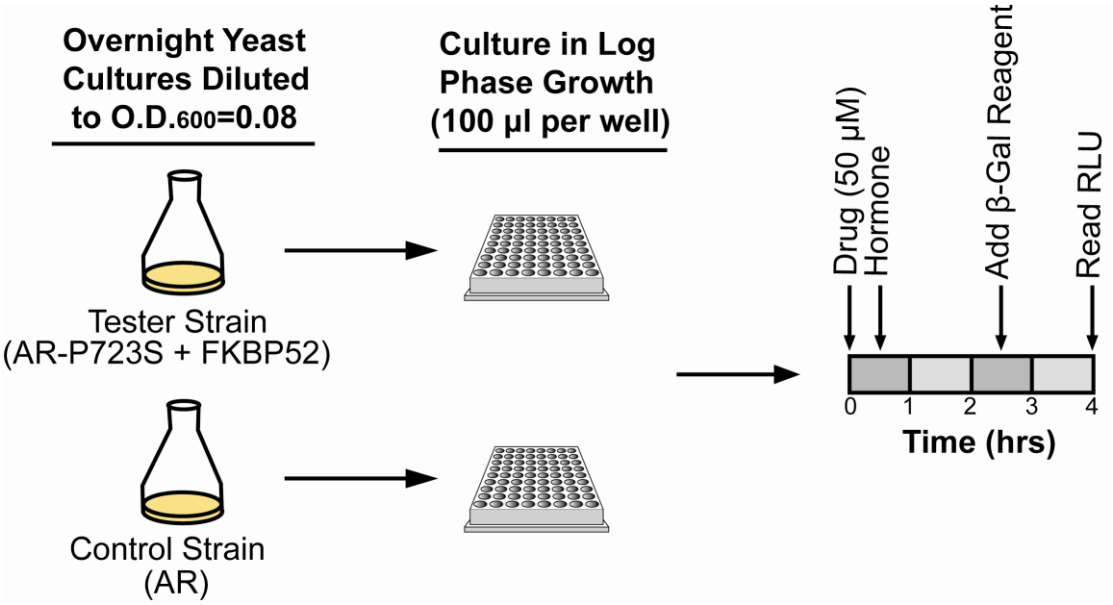

Figure 2.1. Representation of the Yeast-Based Compound Library Screen.

Figure 2.1. Representation of the Yeast-Based Compound Library Screen.

Overnight saturated cultures of the AR-responsive β-galactosidase reporter strains exogenously expressing human FKBP52 and AR-P723S (Tester) or wild type AR alone (control) were diluted back to an OD_{600} of 0.08 and incubated at 30.0 °C with shaking until log phase growth was observed (OD_{600} of approximately 0.1). The cultures were then aliquoted at 100 μl per well followed by the immediate addition (time 0) of library compounds at 50 μM. Hormone was added at 1 hr later followed by the addition of β-galactosidase substrate at 2 hrs after initial compound addition. Luminescence from the wells was measured 2 hours post compound addition. This assay was designed to identify any compound that inhibited FKBP52 regulated receptor activity in the tester strain, but not inhibit AR function alone in the absence of FKBP52. The AR alone control strain also controlled for general toxicity. FK506 was used as positive control.

Mammalian cell lines. Mouse embryonic fibroblast cells isolated from homozygous 52KO embryos (52KO MEF) were previously described [79]. The MDA-kb2 cells, obtained commercially from the American Type Culture Collection (ATCC), were also described previously [80, 81]. HeLa, 22Rv1, and VCaP cells were obtained commercially (ATCC). LNCaP cells were obtained from Dr. Donald Tindall (Mayo Clinic, Rochester, MN). LAPC4 cells were obtained from Dr. Charles Sawyers and Robert Reiter. All cells were maintained in the presence of 10% fetal bovine serum and 5% CO_2 at 37°C with the exception of MDA-kb2 cells. MDA-kb2 cells were grown in the absence of CO_2 at 37.0 °C. 52KO MEF and HeLa cells were maintained in MEM-EBSS medium with 2 mM L-glutamine, LNCaP and 22Rv1 cells were maintained in RPMI-1640 medium, MDA-kb2 cells were maintained in L-15 medium, and LAPC4 and VCaP cells were maintained in DMEM medium. At 24 hrs prior to experiments, cells were culture in medium lacking phenol red and charcoal-stripped FBS.

LNCaP, LAPC4, and VCaP prostate cancer cells all express endogenous AR and are sensitive to androgens. LNCaP cells are characterized by the presence of the AR T877A mutation. Both AR alleles are wild type in LAPC4 cells. VCaP cells are characterized by endogenous AR gene amplification and, although they can respond to androgens, are also capable of androgen-independent growth. The 22Rv1 cell line was derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. The AR mutation occurred during the progression to androgen independence. AR in 22Rv1 is characterized by an in-frame tandem duplication of exon 3 that encodes the second zinc

finger of the AR DNA-binding domain [82, 83]. 22Rv1 cells also express a constitutively active truncated form of AR lacking the C-terminal hormone binding domain. As a result, 22Rv1 cells can both respond to hormone and display hormone-independent growth.

Toxicity Analysis in Mammalian Cells. Two methods were used to measure cell viability of HeLa and Mouse Embryonic Fibroblast (MEF-FKBP52KO) cells. The first one to be used was trypan blue dye exclusion. This method determines cell viability based on cell membrane integrity. Cells were plated in 6-wells plates at a cell density of $2x10^6$ cells/well. After the cells attached they were treated with different concentrations of inhibitor for 24hrs. The cells were trypsinized and centrifuged for 5 minutes at 100xg. The cell pellet was resuspended in 1mL of 1XPBS and 10uL of cell suspension mixed with 10 uL of 0.4% trypan blue. The mixture was incubated for 3 minutes at room temperature followed by cell counting in the hemocytometer (viable cells were unstained unlike nonviable cells). The second method for testing cell viability was the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrzolium bromide) obtained from Invitrogen (Cat. M-6494) which measures mitochondrial redox potential. Cells were plated in a 96-well plate at a density of $5x10^3$ cells/well. After the cells attached they were treated with different inhibitor concentrations for 24 hours. The media was removed and replaced with fresh culture media and 10 uL of 12 mM MTT reagent added to each well. The samples were incubated at 37.0 ^oC for 4 hours followed by the addition of 100 uL sodium dodecyl sulfate Hydrochloric acid solution (SDS-HCL). The samples were incubated again for a period of 4-18 hours and the absorbance read in the spectrophotometer (570 nm).

Luciferase reporter assays. Plasmid transfections in 52KO MEFs were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were plated in 6-wells plates at a cell density of $2x10^6$ cells/well (approximately 80% confluence) and plasmid transfections at a DNA (µg) to lipofectamine ratio of 1:3 in MEM-EBSS lacking fetal bovine serum for four hours. The plasmids used for these assays were: hormone-responsive firefly luciferase reporter (400 ng per well), mammalian expression vector (pCI-neo; Promega, Madison, WI) expressing the androgen receptor (800 ng per well), pCI-neo alone or expressing FKBP52 (800ng), and a constitutive β-galactosidase expression plasmid (50ng) as a transfection control. At 24 hrs post transfection the cells were treated with the indicated inhibitor concentrations for 1 hr followed by treatment with DHT (concentrations used corresponded to the EC_{50} for DHT at each condition) for 16 hrs and 37.0 ^oC. The cells were then lysed in 150 µl of M-PER mammalian protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL) supplemented with Complete mini EDTA-free protease inhibitors (Roche, Indianapolis, IN) per well and incubated at room temperature for 10 min. For luciferase activity, 100 µl of luciferase assay reagent (Promega) was added to 40 µl of cell lysate in an opaque 96-well plate and light emission was measure in the luminescence plate reader (Luminoskan Ascent, Thermo Labsystems). For β-galactosidase activity, 100 µl of Tropix Gal-screen reagent (Applied Biosystems, Foster City, CA) was added to 10 µl of cell lysate in a white 96-well plate and incubated for 2 hrs at room temperature. β-galactosidase activity was measured using a luminescence plate reader. Differences in transfection efficiency were normalized by dividing RLU (relative light units) by β-galactosidase activity. The MDA-kb2 cells stably express an androgen-responsive firefly

luciferase reporter construct as previously described [81]. Thus, no plasmid transfections were necessary in this cell line. MDA-kb2 cells were treated with 0.2 nM DHT (EC_{50} for DHT in this cell line) and the indicated concentrations of inhibitors for 20 hrs followed by cell lysis and luminescence measurement using Bright-Glo (Promega) Luciferase assay reagent according to the manufacturer's instructions. The assays shown for all cell lines are representative of at least three independent assays with each data point measured in triplicate.

Western blot analysis. For the assessment of AR nuclear translocation cells were grown to 50% confluence, washed in serum-free medium and re-cultured for 48 hours in fresh medium followed by addition of MJC13 at the concentrations indicated. After an additional 24 hrs hormone was added (R1881 or fetal bovine serum) and cells cultured for an additional 2 hrs. Cells were lysed and separated into nuclear and cytosolic fractions according to previously established methods. Detection of AR in the nucleus and the cytosol was performed by polyacrylamide gel electrophoresis followed by Western blotting. For Western blots, cells were lysed 48 hrs after transfection with the M-PER mammalian protein extraction reagent (Thermo Fisher Scientific Inc.) as described above. To determine protein concentration the Coomassie Plus protein assay reagent (Thermo Fisher Scientific Inc.) was used. Typically 20 µg of total cellular protein was separarted on a 10-20% Criterion gel (Bio-Rad, Hercules, CA) and transferred to a Polyvinylidene Difluoride membrane. The mouse monoclonal antibody directed against FKBP52 (HI52D) was used as previously described [84]. The mouse monoclonal antibody directed against Hsp90 (H90-10) was generously provided by Dr. David Toft

(Mayo Clinic, Rochester, MN). The polyclonal rabbit antibody directed against AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA), the mouse monoclonal antibody directed against glyceraldehyde phosphate dehydrogenase (6C5; Biodesign International, Saco, MN), and the alkaline phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies (Southern Biotechnology Associates, Birmingham, AL) were all obtained commercially. The bands were visualized using an Immuno-star substrate (Bio-Rad) and exposing to x-ray film.

Electrospray Ionization-Mass Spectrometry (ESI-MS) Analysis. Cell samples were dissolved in methanol containing 0.1% formic acid and injected by infusion (500 nL/min) or static nanospray (EconoTip, New Objective) into an ElectroSpray Ionisation-Time-of-Flight Mass Spectrometry (ESI-QTOF-MS) analyzer (Qtof-1, Micromass, Waters). Spectra was collected in the positive-ion mode at 50-2500 mass-to-charge (*m/z*) range. The nanospray source was set at 1.0-2.5 kV, cone voltage 35 V, and the source and desolvation temperatures at 110 and 150^{o}C, respectively.

III. Results

Identification of Small Molecule Inhibitors of FKBP52-Enhanced AR Function.

A yeast-based screen of a compound library (Figure 2.1) resulted in the identification of two candidate inhibitors (H7 and H8) that specifically inhibited FKBP52-enhanced AR-P723S function (the P723S mutant was used to increase sensitivity in the assay). H8, though functional, was disregarded in this study as it was found to be specific for FKBP52-enhanced AR-P723S, but had no effect on wild type AR (Figure 2.4). In addition to H7, we assessed 28 additional compounds, designed by Johanny Tonos De Leon under the supervision of Dr. Marc Cox, that represented slight chemical modifications of H7 for effects on FKBP52-enhanced wild type AR, AR-P723S, and GR signaling in the yeast-based reporter assay. The complete set of yeast-based SAR data is illustrated in both qualitative (Figure 2.3) and quantitaive (Figure 2.4 and Table 2.1) format. In addition, the results and chemical structures for two of the most promising compounds, MJC01 and MJC13, and the original hit compound H7 are shown in Figure 2.5. The control strain contained only the receptor in the absence of FKBP52 while all other strains contained FKBP52 allowing the results to be normalized to show only FKBP52-specific effects. While H7 produced maximal inhibition at 100 µM (Fig. 2.5A), MJC01 and MJC13 displayed increased potency with maximal inhibition between 5 and 10 µM (Fig. 2.5B-C). All of the FKBP52-specific compounds identified displayed at least some general receptor inhibition at high doses. MJC01 generally inhibited AR signaling in the absence of FKBP52 at the 100 µM dose as evidenced by the upward trend in the dose response curve at that dose (Fig. 2.5B). In contrast, MJC13 was FKBP52-specific at concentrations as high as 100 µM (Fig. 2.5C), although it did produce some general

receptor inhibition at significantly higher doses. To further control for specificity we assessed these compounds for their effect on the expression of a galactose-inducible β-galactosidase reporter (Figure 2.6). Neither MJC13 nor MJC01 affected inducible reporter expression indicating that the inhibition observed is not due to general effects on transcription, translation, or protein stability. Some of the compounds tested, including MJC01 (Figure 2.5B), also differentially affected wild type AR and AR-P723S reporter activity. In addition to the fact that the compounds can affect receptor function alone in the absence of FKBP52 at high doses suggests that the compounds bind the AR hormone binding domain and possibly specifically at the BF3 surface. This hypothesis is also supported by the fact that FKBP52-specific inhibitors are structurally similar to the fenamic acids, which were previously shown to bind the BF3 surface. For comparison, we also assessed the potential FKBP52-specific effects of various fenamic acid derivatives. Figure 2.5D revealed that the prototypical BF3-binding AR inhibitor flufenamic acid displayed no FKBP52-specific effects. Thus, although structurally similar they are functionally distinct. Many of the compounds tested also differentially affected AR and GR signaling. MJC13 was slightly more specific for FKBP52-enhanced AR signaling as compared to GR (Figure 2.5C). It is important to point out that all of the fenamic acids tested, with the exception of mefenamic acid, were not specific for FKBP52 but generally inhibited AR and GR signaling to varying degrees. Mefenamic acid had no effect on receptor function in the presence or absence of FKBP52. MJC13 displayed higher potency and better selectivity for AR than any other compound tested. Although MJC01 has a higher potency than H7 it is less selective for FKBP52 as evidenced by the reduction in FKBP52-specific inhibition at the 100 μM dose. Thus, at

the current time, MJC13 is the most promising lead compound that displays little effect on AR in the absence of FKBP52 and may selectively target AR better than any other compound tested.

