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Androgen Receptor Splice Variants are Resistant to Inhibitors of Hsp90 and FKBP52, which alter Androgen Receptor Activity and Expression

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Abstract

Androgen ablation therapy is the most common treatment for advanced prostate cancer (PCa), but most patients will develop castration-resistant prostate cancer (CRPC), which has no cure. CRPC is androgen-depletion resistant but androgen receptor (AR) dependent. AR is a nuclear receptor whose transcriptional activity is regulated by hormone binding to the ligand-binding domain (LBD). Constitutively active AR splice variants that lack LBDs often are expressed in CRPC. The expression of these variants indicates that methods to inhibit AR activity that do not rely on inactivating the LBD are needed. Heat shock protein 90 (Hsp90), a potential therapeutic target in PCa, is an AR chaperone crucial for proper folding, hormone binding and transcriptional activity of AR. We generated LNCaP cell lines with regulated expression of the AR-V7 variant as well as a cell line expressing artificially truncated AR (termed AR-NTD) to characterize splice variant function. Using an Hsp90 inhibitor, Geldanamycin (GA), and an AR-Hsp90-FKBP52 specific inhibitor, MJC13, we sought to determine if the AR variants also require Hsp90 and associated co-chaperone, FKBP52, for their activity. GA inhibits AR transcriptional activity but has little effect on AR-V7 activity. Moreover, GA decreases the stability of AR protein, with no effect on AR-V7 levels. Full-length AR activity is strongly inhibited by MJC13 while AR-V7 is unaffected. Thus, the variants are resistant to inhibitors of the Hsp90-AR heterocomplex. Although Hsp90 inhibitors will continue to inhibit growth promoting kinases and signaling through activated full-length AR in CRPC, AR signaling through variants will be retained.

Keywords

Androgen Receptor (AR); Prostate Cancer; Splice Variants; Hsp90; FKBP52

1. Introduction

Prostate cancer (PCa), an androgen-dependent disease, is the second leading cause of cancer-related death in American men making it a significant health problem [1]. The

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androgen receptor (AR) plays a critical role in normal prostate development and in PCa initiation and progression [2]. Consequently, androgen ablation therapy is the most common treatment for advanced PCa. Most patients initially respond favorably to this treatment [1]. However, a majority of patients will eventually develop castration-resistant prostate cancer (CRPC) within 18–24 months of initial treatment [3]. There is no cure for CRPC, which is androgen depletion resistant but typically AR-dependent. However, a CYP17 inhibitor, abiraterone acetate [4], and a new anti-androgen, MDV3100 [5], prolong life an average of a few months. Thus, AR is a viable target for treatment of CRPC.

AR is a member of the nuclear receptor superfamily of transcription factors. The AR gene is located on Xq11-12; it contains eight exons that encode a protein of approximately 919 amino acids with variability due primarily to variation in the poly glutamine tract in the amino terminus [2, 6]. Structurally AR is composed of distinct functional motifs: amino-terminal domain (encoded by exon 1), DNA-binding domain (DBD encoded by exons 2 and 3), a hinge region (H encoded by the 5' portion of exon 4), and a ligand-binding domain (LBD encoded by the remainder of exon 4 through exon 8) [6, 7]. The transactivation domain and the LBD also contain the AF-1 and AF-2 transactivation domains respectively [6, 7]. Without ligand, AR is transcriptionally inactive and is bound to chaperone complexes that include heat shock protein 90 (Hsp90) and the Hsp90-associated co-chaperones FKBP52 and p23. Hormone binding induces a conformational change in the receptor allowing for dissociation of chaperone complex, nuclear translocation, dimerization of receptors, DNA binding and interaction with transcriptional coregulators to alter gene expression [7].

Numerous mechanisms have been implicated in aberrant AR reactivation in the castration resistant environment. These include AR mutations, AR amplification, and local androgen synthesis [8]. AR hypersensitivity, activation of multiple kinase pathways, and altered coregulator expression have also all been implicated in reactivation of AR [8, 9]. Recently, multiple alternatively spliced AR isoforms have been identified in CRPC [10–13]. These isoforms typically contain exons 1–3, lack the hormone-binding domain, and contain a small amount of unique sequence derived from cryptic exons. Thus, their activities are independent of hormone. The best characterized of these is AR-V7 (also termed AR3), which contains exons 1–3 and 16 unique amino acids [11, 13]. Antibodies to this sequence have been developed and AR-V7 is detected in the CRPC cell line, 22RV1, and in many CRPC cancers. Cells expressing the AR variants have CRPC characteristics including growth in androgen-depleted medium or as xenografts in castrated mice and androgen-independent activation of AR target genes [8, 10–13]. Ratios of expression of the variants relative to AR range from much less variant than full-length [11] to equivalent or slightly higher levels of variants as seen in 22RV1 cells [11] or in bone metastases in CRPC patients [14]. Attaining a better understanding of the role and function of these constitutively active splice variants in CRPC will help identify potential therapeutic targets.