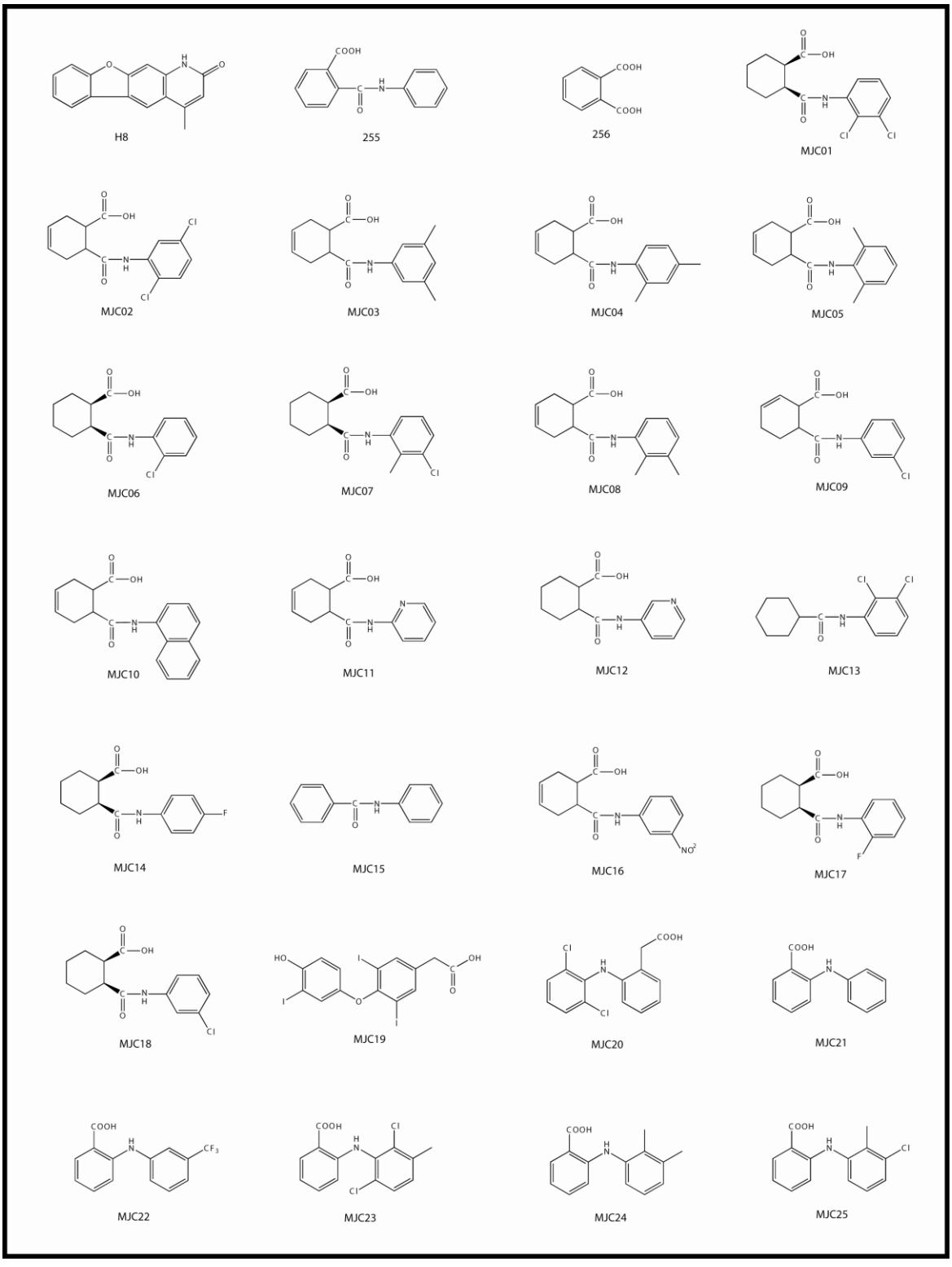

Figure 2.2. Structure of FKBP52-Specific Inhibitors.

Figure 2.2. Structure of FKBP52-Specific Inhibitors.

FKBP52 inhibitory molecules are structurally similar to fenamic acids [85, 86] and some small molecules known to bind the androgen receptor BF3 surface [87]. However, unlike the known BF3 binding molecules which generally inhibit AR function in the 100 uM range, our compounds specifically inhibit FKBP52-enhanced AR function at 100 fold lower concentration and, thus, are effective at concentrations that are below those required for AR function in the absence of FKBP52. The result of the initial high-throughput screen was the identification of one compound (H7) that inhibits FKBP52-enhanced AR function, but not AR function alone in yeast (figure 2.5). However, the compound showed weak inhibitory activity in our assay. In order to find compounds with higher activity and better AR selectivity, we analyzed this hit and identified 28 additional compounds that represent slight structural modifications to H7. Note that compounds MJC19 through MJC25 are small molecules that have been previously described to bind to the BF3 surface. H7 derivatives include modification of the phenyl moiety by introducing various substituents including nitro, hydroxyl, alkoxy, aryl and alkyl groups or aromatic heterocycles and modification of the spacer between the cyclohexyl and phenyl moieties.

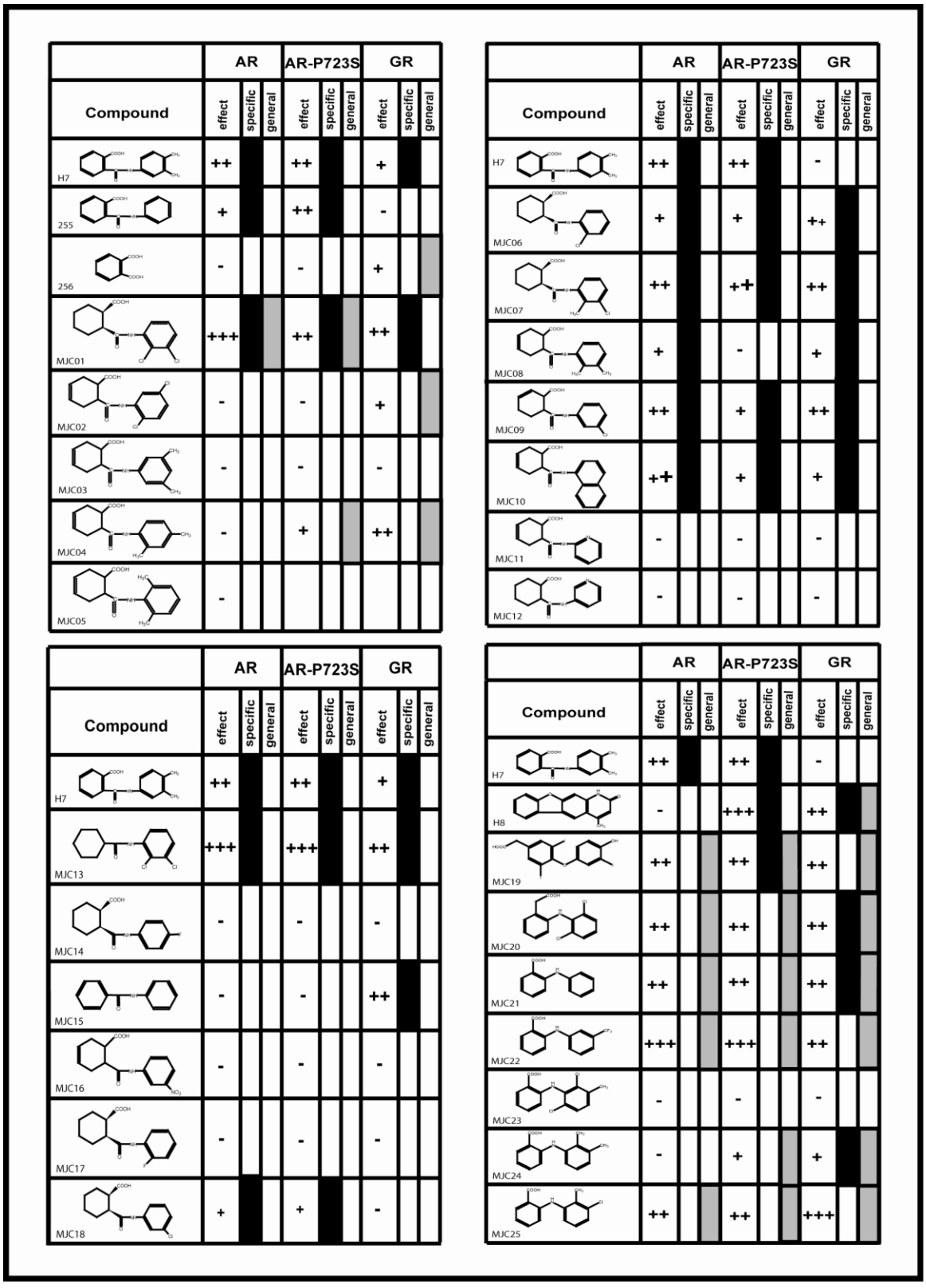

Figure 2.3. Qualitative Analysis of SAR Data.

Figure 2.3. Qualitative Analysis of SAR Data. All H7 derivatives tested were assigned an MJC number and placed on qualitative tables. The degree of inhibition is denoted but positive signs with +++ equally maximal inhibition. The specificity of inhibition towards FKBP52-enhanced receptor function is denoted by a black box and general inhibition of the receptor in the absence of FKBP52 is denoted by a gray box. When a compound has both, black and grey boxes, the molecule shows specific inhibition at low doses and a general inhibitory effect at higher doses.

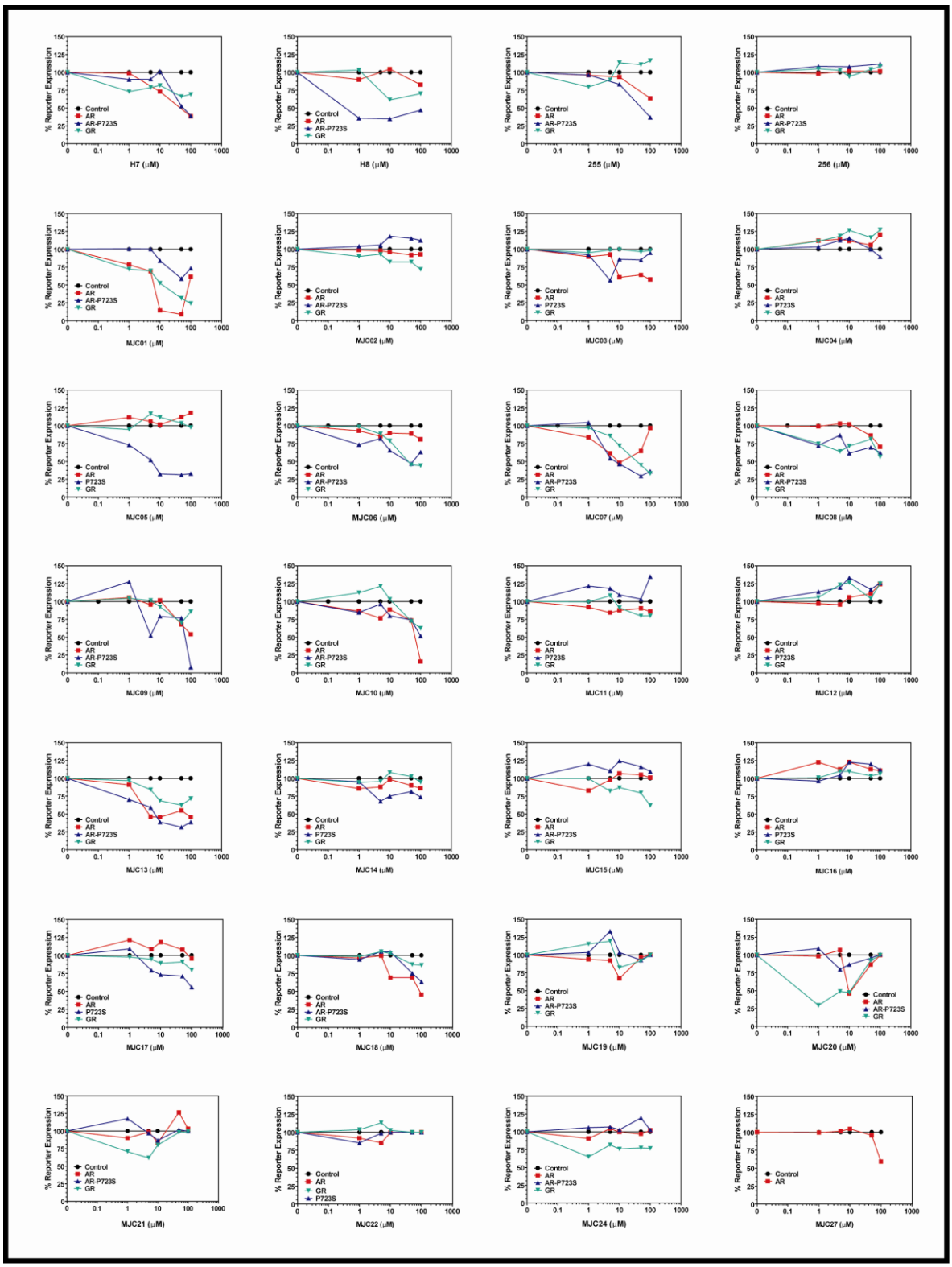

Figure 2.4. Normalized SAR Data.

Figure 2.4. Normalized SAR Data. Yeast reporter strains expressing wild type AR in the absence (control, black closed circles) or presence (AR, red closed squares) of FKBP52, and the AR-P723S point mutant in the absence (control, black closed circles) or presence of FKBP52 (AR-P723S, blue upright triangles), and GR in the absence (control, black closed circles) or presence of FKBP52 (GR, green down-facing triangles) were treated with a range of concentrations of the indicated compounds in the presence of DHT. The data were normalized to show only effects on FKBP52-enhanced receptor function by calculating the percent reduction in the control strain for each data point and adding that back to each data point for both the control and FKBP52 tester strains.

Table 2.1 IC_{50} Values Determined in SAR Assays

Inhibitor	IC_{50} WT AR (μM)	IC_{50} P723S (μM)	IC_{50} GR (μM)
H7	48.37	63.10	3062
H8	-	0.305	324.3
255	194.4	56.31	-
256	-	-	-
MJC01	7.334	255.1	12.79
MJC02	62397	-	329.08
MJC03	176.1	6.249	-
MJC04	-	125.7	-
MJC05	-	6.461	-
MJC06	169259	51.23	54.85
MJC07	9.067	14.26	39.16
MJC08	168.5	408.5	1525
MJC09	105.7	41.40	786.9
MJC10	64.95	165.5	126.9
MJC11	-	-	-
MJC12	-	-	-
MJC13	4.31	9.41	502.8
MJC14	863.4	10174	-
MJC15	-	-	369.9
MJC16	-	-	-
MJC17	-	166.5	3626
MJC18	97.03	137.6	458.7
MJC19	-	-	9154
MJC20	9.29	-	0.256
MJC21	-	-	-
MJC22	-	-	-
MJC23	-	8421	-
MJC24	-	-	733.60
MJC25	-	647.20	-
MJC26	-	11634	-
MJC27	109.8	0.553	15.79

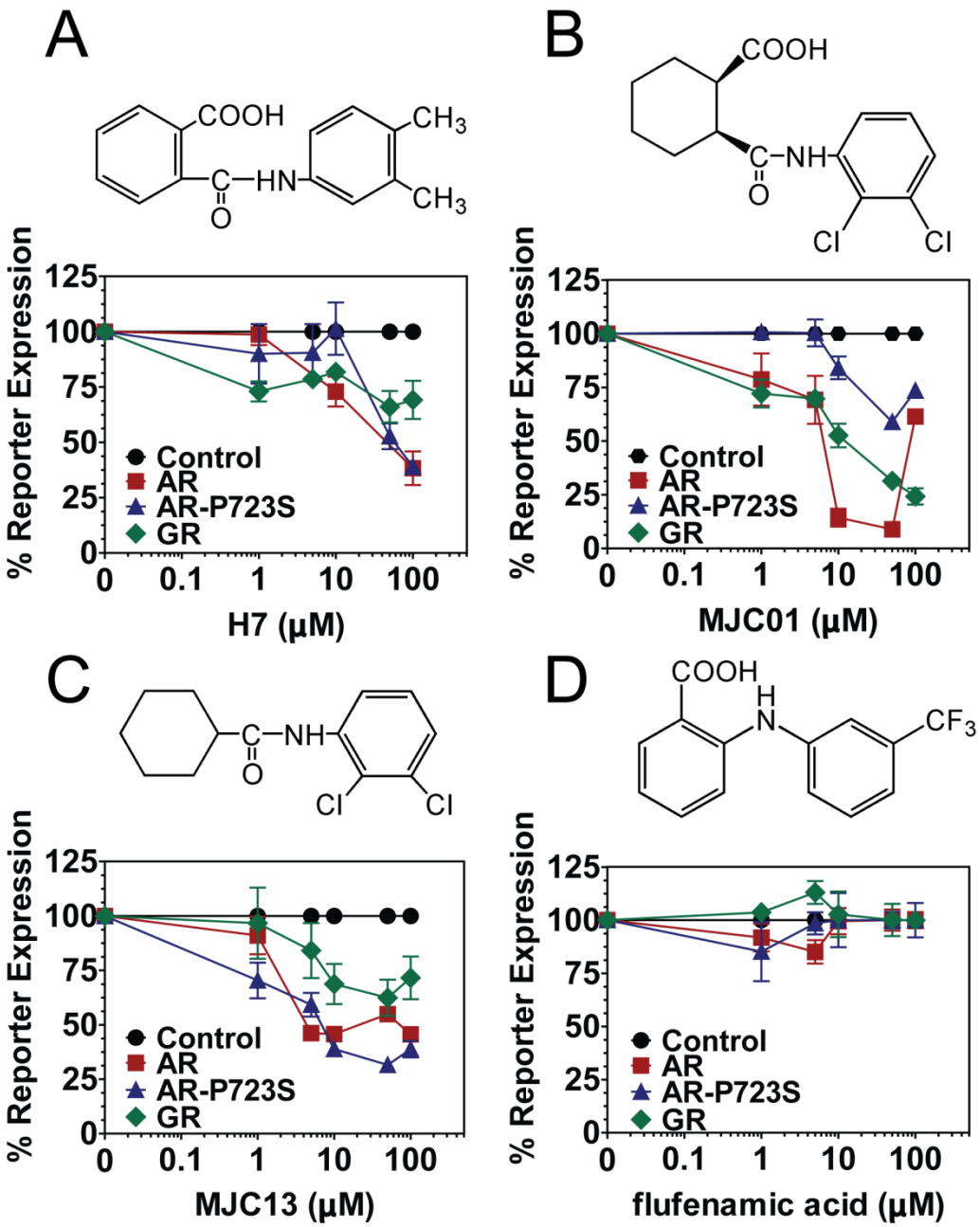

Figure 2.5. Analysis of Inhibitors Specific for FKBP52-Regulated Receptor Function in Yeast.

Figure 2.5. Analysis of Inhibitors Specific for FKBP52-Regulated Receptor Function in Yeast. Yeast reporter strains expressing wild type AR in the absence (control, closed circles) or presence (AR, closed squares) of FKBP52, and the AR-P723S point mutant in the absence (control, closed circles) or presence of FKBP52 (AR-P723S, closed triangles) were treated with a range of concentrations of the indicated compounds in the presence of DHT. H7 (A) is the original hit identified from the library screens, MJC01 (B) and MJC013 (C) are the current lead compounds, and flufenamic acid (D) is a known AR inhibitor that associates with the BF3 surface. The structures of the molecules are illustrated above each respective graph. The data were normalized to show only effects on FKBP52-enhanced AR function by calculating the percent reduction in the control strain for each data point and adding that back to each data point for both the control and FKBP52 tester strains. Thus, a hormesis-like effect as seen for MJC01 at 100 μM (B) indicates that the receptor in the absence of FKBP52 was generally inhibited at that dose (FKBP52 independent effects).

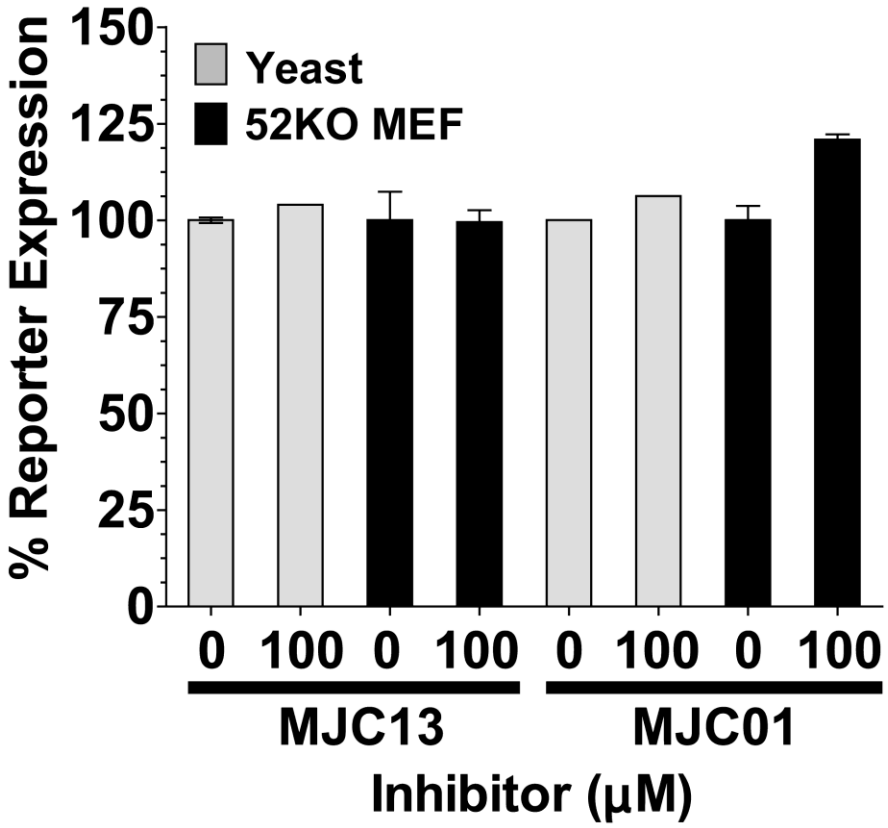

Figure 2.6. The FKBP52 Inhibitors do not Affect General Transcription, Translation, or Protein Stability.

Figure 2.6. The FKBP52 Inhibitors do not Affect General Transcription, Translation, or Protein Stability. Galactose-inducible β-galactosidase reporter expression in yeast (gray bars) and constitutive *renilla* luciferase reporter expression in FKBP52-deficient mouse embryonic fibroblasts (black bars) was assessed in the presence or absence of the indicated inhibitors at 100 μM.

The AR BF3 Surface is a Putative FKBP52 Interaction/Regulatory Surface

The stoichiometry and identity of proteins forming cytoplasmic chaperone complexes with androgen receptor are not known in detail. FKBP52, a cochaperone, associates with steroid hormone receptor-chaperone complexes and is a specific positive regulator of AR, PR and GR function [25, 33, 88]. Although FKBP52 functionally associates with three steroid hormone receptors indirectly through Hsp90 binding several lines of evidence suggest that FKBP52, in particular the FKBP52 FK1 domain, directly binds to the receptor hormone binding domain within the context of the Hsp90 chaperone complex [89]. To identify this potential FKBP52 interaction surface on AR we aligned receptor amino acid sequences and identified seven amino acid residues (L805, C806, K845, R846, R840, F673, and P723) within the AR hormone binding domain that are conserved in the FKBP52-regulated receptors, PR and GR, but differ in mineralocorticoid receptor (MR). Interestingly, all seven have been found mutated in prostate cancer and/or androgen insensitivity syndrome (AIS) patients (McGill Androgen Receptor Gene Mutations Database, http://androgendb.mcgill.ca/). An analysis of the AR hormone binding domain crystal structure revealed that these residues delineate a surface region that overlaps with the recently described AR BF3 surface [90] (Figure 2.7B-C). In addition, mutation of P723, within the BF3 surface, results in a receptor that is hypersensitive to FKBP52 potentiation [33]. To assess the effects of the additional residues on FKBP52 regulation of AR function we systematically mutated each of the residues and assessed the mutant receptors for their ability to respond to FKBP52 potentiation in yeast-based AR-mediated reporter assays. In all cases the specific mutations made were based on mutations that have been identified in prostate cancer

and/or AIS patients. Many of these mutant receptors lost activity and did not respond to FKBP52. However, we identified two additional mutations, F673P and C806Y, which resulted in AR hypersensitivity to FKBP52 potentiation (Figure 2.6A), although not to the same degree as P723S. As highlighted in Figure 2.7A F673 contacts P723 within the BF3 surface and C806, although not a surface residue, is buried directly below p723 and F673. Thus, the BF3 surface, particularly the region containing F673 and P723, is a putative FKBP52 interaction and/or regulatory surface.