Most efforts to develop AR targeted treatments have aimed to further reduce residual androgen levels or to develop more potent AR antagonists [8, 9]. Neither of these approaches will affect splice variant activity. Hsp90 inhibitors are being tested in clinical trials for treatment of cancer [15]. Hsp90 is a molecular chaperone critical for maintaining proper folding of numerous nuclear receptors including AR as well as a variety of other proteins including some kinases. Geldanamycin (GA), an Hsp90 inhibitor, binds to the ATP-binding pocket of Hsp90 and inhibits Hsp90 activity resulting in improper folding and degradation of client proteins [7, 9, 10, 15, 16]. In addition to its well-characterized role for maintaining steroid receptors in a conformation capable of binding hormone, there is some evidence that Hsp90 plays a role in receptor action in the nucleus. GA inhibits glucocorticoid receptor (GR) release from chromatin during hormone withdrawal,

suggesting that the Hsp90 chaperone has additional functions in steroid receptor action [17]. The Hsp90 co-chaperone, FKBP52, plays a role in controlling transactivation functions of AR but does not alter androgen binding [18–20]. FKBP52^{-/-}MEF cells have compromised AR transcriptional activity and there is tissue selective loss of AR activity in FKBP52-deficient mice resulting in phenotypes consistent with partial androgen insensitivity including dysgenic prostate. Hsp90 inhibitors target AR activity decreasing AR regulated gene expression [9, 17]. Effects limited to regulating hormone binding should not alter the activity of AR variants. However, other functions of Hsp90 and the activities of FKBP52 have the potential to alter AR variant activity. Thus, in this study, we have compared effects of two inhibitors, an Hsp90 inhibitor (GA) and an inhibitor of FKBP52-regulated AR (MJC13) on the activities of full-length AR and the AR-V7 splice variant.

2. Experimental

2.1 Cell Culture and Supplies

HeLa, LNCaP, and 22Rv1 cells were purchased from the American Type Culture Collection (Manassas, VA). LNCaP-V7 and LNCaP-NTD cell lines, prepared using the ViraPower™ T-REx™ Lentiviral Expression System (Gateway), are LNCaP cell lines that inducibly express either the AR-V7 or AR-NTD variant in response to doxycycline. The cell lines were maintained in a humidified atmosphere with 5% CO₂ at 37°C. HeLa cells were maintained in DMEM (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS) (Intergen Co., Purchase, NY), and penicillin/streptomycin (Invitrogen). LNCaP cells were grown in RPMI 1640 (Invitrogen), 10% FBS, and penicillin/streptomycin. LNCaP-V7 were maintained in similar conditions but were kept under blasticidin and geneticin (G418) selection. 22Rv1 cells were maintained in the supplier's prescribed RPMI media supplemented with 10% FBS. For assays of transcriptional activity, cells were transferred to medium containing charcoal stripped serum. Methyltrienolone (R1881) was purchased from Perkin-Elmer (Boston, MA) and Geldanamycin (GA) from Calbiochem (Darmstadt, Germany). MJC13 was obtained from Marc Cox (UT-El Paso, TX) [21]. In this paper, the concentrations of 1 μM GA and 30 μM MJC13 were used since they have already been previously reported to optimally affect AR [16, 21].

2.2 Transient Transfections

HeLa cells were transiently transfected with the previously described plasmids: GRE-luciferase reporter, pCR3.1-β-galactosidase, and pCR3.1-AR plasmids [22] and pCR3.1-ARV7 plasmid, which was a gift from Manjula Nakka and William Krause (BCM). Poly-L-lysine coupled adenovirus was used as a carrier for the plasmids to transiently transfect HeLa cells as described previously [22]. Luciferase and β-galactosidase activity assays were performed as previously described [23].

2.3 Western Blotting

HeLa, LNCaP, LNCaP-V7, LNCaP-NTD, and 22RV1 cells were lysed by four rounds of freeze/thaw using 1XReporter Lysis Buffer (Promega) containing 0.4 M NaCl. A 7.5% SDS gel was used to resolve proteins (20 μg), which were then transferred to nitrocellulose. Proteins were detected using the previously described AR441 antibody [22] at a 1:1000 dilution, actin antibody (GE Healthcare, United Kingdom) at a 1:10,000 dilution, and β-tubulin antibody (Upstate, Lake Placid, NY) at a 1:10,000 dilution and ECL reagents (GE Healthcare). ImageJ software was used to quantify protein expression.

2.4 Quantitative RT-PCR (qPCR)

TRIzol reagent (Invitrogen) was used to prepare RNA from the treated LNCaP and LNCaP-V7 cells. cDNA Synthesis Master Mix (GenDEPOT) was used to reverse transcribe the total

RNA (1µg) to make cDNA to measure target genes. Target gene expression was analyzed using SYBR green PCR Master mix and an ABI 7500 Fast sequence detection system. The primer sets for *PSA*, *TMPRSS2*, *AR*, and *18S* have been described previously [24]. The primer set for FK506 binding protein 5 (*FKBP5*) is sense 5'-GGATATACGCCAACATGTTCAA-3' and antisense 5'-CCATTGCTTTATTGGCCTCT-3'.