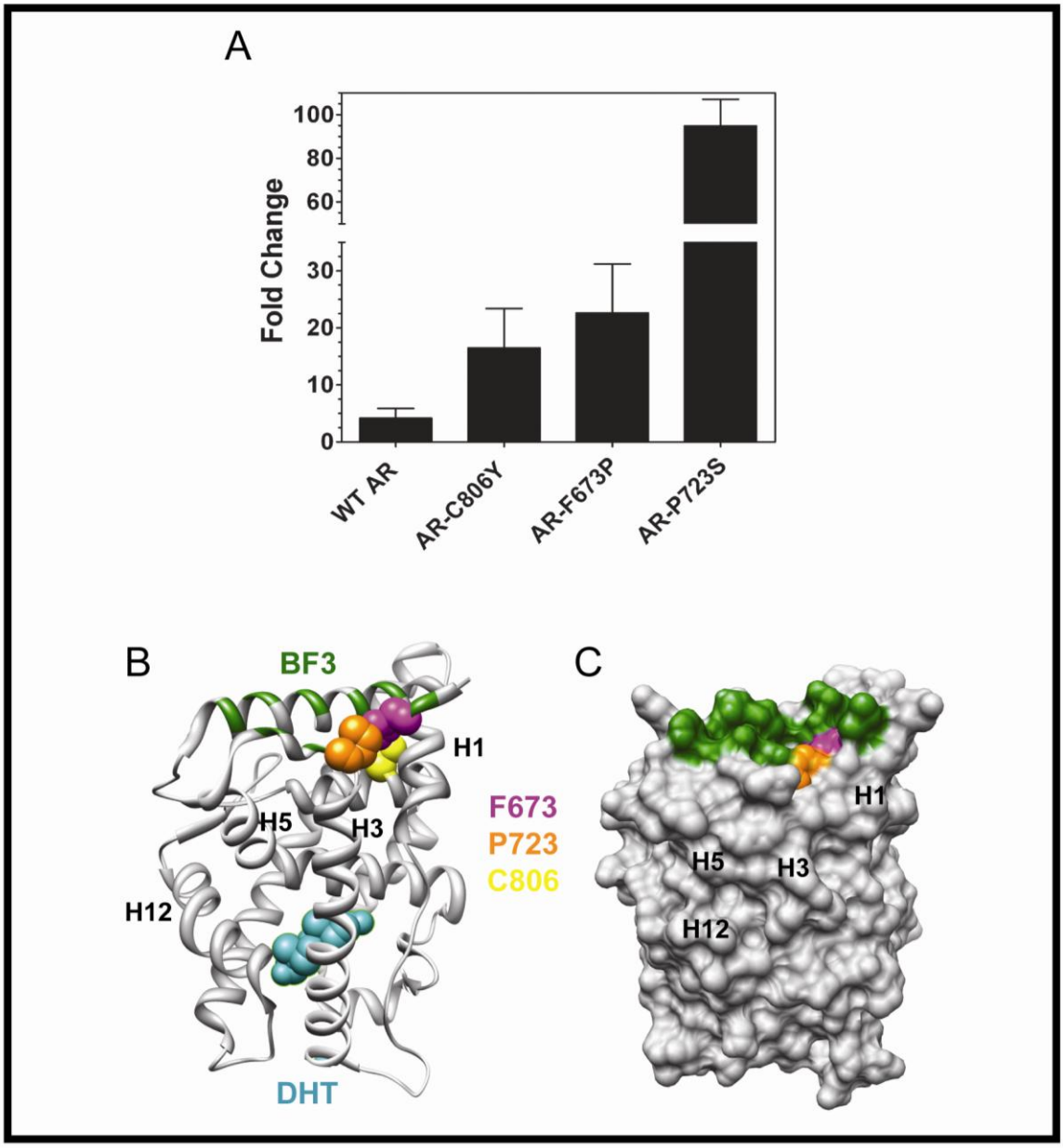

Figure 2.7. Mutations in the AR BF3 Surface Result in Increased Dependence on FKBP52 for Function.

Figure 2.7. Mutations in the AR BF3 Surface Result in Increased Dependence on FKBP52 for Function. A. Yeast reporter strains for wild type AR or the indicated AR point mutants were transformed with an empty plasmid vector or plasmid expressing human FKBP52. The yeast were induced with DHT and assessed for β-galactosidase expression. The data are plotted as fold change of the activity measured in the presence of FKBP52 over the activity in the absence of FKBP52 and are representative of at least five independent experiments. B-C. The AR hormone binding domain crystal structure showing the location of the mutated residues in relation to the BF3 surface is shown. The BF3 surface is highlighted in green, bound DHT is highlighted in blue, and the F673, P723, and C806 residues are highlighted in purple, orange and yellow respectively.

Compounds Effectively Target FKBP52-Enhanced AR Signaling in Mammalian Cells

The compound library screen and subsequent structure activity relationship (SAR) analysis were all performed in yeast-based assays. To assess the effects of the compounds in higher vertebrate model systems we first tested the compounds for the ability to inhibit AR signaling in MDA-kb2 cells (Figure 2.8A-B). This cell line contains a stable androgen-responsive luciferase reporter construct and serves as a rapid mammalian assay for assessing AR inhibition [81]. This cell line expresses endogenous FKBP52 and can't be used to distinguish FKBP52-specific effects. MDA-kb2 cells were treated with a range of concentrations of the indicated compounds for 20 hrs and assessed for both cell viability and AR-dependent expression of the luciferase reporter gene. The compounds displayed no cellular toxicity in MDA-kb2 cells as assessed by ATP quantification (Figure 2.8A). H7, MJC01, and MJC13 all inhibited AR-mediated expression of the luciferase reporter with half maximal inhibitory concentrations (IC_{50}) of 24.56, 3.60, and 1.79 μM respectively (Figure 2.8B). These data correlate well with those observed in the yeast-based assays.

Mouse embryonic fibroblasts (MEFs) derived from FKBP52-deficient mice (52KO) [88] is the only higher vertebrate model system that contains a true FKBP52-negative background in which to test the compounds for FKBP52-specific effects. Thus, we setup AR-mediated luciferase reporter assays in 52KO MEF cells in the presence or absence of an FKBP52 expression vector and assessed the compounds for cellular toxicity and FKBP52-specific inhibition of androgen-dependent luciferase expression. None of the compounds produced cellular toxicity up to the maximum soluble

concentration of 250 µM as assessed by trypan blue dye exclusion & MTT assays (Figure 2.8C). MJC01 and MJC13 specifically inhibited FKBP52-enhanced AR-mediated expression of the luciferase reporter gene with IC_{50} values of 0.62 and 0.45 µM respectively (Figure 2.8D and E). Consistent with the data obtained in the yeast-based assays MJC01 displayed significantly higher FKBP52-independent inhibition of AR function (Figure 2.8D) as compared to MJC13. MJC13 also produced general AR inhibition in this system at concentrations above 50 µM (data not shown). To further evaluate compound specificity, the compounds were assessed for effects on constitutive *renilla* luciferase expression in the 52KO MEF cells (Figure 2.6). Neither MJC13 nor MJC01 affected the constitutive expression of *renilla* luciferase.

Western blots using lysates prepared from the cells tested as described in Figure 5E showed increasing levels of AR and FKBP52 protein that directly correlated with increasing concentrations of MJC13 (Figure 2.8F). Repeated experiments revealed that the degree of stabilization varied between experiments and one of the more dramatic examples is shown in Figure 2.8F. Variable stabilization of Hsp90 and p23 protein levels was also observed, but to a lesser degree than that seen for FKBP52 and AR.

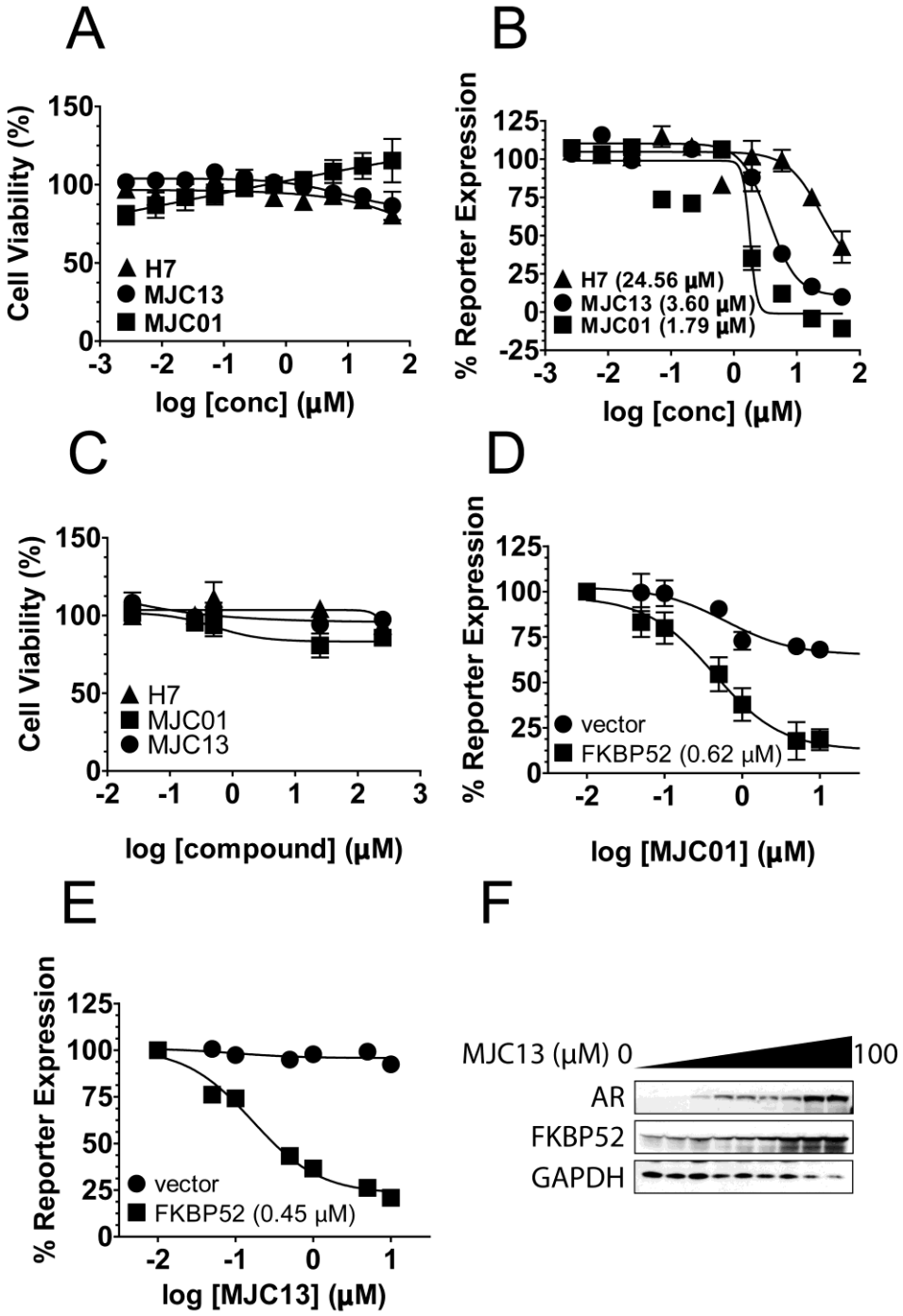

Figure 2.8. Effects of the Inhibitors on FKBP52-Regulated AR Function in Mammalian Cells.

Figure 2.8. Effects of the Inhibitors on FKBP52-Regulated AR Function in Mammalian Cells. A-B. MDA-kb2 cells carrying a stably transfected AR-responsive luciferase reporter were treated with 0.2 nM DHT and a range of concentrations assessed for cell viability (A) and AR-dependent expression of the luciferase reporter (B). The IC_{50} values for the compounds are shown in the legend. C-E. Luciferase reporter assays in 52KO mouse embryonic fibroblast cells in the presence or absence of FKBP52 were performed. Transfected cells were treated with DHT and a range of concentrations of the indicated compounds and assessed for cell viability (C) and AR-dependent expression of the luciferase reporter (D-E). The IC50 values for MJC01 (D) and MJC13 (E) are shown in the legends. F. Lysates were prepared from 52KO MEF cells transfected with AR and FKBP52 expression vectors that were treated with DHT and a range of MJC13 concentrations (0, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 μM) for 24 hours. Lysates were immunoblotted for AR, FKBP52, and GAPDH as a loading control.

Purity of MJC Molecules. One concern that often arises when working with small molecule inhibitors is the fact that the inhibitory activity observed could be due to impurities within the sample. Thus, mass spectrometry was performed with the lead compounds to assess purity. The compounds tested showed approximately 95% purity (Figure 2.9).

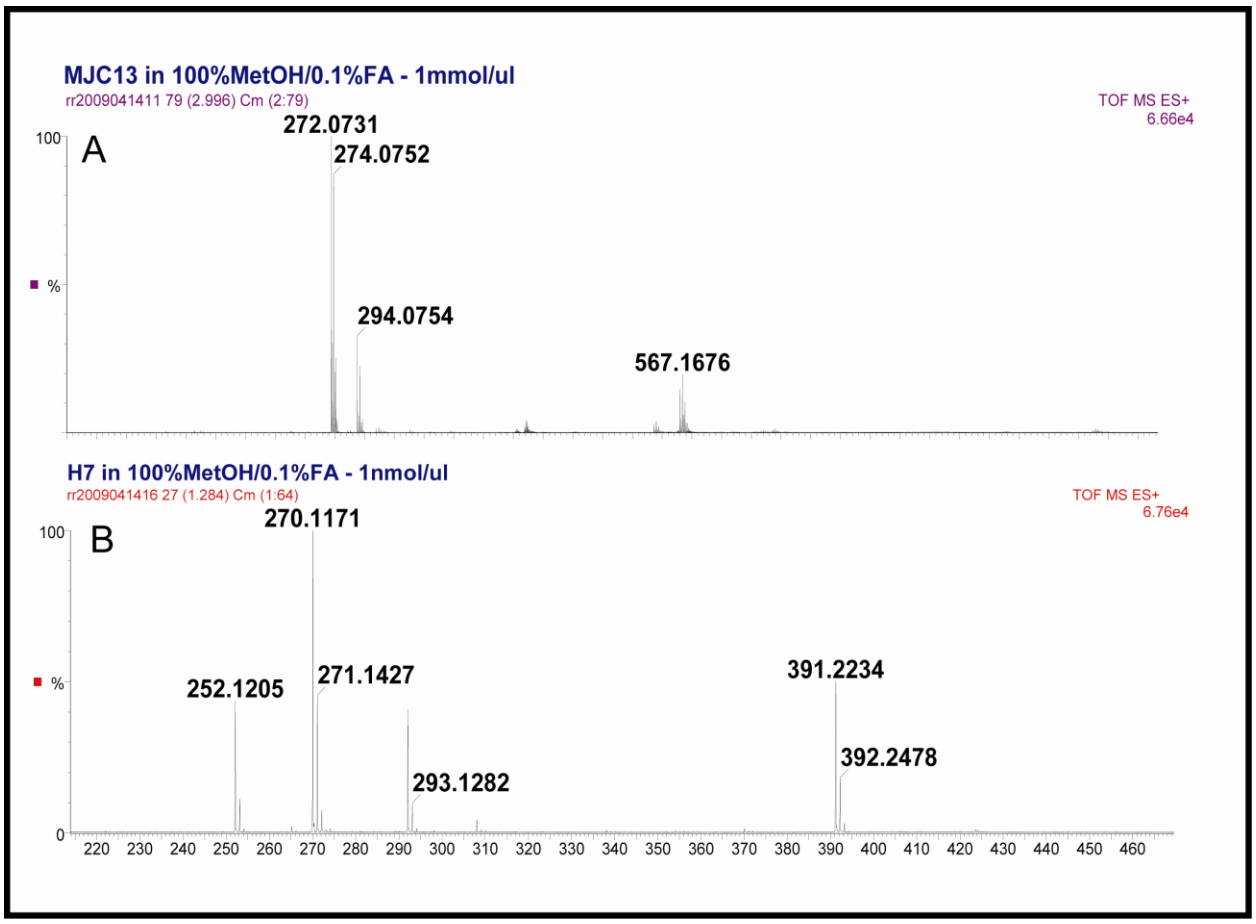

Figure 2.9. Purity of MJC Molecules.

Figure 2.9. Purity of MJC Molecules. Electrospray ionization-mass spectrometry (ESI-MS) analysis was used to assess compound purity. The compounds tested showed approximately 95% purity. A. MJC13 and B. H7.

CHAPTER 3

CHARACTERIZATION OF ANDROGEN RECEPTOR-FKBP52 INTERACTIONS IN THE PRESCENCE OF THE INHIBITORS AND THE EFFECTS ON RECEPTOR LOCALIZATION

I. Rationale

We have identified a series of small molecules that specifically inhibit FKBP52-enhanced receptor function. Given that a functional screen was used for candidate inhibitor identification, the target site(s) are unknown. Whether MJC13 and active derivatives bind directly to FKBP52 and disrupt FKBP52 interaction with the receptor-Hsp90 complex remains to be determinate. The fact that the MJC molecules are structurally similar to fenamic acid molecules previously shown to bind the BF3 surface and the findings that they can differentially affect wild type AR and AR-P723S function suggest that the molecules may target the AR BF3 surface. This notion is further supported by the fact that the AR BF3 surface is hypothesized to be an FKBP52 interaction/regulatory surface. The studies detailed in this chapter sought to characterize the inhibitor target site and to use that information to identify the mechanism of inhibition.

II. Materials and Methods

Co-Immunoprecipitation. Cells were plated in 10 cm dishes at 10 % confluency in RPMI 1640 medium containing 10% charcoal-stripped fetal bovine. After 24 hours, MJC13 or vehicle was added to a final concentration of 30 μM. After an additional 24 hours, R1881 was added to a final concentration of 300 pM and the cells were lysed two hours later in TMNSV buffer [50 mM Tris·HCl (pH 7.4), 0.1% Nonidet P-40, 20 mM Na_2MoO_4, 150 mM NaCl, 2 mM Na_3VO_4] supplemented with a Complete™ protease inhibitor tablet (Roche). Protein concentrations were measured with a BCA™ assay kit (Thermo Scientific) and 1 ml of 1 mg/ml cell lysates prepared. Thirty microliters of recombinant Protein G agarose beads were added to each sample and rotated for one hour at 4 ^{o}C; 10 μg of immunoprecipitating antibody was then added to each sample and rotated for an additional two hours at 4 ^{o}C. Beads were washed four times with TMNSV buffer and boiled in SDS sample buffer for five minutes. Eluted proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and blotted with specific antibody for the protein of interest. Anti-AR (N-20, Santa Cruz), anti-Hsp90 (SPA-835,Stressgen), and anti-FKBP59 (SRA-1400, Stressgen) were used for both immunoprecipitation and immunoblotting.

Androgen Receptor . Early stage Prostate Cancer cells (LNCaP cells) were maintained in RPMI media supplemented with 10% fetal bovine serum and 0.1% L-glutamine (GIBCO). At 50% confluence, cells were washed several times in serum-free RPMI-1640 medium and re-cultured for 48 hours in RPMI-1640 medium containing 10% charcoal-stripped fetal bovine serum. At that time, MJC13 was added to a final

concentration of 30 μM. After an additional 24 hours, hormone was added (0.1 nM) and cells cultured for an additional 2 hours. Cells were lysed and separated into nuclear and cytosolic fractions according to previously established methods [91]. Quantification of AR in the nucleus and the cytosol was performed by Western blotting with an AR-specific antibody (N-20 from Santa Cruz) followed by SDS polyacrylamide gel electrophoresis.

Biacore/Binding Affinity of MJC13 to the AR-HBD. These experiments were performed by Dr. Robert Fletterick's laboratory at the Univiersity of California at San Francisco. There are only a few laboratories in the world that can perform Biacore studies with the AR hormone binding domain (AR-HBD) since this structure is very unstable. Dr. Fletterick kindly offered to perform this experiment for us; therefore there is no detailed protocol for this methodology.

Scintillation Proximity Binding Assays. The basic protocol for the scintillation proximity binding assay with AR LBD was performed as previously described [92]. Liquid handling was carried out using an automated liquid handling system (Biomek FX). To a 384-well Ni-chelate coated Flashplate® (PerkinElmer) 50 μl of 5 μM recombinant AR LBD was added in assay buffer (50 mM HEPES, 150 mM Li_2SO_4, 0.2 mM TCEP, 10% glycerol, 0.01% Triton X-100, pH 7.2). After 60 min incubation the protein solution was discarded, followed by washes with assay buffer. Next, 25 μl of the serially diluted inhibitors in assay buffer containing 10% DMSO were added to each well

followed by an additional 25 µl of a 3H DHT radioligand solution in assay buffer with a final assay solution of 5% DMSO. The plates were sealed and allowed to equilibrate for 5 hrs at 4°C. [^{3}H]-DHT was used at a final concentration of 20 nM. Radiocounts were measured using a TopCount Microplate Scintillation and Luminescence Counter (Packard Instrument Company). All samples were tested in triplicates.

Fluorescence Polarization Assay. The fluorescence polarization assay was previously described [93]. In short, 20 µl of protein mixture (6.25 µM recombinant AR LBD plus DHT and 0.0125 µM SRC2-3 peptide in dilution buffer; final concentration 50 µM compound, 4% DMSO) was added to 1.2 µl of respective compound solubilized in DMSO in a 384-well plate (Costar 3710). The plated conditions were equilibrated for 5 hrs before total fluorescence and fluorescence polarization measurements (excitation 485 nm, emission 530 nm) on an Envision (Perkin Elmer).

AR BF3 Docking Simulations. MJC01 and MJC13 were docked into the BF3 pocket of androgen receptor using DOCK 3.5.54 [94, 95]. Seven AR complex structures (pdb IDs, 2PIT, 2PIU, 2PIV, 2PIW, 2PIX, 2PKL and 2QPY) were docked individually to capture some receptor conformational flexibility. The protein in the complex structure was used as rigid receptor, without water molcules. The original crystal ligand was used to generate matching spheres to place the new compound. Multiple conformations of the compound were pre-generated and placed in the binding pocket guided by the matching spheres. Ligand poses were scored by their interactions with the protein, through a grid-based method calculating van der Waals, electrostatic

interactions and ligand desolvation energy. The top scoring poses against different receptor conformations were visually examined. The pose selected for each ligand was the one with the best docking scores and also the best complementary shape with the protein binding pocket (pdb ID, 2PIX).

III. Results

Defining the Binding Locus for MJC13

AR likely adopts an altered conformation when it forms complexes with FKBP52 and HSP90. MJC13 may bind to these proteins directly or indirectly via protein complexes. In collaboration with Robert Fletterick's laboratory (University of California San Francisco) we first assessed the case for direct association of FKBP52 to DHT bound and apo AR using surface plasmon resonance (SPR) studies. We could not identify any interactions between the two proteins. Thus, we next tested binding interaction between FKBP52 and compound H7 or MJC13 and as well found no interaction (data not shown).

To rule out interaction of these compounds with the hormone binding pocket the compounds were assessed for their ability to compete with DHT binding in scintillation proximity binding assays with the recombinant AR hormone binding domain (Fig. 3.1A) . This work was performed in collaboration with Kip Guy's laboratory (St. Jude Children's Research Hospital). Data from this work revealed that unlabelled DHT was able to effectively compete for tritiated DHT. However H7, MJC01, and MJC13 did not compete with DHT for binding within the AR hormone binding domain at relevant concentrations. Interestingly a slight inhibitory effect was observed in the presence of 100 μM MJC01, which may explain the general inhibition of receptor function at high doses. Fluorescence polarization assays of fluorescently labeled peptides from coactivators with the recombinant AR hormone binding domain were also performed to rule out interaction at the AF2 site (Fig. 3.1B-C). Fluorescence polarization is shown in Figure

3.1B and total fluorescence in Figure 3.1C. None of the compounds tested were able to compete with the SRC2-3 peptide for binding the AF2 site.

In the absence of data directly demonstrating interaction with the AR hormone binding domain we performed *in silico* docking simulations to predict the possible orientation of the molecules on the BF3 surface in collaboration with Yu Chen's laboratory (University of South Florida). The poses chosen for illustration in Figure 3.2 had some of the best docking scores (including electrostatic, van der Waals interactions and ligand desolvation energy) and the best shape complementarity with the binding pocket. Both ligands make extensive non-polar contacts with residues P723, F673, L830 and Y834 on the BF3 surface (Fig 3.2A-B). The amide group of the ligand appears to have favorable electrostatic interactions with the backbone oxygen atom of P723, although the N-O distance is too far for a hydrogen bond in the current binding poses (3.7 and 4.5 Angstrom for MJC01 and MJC13, respectively). The poses resemble that of flufenamic acid in its AR complex structure (pdb ID 2PIX) (Fig. 3.2C). Like flufenamic acid, the carboxylate group of MJC01 does not form any hydrogen bond with the protein in the selected pose. It is clear that the poses shown are one of many that are possible and should be viewed with caution. However, it is interesting that the poses with the highest docking scores all contained contacts with and/or around the P723 and F673 residues.

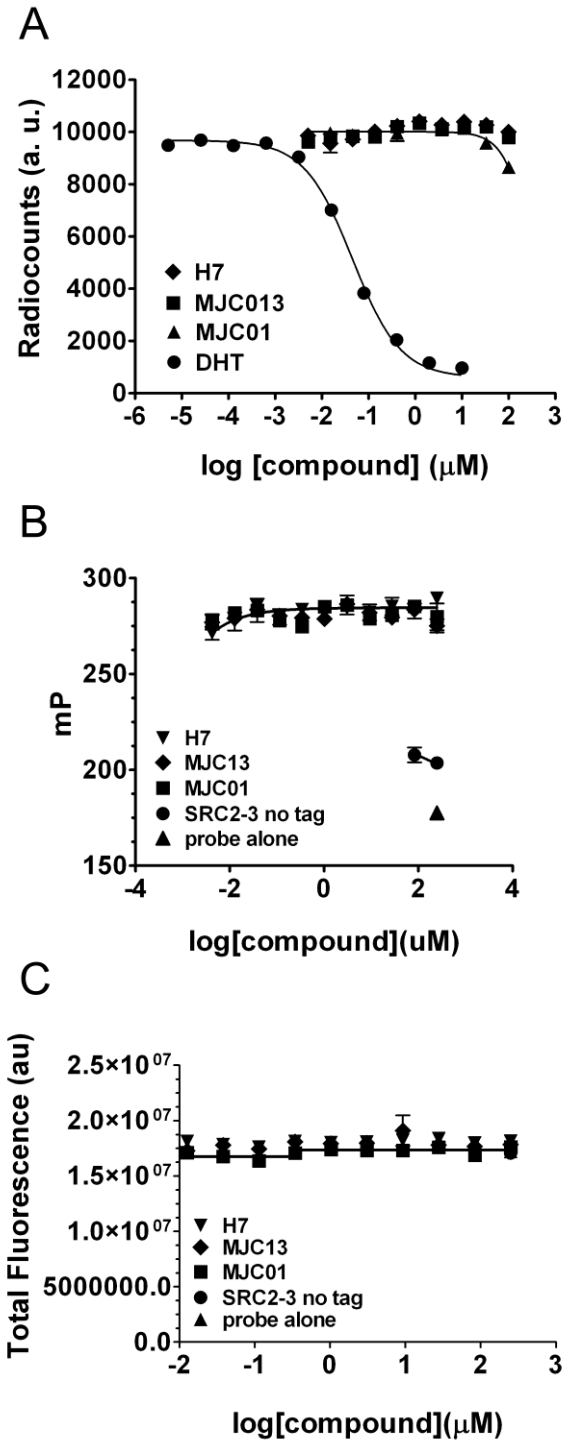

Figure 3.1. FKBP52 Inhibitors Do Not Bind the AR Hormone Binding Pocket or AF2.

Figure 3.1. FKBP52 Inhibitors Do Not Bind the AR Hormone Binding Pocket or AF2. A. A scintillation proximity binding assay using purified AR LBD is shown. Nickel-coated wells preincubated with 6xhistidine-tagged AR LBD were treated for 5 hours with a range of concentrations of the indicated compounds or cold DHT in the presence of 20 nM tritiated DHT and assessed for bound radioactivity. B. A fluorescence polarization assay using purified AR LBD is shown. Purified AR LBD preincubated with the fluorescently labeled SRC2-3 peptide was treated for 5 hours with a range of concentrations of the indicated compounds or non-labeled SRC2-3 peptide and assessed for fluorescence polarization and total fluorescence.

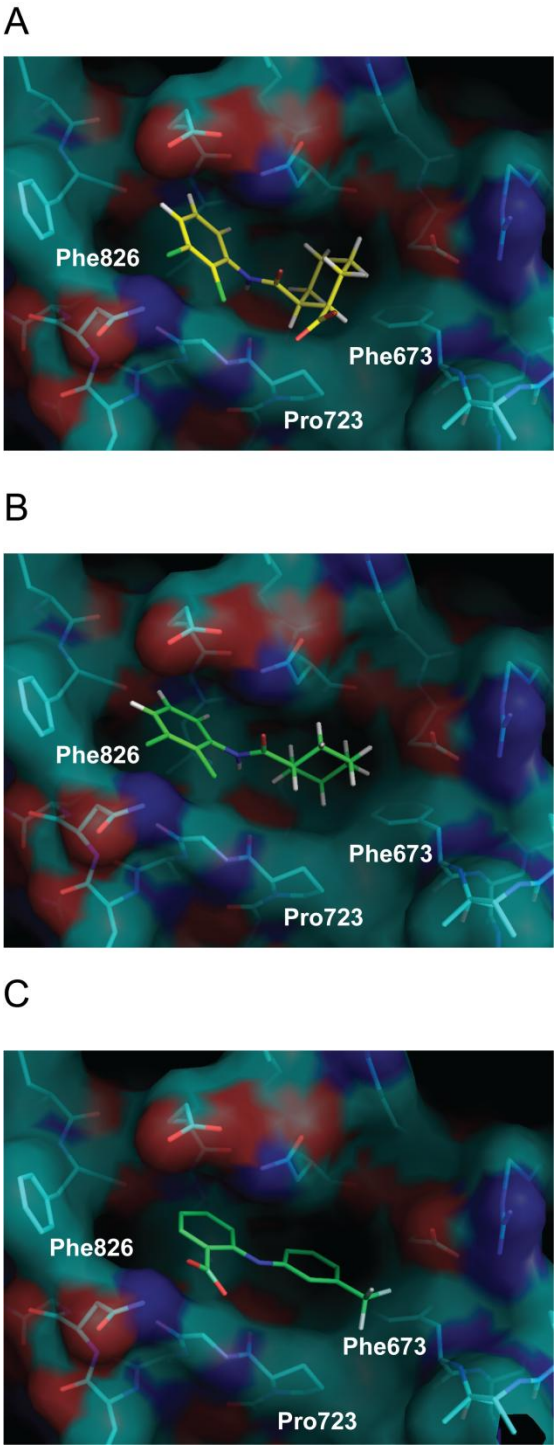

Figure 3.2. AR-Inhibitor Docking Simulations.

Figure 3.2. AR-Inhibitor Docking Simulations. The docking pose of the lead compounds MJC01 (A), MJC13 (B) and the crystal pose of flufenamic acid (C) on the BF3 surface of androgen receptor (protein databank number 2PIX). The poses shown were chosen based on the best docking score (including electrostatic, van der Waals interactions and ligand desolvation energy) and the best shape complementarity with the binding pocket. The carbon atoms of the protein and ligand are shown in cyan and yellow (MJC01)/green (MJC13)/light green (flufenamic acid) respectively. Nitrogen, oxygen, hydrogen and fluorine atoms are shown in blue, red, white and light blue, respectively.

MJC13 Prevents Receptor-Hsp90 Complex Dissociation and Nuclear Translocation in Cellular Models of Prostate Cancer

The effects of MJC13 on the stability of AR and associated chaperones is similar to that observed in the presence of non-hydrolyzable ATP analogues or sodium molybdate, which prevent hormone-dependent receptor-Hsp90 complex dissociation. To test the effects of MJC13 on complex formation and/or hormone-dependent complex dissociation we performed co-immunoprecipitations of FKBP52, AR and Hsp90 in lysates from cellular models of androgen-responsive (LNCaP and LAPC4) and hormone-refractory (22Rv1) prostate cancer cells grown in the presence or absence of hormone and MJC13 (Fig. 3.3). The ability of FKBP52 to bind the AR-Hsp90 complex in co-immunoprecipitations from prostate cancer cell lysates was unaffected while the addition of hormone resulted in complex dissociation as determined by a loss in the ability of FKBP52, AR, and Hsp90 co-precipitating. However, complex dissociation in the presence of hormone was abrogated by the addition of MJC13 in LNCaP, LAPC4, and 22Rv1 cells (Fig. 3.3A, C, and D respectively). In addition, Western immunoblots of fractionated lysates prepared from LNCaP, LAPC4, and 22Rv1 cells grown in the presence or absence of hormone and MJC13 revealed that hormone-induced AR translocation to the nucleus was blocked by the drug (Fig. 3.3B, D, and F respectively). MJC13 inhibition of AR nuclear translocation could be overcome by high hormone concentrations in LNCaP cells (Fig. 3.3B), which may reflect the lack of receptor dependence on FKBP52 for function at higher hormone concentrations [25].

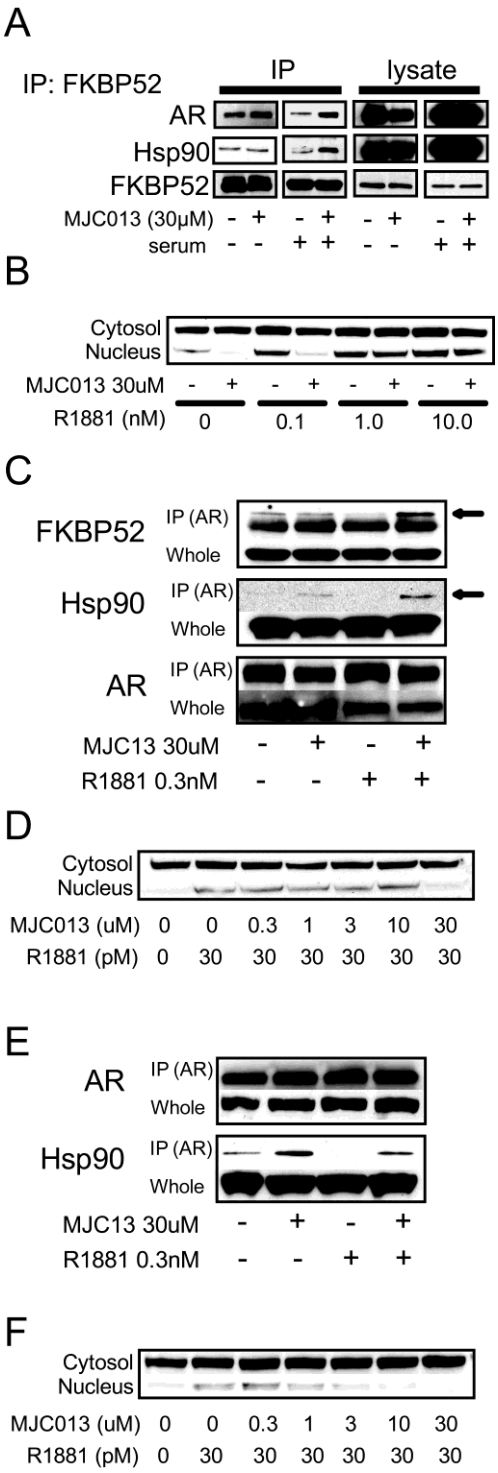

Figure 3.3. Effects of MJC13 on Receptor-Hsp90 Complex Dissociation and Nuclear Translocation in Early and Late Stage Prostate Cancer Cells.

Figure 3.3. Effects of MJC13 on Receptor-Hsp90 Complex Dissociation and Nuclear Translocation in Early and Late Stage Prostate Cancer Cells. The effects of MJC13 on hormone-dependent AR-Hsp90 complex dissociation and AR nuclear translocation were assessed in LNCaP (A-B), LAPC4 (C-D), and 22Rv1 (E-F) cells by co-immunoprecipitation and Western blot respectively. Lysates from cells grown in the presence or absence of the indicated concentrations of hormone and MJC13 for 24 hours were subjected to immunoprecipitation with either an antibody directed against FKBP52 (A) or AR (C and E) and immunoblotted for the indicated proteins. Fetal bovine serum served as the source of hormone in A. Lysates prepared from cells treated with the indicated concentrations of ligand and MJC13 for 24 hours were also fractionated and immunoblotted for AR in both the cytosol and nucleus (B, D, and F). These experiments were performed in triplicates.

CHAPTER 4

CHARACTERIZATION OF THE EFFECTS INHIBITORS HAVE ON HORMONE-DEPENDENT GENE EXPRESSION AND CELL PROLIFERETION IN PROSTATE CANCER CELLS

I. Rationale

Prostate cancer progression mostly depends on the availability of viable androgen receptor. The inhibitory molecules described herein not only impaired FKBP52-dependent AR function and signal transduction but also prevented the receptor from translocating into the nucleus and promoting gene expression. FKBP52 inhibitors identified were characterized and assessed for their ability to inhibit androgen-dependent prostate specific antigen secretion (PSA), in addition to measuring the effect each compound has on human prostate cancer cell proliferation (early and late stage). PSA is a serine protease that is secreted in small amounts by healthy prostate cells and is a major component of the seminal fluid; therefore it is used as a biomarker for PCa development [41]. PSA in serum is also used to monitor the success of therapies currently used for the treatment of PCa such as radiation, androgen ablation or prostatectomy and for monitoring the recurrence in PCa therapy. PSA has been detected in serum since 1990 for screening of PCa because it is an AR-regulated gene [42]. In addition to PSA, we also chose to assess the androgen-dependent expression of FKBP51, which is related to FKBP52, but does not regulate receptor function in

cellular assays. FKBP51 has recently emerged as a potential prostate cancer biomarker due to its hormone-dependent expression.

II. Materials and Methods

Prostate Cancer Cell Lines. 22Rv1 and VCaP cells were obtained commercially (ATCC). LNCaP cells were obtained from Dr. Donald Tindall (Mayo Clinic, Rochester, MN). LAPC4 cells were obtained from Dr. Charles Sawyers and Robert Reiter. All cells were maintained in the presence of 15% fetal bovine serum and 5% CO_2 at 37 °C. LNCaP and 22Rv1 cells were maintained in RPMI-1640 medium and LAPC4 and VCaP cells were maintained in DMEM medium. At 24 hrs prior to experiments, cells were switched to medium lacking phenol red and containing charcoal-stripped FBS. LNCaP, LAPC4, and VCaP prostate cancer cells all express endogenous AR and are sensitive to androgens. LNCaP cells are characterized by the presence of the AR T877A mutation. Both AR alleles are wild type in LAPC4 cells. VCaP cells are characterized by endogenous AR gene amplification and, although they can respond to androgens, are also capable of androgen-independent growth. The 22Rv1 cell line was derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. The AR mutation occurred during the progression to androgen independence. AR in 22Rv1 is characterized by an in-frame tandem duplication of exon 3 that encodes the second zinc finger of the AR DNA-binding domain. See Table 3.1 for details on the AR mutations found in the cell lines.

Table 4.1. Prostate cancer cell lines used.

Cell line	AR mutation	p53 mutation	TP53 mutation	Identity	References
22Rv1	H874Y	Q331R	-	CWR22-R1 cell line	[82, 83]
LAPC4	WT	A175H, P72R, R175H	G11827C	Unique	[82, 83]
LNCaP	T877A	-	WT	Unique	[82, 83]
VCaP	WT	A248W	-	Unique	[82, 83]

Enzyme-Linked Immunoadsorbant Assays (ELISA) for PSA

The indicated cells were plated at a density of $2x10^6$ cells/well and treated with a range of inhibitor concentrations 24 hrs after plating, followed by treatment with 500 pM DHT for LNCaP cells. VCaP cells were not treated with DHT. Media was collected from each well daily and stored at -20^oC until all the treatments were completed. ELISA plates (Alpha Diagnostic International) previously treated for the detection of human PSA by the manufacturer were used. A 25 µl of sample or standard was added to each well with 100 µl of AB-enzyme conjugate and incubated for 30 min at room temperature. The wells were washed with 300 µl of wash buffer followed by the addition of 100 uL TMB substrate per well and an incubation period of 15 min at room temperature. The reaction was stopped by adding 50 µl of stop solution to all wells and the absorbance measured at 450 nm using the Versa Max microplate spectrophotometer.

Cell Proliferation Assays. Cells were plated in a U-shaped 96-well plate at a density of $3x10^3$ cells/well. After the cells attached they were treated with inhibitor for 1 hour followed by the addition of 500 pM DHT. The wells were treated with 20 uL of tritiated thymidine (Isotype - [^{3}H] from Perkin Elmer) for 18 hours. Cells were lysed using a Cell Harvester (Micro96 Harvester from Skatron Instruments) and lysates were transferred to a filter paper (FilterMAT Cat # 11731 from Skatron Instruments) and incubated for 1 hour at room temperature. Samples were diluted in 3 ml of scintillation fluid (Scinti SAFE Econo F (LSC Cocktail) SX-22-5 from Fisher) and subjected to scintillation counting. All experimental measurements were performed in triplicate.

Quantitative Real-Time PCR. RNA was isolated using the RNeasy RNA Isolation kit according to manufacturer's instructions (Qiagen). For reverse transcription, 200 ng of total RNA was used in a reaction mixture containing 1X TaqMan RT buffer (Applied Biosystems) and Multiscribe Reverse Trasnscriptase. Reverse transcription was performed for 10 min at 25 °C, 30 minutes at 48 °C and 5 minutes at 95 °C using the PE9700 thermal cycler (Applied Biosystems). Real-time PCR primers were designed using Primer Express software (Applied Biosystems). The number of PCR cycles needed to reach the fluorescence threshold value is the cycle threshold (Ct). Ct values for the control (18S rRNA) and PSA gene were determined and relative RNA levels were calculated by the comparative Ct method as described by the manufacturer. Experiments were performed in duplicate; data are shown as PSA expression relative to 18S rRNA.

III. Results

MJC13 Effectively Blocks AR-Dependent Gene Expression and Proliferation in Prostate Cancer Cells

The effects of MJC13 on endogenous AR-dependent gene expression were assessed by analysis of prostate specific antigen (PSA) expression in LNCaP and VCaP cells. We also assessed the impact of MJC13 on expression of the 51 kDa FK506 binding protein (FKBP51) in these cell lines. FKBP51 has emerged as a potential hormone-dependent cancer biomarker [96, 97] due to its hormone-inducible expression (Figure 4.1). However, unlike PSA, FKBP51 is a component of steroid hormone receptor-chaperone complexes [84, 98, 99] and has recently been shown to promote AR function in LNCaP cells in a similar manner as FKBP52 [100]. Thus, inhibition of FKBP51 expression by MJC13 may have therapeutic implications. ELISA analysis of PSA secretion from LNCaP cells (Figure 4.2A) and VCaP cells (Figure 4.2B) demonstrated that MJC13 effectively inhibits PSA secretion from both cell lines. Inhibition of hormone-stimulated PSA secretion from LNCaP cells was more potent as compared to hormone-independent secretion from VCaP cells. However, in the presence of hormone MJC13 inhibited PSA secretion from VCaP cells to a similar degree as in LNCaP. The effects of MJC13 on endogenous levels of PSA and FKBP51 in LNCaP and VCaP cells were assessed by Western immunoblot and densitometry (Figure 4.2C and D). Representative blots for FKBP51, PSA, and the loading control GAPDH are shown (Figure 4.2C and D; *upper panels*). The normalized and averaged densitometry data from three independent experiments demonstrate that MJC13 reduced endogenous FKBP51 and PSA expression in a dose-dependent manner

(Figure 4.2C and D; *lower panels*). To further characterize the effect of MJC13 on AR transcriptional activity in LNCaP cells, we assessed both endogenous *PSA* gene expression and expression of the AR-responsive gene *TMPRSS2* by quantitative real time PCR (Q-PCR). MJC13 effectively abrogated constitutive expression of both AR-driven genes (Figure 4.2E, left panel). In contrast, in 22Rv1 cells, MJC13 was only able to block androgen-induced (but not constitutive) PSA expression (Figure 4.2E, right panel). These data are consistent with the fact that 22Rv1 cells express both full length AR and a constitutively active, truncated AR lacking the LBD (and so predicted to be insensitive to androgen, FKBP52, and MJC13).

Finally, the effect of these compounds on androgen-dependent prostate cancer cell proliferation was assessed by tritium incorporation in LNCaP, LAPC4, and 22Rv1 cells (Figure 4.3). MJC01 and MJC13 both inhibited androgen-dependent cell proliferation at concentrations consistent with those observed to be effective in reporter assays. MJC01 was less effective at inhibiting androgen-dependent LNCaP cell proliferation compared to MJC13 (Figure 4.3A). However, the effects of MJC01 and MJC13 in LAPC4 and 22Rv1 cells were similar. For comparison, the effect of a known AR antagonist, bicalutamide, which interacts with the hormone binding pocket, was assessed. Both MJC01 and MJC13 were more potent than bicalutamide in these assays.

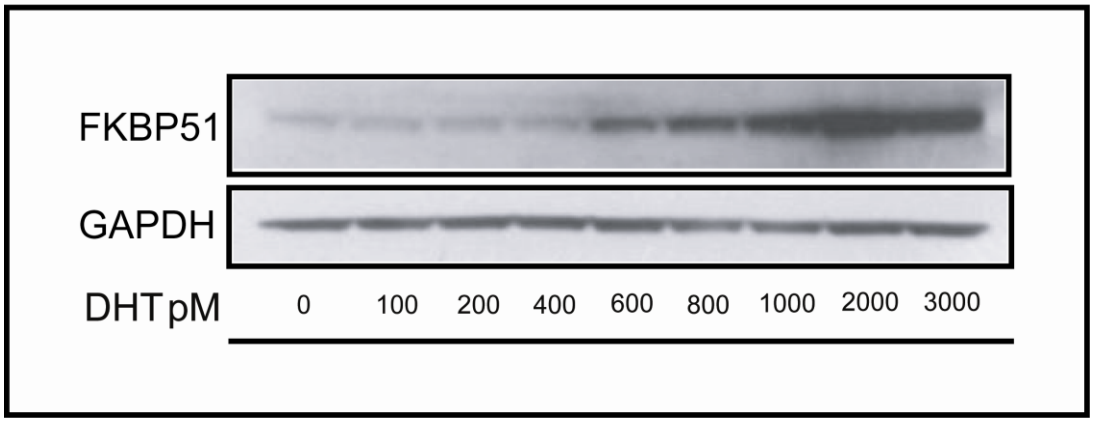

Figure 4.1. FKBP51 Expression is Androgen-Dependent.

Figure 4.1. FKBP51 Expression is Androgen-Dependent. Western blot in LNCAP cells treated with the indicated range of DHT concentrations demonstrates that FKBP51 expression is increase in a dose-dependent manner after 16 hours of treatment with DHT.

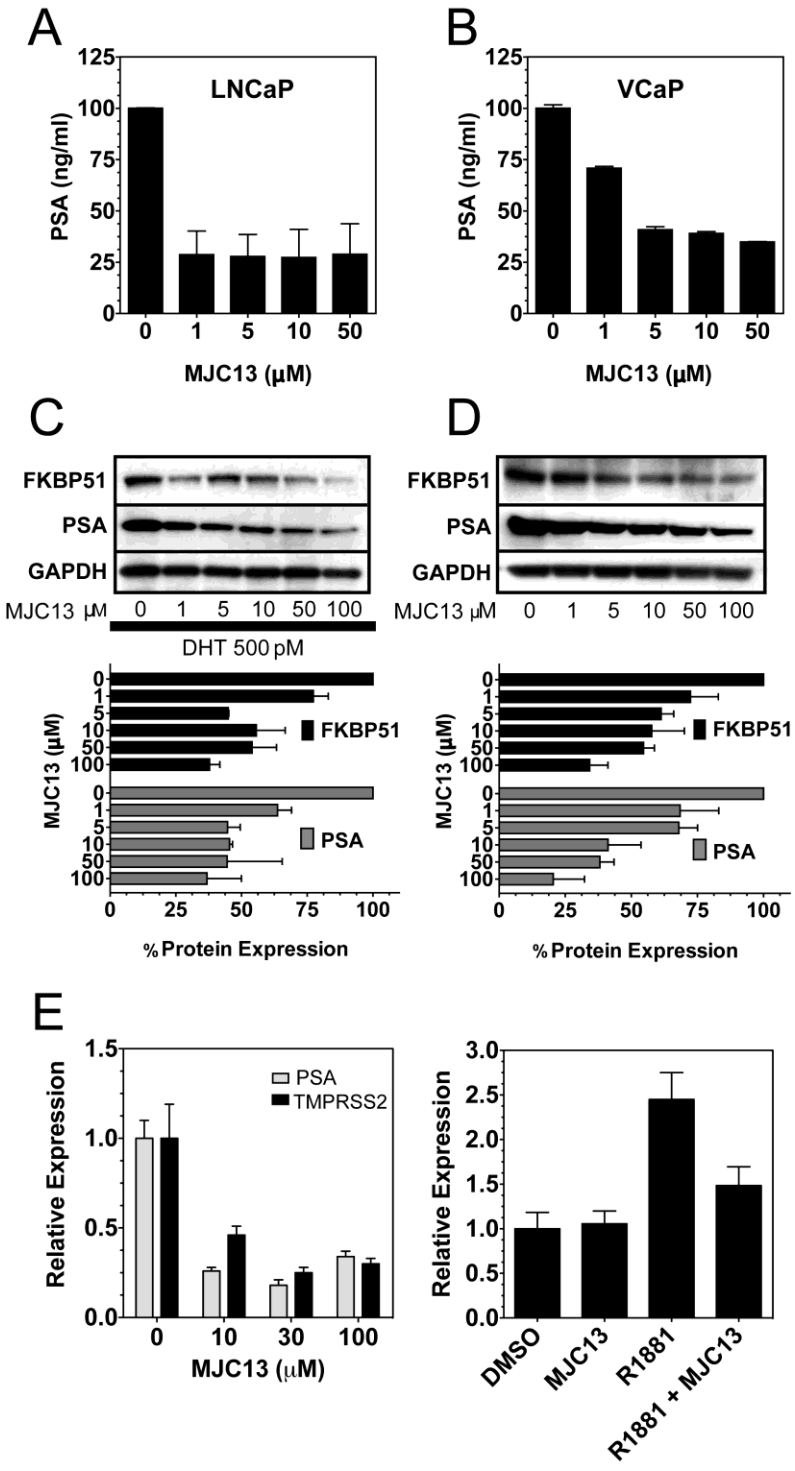

Figure 4.2. Effects of MJC13 on AR-Dependent Gene Expression in Early and Late Stage Prostate Cancer Cells.

Figure 4.2. Effects of MJC13 on AR-dependent Gene Expression in Early and Late Stage Prostate Cancer Cells. A-B. ELISA assays to measure PSA secretion were performed in LNCaP (*A*) and VCaP (*B*) cells. Cells were treated with the indicated MJC13 concentrations in the presence (*A*) or absence (*B*) of DHT for 24 hrs and PSA levels in the media were quantified. (*C-D*) Western blots to measure AR-dependent expression of PSA and FKBP51 were performed in LNCaP (*C*) and VCaP (*D*) cells. Cells were treated with the indicated concentrations of MJC13 in the presence (*C*) or absence (*D*) of DHT for 24 hrs, lysed, and lysates were electrophoresed and immunoblotted for FKBP51, PSA and GAPDH (loading control). The upper panels show representative Western blots. The lower panels represent averaged densitometry data from at least 3 independent experiments. (*E*) left panel: *PSA* and *TMPRSS2* gene expression in LNCaP cells was assessed by Q-PCR. Cells were treated for 24 hours with increasing concentrations of MJC13 in the presence of 10% fetal bovine serum. Data are displayed as expression relative to that of 18S rRNA; right panel: R1881-dependent and independent *PSA* gene expression in 22Rv1 cells was assessed by Q-PCR. Cells (in the presence of charcoal-stripped serum) were untreated, treated for 24 hours with MJC13 alone, or treated for 24 hours with 0.5 nM R1881 in the presence and absence of 30 μM MJC13. Data are displayed as *PSA* mRNA expression relative to that of 18S rRNA. Each experiment was performed, at least, three times.

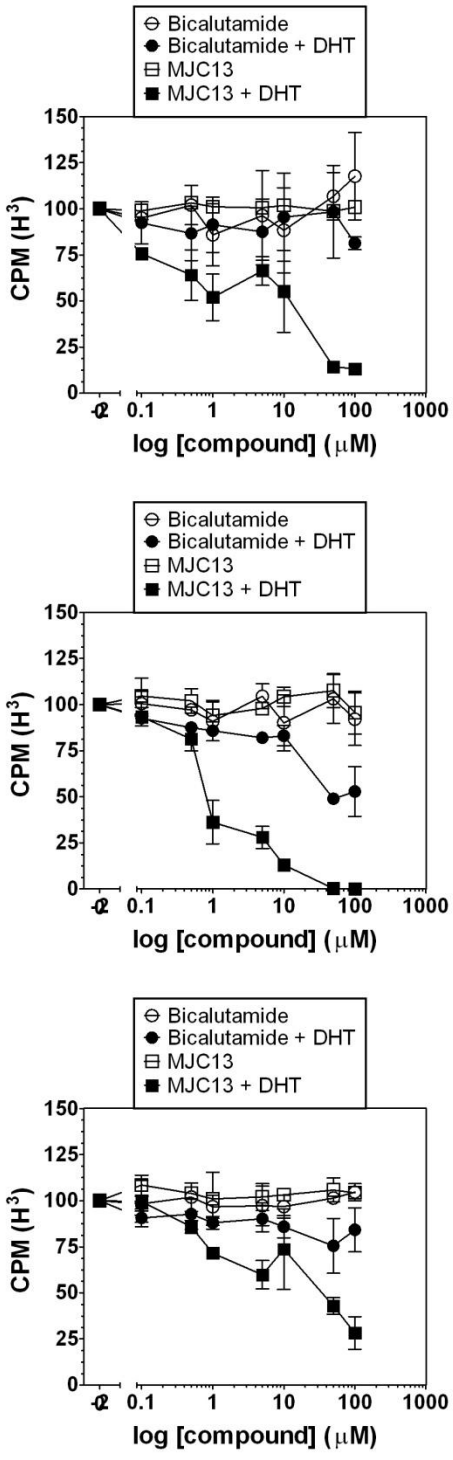

Figure 4.3. MJC compounds Effectively Inhibit Androgen-Dependent Prostate Cancer Cell Proliferation.

Figure 4.3. MJC Compounds Effectively Inhibit Androgen-Dependent Prostate Cancer Cell Proliferation. Tritium (tritiated thymidine) incorporation assays were performed on LNCaP (*A*), LAPC4 (*B*), and 22Rv1 (*C*) cells treated with a range of compound concentrations in the presence (closed symbols) or absence (open symbols) of 0.5 nM DHT. The known AR antagonist bicalutamide (circles) was included for comparison with MJC13 (squares). All data are expressed as a percentage with the level of tritiated thymidine incorporation in the absence of compound for each condition set to 100%.

CHAPTER 5

DISCUSSION

Previous studies have shown that Hsp90-receptor complexes assembled from purified components *in vitro* have abnormal compositions, lack dynamics, and fail to dissociate upon hormone binding due to the active nature by which the receptors achieve their hormone-bound conformations to which FKBP52 associates. Also because of this dynamic process, these receptors are unstable in the absence of Hsp90 and difficult to purify *in vitro*. Consequently, it is impractical to use a simple purified protein system to assess direct interactions between FKBP52 and its receptors. Any potential interactions between FKBP52 and the receptors would occur within the context of the Hsp90 complex given that FKBP52-mediated potentiation of receptor function requires FKBP52 binding to Hsp90 [25].

Functional domain mapping approaches in yeast suggested that the FKBP52 FK1 domain, the proline-rich loop overhanging the PPIase catalytic pocket, is critical for regulation of receptor function through interaction with the receptor hormone binding domain [101]. In support of this idea we have identified a surface region on the AR hormone binding domain that displays increased dependence on FKBP52 for normal function when mutated (also termed FKBP52 hypersensitivity) (Figure 2.6). This surface directly correlates with the recently identified BF3 surface. Although we do not provide direct evidence for FKBP52 interaction with BF3, the data presented here indicate, that FKBP52 can indirectly influence receptor function through this surface. In addition, we

demonstrate that FKBP52 regulation of receptor function can be blocked by small molecules that are predicted to bind the BF3 surface.

In this dissertation we studied the role of the FKBP52 immunophilin in AR action by measuring the effects FKBP52-specific inhibitors have in AR-mediated processes such as receptor nuclear translocation and prostate cancer cells proliferation. We demonstrated that the lead molecules, MJC13 and MJC01, specifically inhibited FKBP52-enhanced receptor activity in both yeast and mammalian cell lines (Figures 2.4 and 2.7). MJC13 prevented hormone-induced receptor-Hsp90 complex dissociation in the presence of FKBP52, which resulted in less receptor translocation to the nucleus (Figure 3.3). As a consequence AR-dependent gene expression and cell proliferation in cellular models of prostate cancer were inhibited (Figure 4.2). Taken together, these data suggest that FKBP52 regulates AR function through the BF3 surface and that FKBP52-mediated receptor potentiation can be inhibited by targeting the BF3 surface with small molecules.

Even though our data suggest that MJC13 binds the AR BF3 surface, efforts to provide direct evidence of this interaction through SPR analysis and co-crystalization have not been instructive. These difficulties are not unique among molecules known or thought to bind the BF3 region since providing direct evidence of BF3 binding for some of the fenamic acid-derived AR inhibitors have also proven difficult. In addition, some of these molecules bind weakly to BF3, therefore we postulate that these molecules might associate weakly with multiple sites on the receptor hormone binding domain at high concentrations. It is also possible that the BF3 surface on the purified AR hormone binding domain is not in an optimal conformation for MJC13 binding in the absence of

the chaperone proteins that actively assist with the receptor folding. Despite these difficulties, multiple lines of evidence suggest that MJC13 inhibits FKBP52-mediated AR function through binding BF3.

We also demonstrated that FKBP52 can influence at least a portion of the BF3 surface (Figure 2.6), and molecules that specifically inhibit FKBP52 regulation of receptor function are structurally similar to known BF3 binding molecules. In addition, many of the compounds tested in the SAR studies differentially affected AR-P723S as compared to wild type AR and some of the molecules inhibitory action was specific for AR-P723S (Figure 2.3). Thus, mutations within the putative BF3 binding surface accentuate inhibitor activity. Finally, MJC13 effectively blocked hormone-dependent PSA expression but failed to block hormone-insensitive PSA expression in 22Rv1 cells (Figure 4.2). Given that MJC13 is predicted to target the BF3 surface of the AR LBD, MJC13 would not be expected to affect the expression of PSA in these cells that results from a constitutively active AR protein lacking the LBD. As a final point, *in silico* docking simulations support the idea that MJC13 binds BF3 (Figure 3.2). Taken together these data suggest that MJC13 binds the BF3 surface. However, in the absence of direct evidence, we must consider the possibility that MJC13 and its derivatives do not bind AR. This possibility does not make the data and the compounds presented here any less significant since their inhibitory effect is specifically for FKBP52-enhanced receptor function.

Although elucidating the structural components that confer FKBP52-specificity is of importance for understanding FKBP52 function, compounds that generally inhibit receptor in the absence of FKBP52 are no less interesting from a therapeutic

perspective. However, the most potent compounds identified in these studies, MJC01 and MJC13, were more specific for FKBP52-regulated receptor activity, which may reflect the receptor's requirement for FKBP52 at low hormone concentrations. Based on available evidence it is logical to assume that at physiological concentrations *in vivo* AR dependence on the presence of FKBP52 is heightened, especially in the case of hormone refractory prostate cancer. Thus, compounds that target the regulation of AR by FKBP52 may display increased potency in these settings and represent attractive therapeutic candidates for the treatment of prostate cancer.

Conversely, FKBP52 is not a specific regulator of AR, but also regulates GR and PR [25, 26, 33]. Therefore, pharmacological inhibition of FKBP52 would lead to the inhibition of activity from all three receptors. However, MJC13 and its derivatives are proposed to associate with the FKBP52 regulatory site on the receptor hormone binding domain, which provides an opportunity for receptor-specific targeting of FKBP52 regulation. We showed that FKBP52 inhibitors have distinct effects depending upon which receptor was being analyzed. Although none of the compounds we tested displayed complete AR specificity, many of them, including MJC13 (Figures 2.2 and 2.3), were more potent inhibitors of AR as compared to GR. This data suggests that the design of receptor-specific FKBP52 inhibitors may be possible. Further studies to characterize and specifically target analogous surfaces on other steroid hormone receptors are necessary. Nevertheless, given the role of FKBP52 in other nuclear receptor signaling these compounds also show promise for the treatment of hormone related diseases, male contraception, and research reagents for the study of steroid hormone receptor interactions and function.

In summary, we have identified a surface region on the AR hormone binding domain that, when mutated, displays a greater dependence on FKBP52 for normal function. This surface directly correlates with the recently characterized BF3 surface. In addition, we have developed a series of small molecules that specifically inhibit FKBP52 regulation and enhancement of AR function. These small molecules are predicted to mediate their effects through binding BF3, a surface that we propose to be the FKBP52 interaction and/or regulatory surface on AR. The most promising compound, MJC13, inhibits hormone-dependent AR-Hsp90 complex dissociation and effectively blocks AR-dependent gene expression in cellular models of prostate cancer at low micromolar concentrations.

Further studies to characterize the MJC13 target sight, improve compound efficacy, and improve receptor specificity are needed. MJC13 is the first example of an inhibitor that specifically targets the regulation of receptor function by an Hsp90-associated cochaperone and serves as an excellent starting point for the development of FKBP52-specific compounds for the treatment of hormone-dependent diseases such as prostate cancer. Although we do not know the exact mechanism of action of the inhibitory molecules, we can conclude that AR signaling is targeted through FKBP52. Further understanding of the mechanism involve in FKBP52 inhibition and the role this immunophilin protein has in prostate cancer development, tumor formation and progression will require more advance approaches such as in mouse models of prostate cancer.

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APPENDIX

Yeast strains used in these studies.

Yeast Strain	Receptor	Immunophilin Protein	Reporter Plasmid	Growing Media
1553	WT AR	None	β-gal	Synthetic complete media minus leucine, uracil and tryptophan (SC-LUW)
1555	WT AR	FKBP52	β-gal	Synthetic complete media minus leucine, uracil and tryptophan (SC-LUW)
1483	WT AR	None	β-gal	Synthetic complete media minus leucine, uracil and histidine (SC-LUH)
1496	AR-P723S	FKBP52	β-gal	Synthetic complete media minus leucine, uracil and histidine (SC-LUH)
1347	GR	None	β-gal	Synthetic complete media minus uracil, histidine and tryptophan (SC-UHW)
1345	GR	FKBP52	β-gal	Synthetic complete media minus uracil, histidine and tryptophan (SC-UHW)

CURRICULUM VITA

Johanny Tonos De León was born in San Juan, Puerto Rico. She earned her Bachelor of Science degree with a double major in Biology and Industrial Microbiology from the University of Puerto Rico, Mayaguez campus in 2005. In 2006 she was accepted in the Biochemistry Masters of Science program at the Universidad Central del Caribe Medical School. In 2008 she joined the Pathobiology Doctoral program at the University of Texas at El Paso.

As a graduate student she received several scholarships, fellowships and awards such as: MARC, RISE, SNURP, NSF-ADVANCE, NSF-LSAMP, UTEP Graduate School Research Award and recognition from the Academy of Distinguished Scholars and the White House for Academic Excellence and Leadership. Her dissertation project resulted in the acquisition of national and international patents.

While in the Doctoral program, Johanny Tonos De León worked as a Research Assistant for Dr. Marc Cox and as a Lecturer at the International Business College.

Meeting Presentations & Abstracts

Tonos De Leon, J., Iwai, A., Trepel, J., Feau, C., Guy, K., Fletterick, R., Neckers, L., Cox, MB**.** Targeted Disruption of the Androgen Receptor Regulation by the Immunophilin FKBP52. Emerging Researchers National Conference in Science, Technology, Engineering and Mathematics, February 242-8, 2011. Washington, DC.

Meneses De Leon, J., Iwai, A., Trepel, J., Feau, C., Guy, K., Fletterick, R., Neckers, L., Cox, MB**.** Targeted Disruption of the Androgen Receptor Regulation by the Immunophilin FKBP52. Cold Spring Harbor Laboraroty Molecular Chaperones and Stress Responses Meeting, May4-8, 2010. Cold Spring Harbor, NY.

Meneses De Leon, J. Storer, C., Iwai, A., Trepel, J., Feau, C., Guy, K., Fletterick, R., Neckers, L., Cox, MB. A Distinct Class of Nuclear Receptor Alternate Site Modulators (NRAMs) that Target a Novel Androgen Receptor Regulatory Mechanism Involving FKBP52 and ß-Catenin. Cold Spring Harbor Laboraroty Molecular Chaperones and Stress Responses Meeting, May4-8, 2010. Cold Spring Harbor, NY.

Meneses De León, J., Aki, C., Neckers, L. and Cox, M. 2009. Identification and Characterization of FKBP52-Specific Inhibitors for the Treatment of Prostate Cancer. Midwestern Stress Response and Molecular Chaperones Meeting. January 14-16, 2010. Evanston, IL

García, Y., **Meneses De León, J.** and Cox, M. Development of a Cellular Assay for Testing FKBP52 Inhibitors. Midwestern Stress Response and Molecular Chaperones Meeting. January 14-16, 2010. Evanston, IL

Meneses De León, J., Aki, C., Neckers, L. and Cox, M. 2009. Identification and Characterization of FKBP52-Specific Inhibitors for the Treatment of Prostate Cancer. American Society of Cell Biology National Meeting. December 1-5, 2009. San Diego, CA.

García, Y., **Meneses De León, J.** and Cox, M. Development of a Cellular Assay for Testing FKBP52 Inhibitors. American Chemical Society Southwest Regional Meeting. November 4-7, 2009. El Paso, TX.

Meneses De León, J., Aki, C., Neckers, L. and Cox, M. 2009. Identification and Characterization of FKBP52-Specific Inhibitors for the Treatment of Prostate Cancer. SACNAS National Annual Meeting. October 14-18, 2009. Dallas, TX.

Lopez, J., **Meneses De León, J.,** Cox, M. Functional Comparison of a Novel FKBP52-Specific Inhibitor to the Classic anti-androgen Inhibitor Bicalutamide. SACNAS National Annual Meeting. October 14-18, 2009. Dallas, TX.

García, Y., **Meneses De León, J.,** and Cox, M. Development of a Cellular Assay for Testing FKBP52 Inhibitors. SACNAS National Annual Meeting. October 14-18, 2009. Dallas, TX.

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Permanent Address: 1757B Sheets Place

Frederick, MD 21702

This dissertation was typed by Johanny Tonos De León.