2.5 Statistical Analysis

All data for the experiments are presented as mean $\pm$ SEM. The mean displayed is the average of triplicate biological samples in a single experiment. Each experiment was repeated at least three independent times and a representative example is shown. One-way ANOVA followed by a Tukey post-hoc statistical analysis was performed to analyze the results using GraphPad PRISM. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1 Characterization of Full-Length AR and Variants (V7 and NTD)

Full-length AR is composed of four functional domains: amino-terminal transactivation domain, DNA-binding domain (DBD), hinge region (H), and the ligand-binding domain (LBD) (Fig. 1A). Endogenous V7 is spliced at exon 3 followed by 16 unique amino acids from a cryptic exon 3b and composed of the transactivation domain and the DBD. Our lab has also generated an artificial variant termed NTD composed of the transactivation domain, DBD, and a portion of the hinge region (amino acids 1–660). A comparison between these two forms and full-length AR allows us to distinguish between changes due to the elimination of the LBD and those that might be due to the unique amino acids in V7. Using LNCaP cell lines, which express AR-V7 or AR-NTD in response to doxycycline (Dox), we chose a dose of Dox that induced V7 or NTD expression to levels similar to full-length AR (Fig. 1B) and compared the induction of AR target genes (Fig. 1C). *PSA* was chosen because it is the best characterized AR-dependent gene and serum PSA is used to monitor prostate cancer. *TMPRSS2* was chosen because its promoter regulates the expression of *TMPRSS2*:ETS factor fusions found in the majority of prostate cancers [25]. As expected, we observed hormone-dependent induction of *TMPRSS2* and *PSA*. V7 and NTD also induced expression of *TMPRSS2* and *PSA* although the fold induction was diminished relative to full-length AR. Thus, these cells are suitable models to examine the effects of inhibitors on protein stability and activity of the various AR forms.

3.2 Differential Responses of AR and Variants to Hsp90 Inhibitor Treatment

Treatment of LNCaP-V7 cells with GA for 24 hours robustly inhibited R1881 induced expression of *TMPRSS2*, *PSA*, and *FKBP5* mRNA as expected (Fig. 2A). However, V7 dependent induction of these genes was relatively resistant to GA treatment. The basal expression of *PSA* was reduced whereas that of *TMPRSS2* was increased. Basal level of *FKBP5* was unaffected and the apparent minor reduction in activity in response to V7 induction was not statistically significant. Hsp90 serves as a chaperone for many proteins including kinases and the changes in basal activity likely are a result of effects on other proteins. The slight reduction in *PSA* expression in Dox treated cells may be a modest effect on V7, but is equally likely to be an effect on other proteins required for transcription. In earlier studies, we found that GA treatment reduces activity of a transiently transfected beta galactosidase reporter used for normalization in HeLa cells (data not shown). The decrease in full-length AR activity was due at least in part to decreased protein expression. GA strongly decreased full-length AR protein expression while AR variants' (V7 and NTD) protein expression was unaffected (Fig. 2B). The inhibition was post-transcriptional and

presumably an effect on protein stability because GA did not reduce AR mRNA levels in the LNCaP cells (Fig. 2C). Note that we saw a modest increase in AR mRNA in response to R1881 at the 16-hour time point, which was chosen to match the activity and protein treatment times. However, longer treatment with R1881 results in a reduction in AR mRNA as reported by others [26].

3.3 Effect of GA on AR and V7 Protein Stability

To test whether reduced AR expression was a result of reduced protein stability as we expect, we treated cells with cycloheximide to block translation and compared AR protein levels as a function of time in the presence of GA (Fig. 3A–B) and in the absence of GA (Fig. 3C–D). As previously reported, AR is relatively stable and 16 hours of cycloheximide reduced AR expression by less than 50%. However, in GA treated cells AR expression is reduced by 50% in a little more than 2 hours. In contrast, V7 protein expression was quite stable in the presence of GA and even appeared to increase very slightly suggesting it may be more stable than the tubulin control (Fig. 3A–B). To be certain that GA did not alter V7 stability, we also measured V7 and AR stability in the absence of GA (Fig. 3C–D) and found that V7 stability was similar to or greater than full-length AR. We also examined effects of GA on overall AR and variant expression in 22Rv1 cells, which endogenously express variants. GA treatment decreased full-length AR protein expression by more than 50% while V7 protein was unaffected (Fig. 3E).

3.4 MJC13 Decreases AR Activity but does not affect V7 Activity

We next investigated the effect of a specific FKBP52-Hsp90-AR inhibitor, MJC13 [21], on AR activity. In HeLa cells, 24 hours of MJC13 treatment decreased hormone-induced androgen-dependent luciferase reporter activity while V7 activity was unaffected (Fig. 4A). In LNCaP-V7 cells, MJC13 also greatly reduced androgen-dependent induction of TMPRSS2 and PSA mRNA, while it had no effect on V7 activity (Fig. 4B). Treatment of LNCaP-V7 cells with MJC13 for 24 hours increased expression of endogenous full-length AR presumably due to protein stabilization as previously reported [21] while the V7 protein level was unaffected (Fig. 4C). In the 22RV1 cells, MJC13 treatment for 24 hours also increased the level of full-length AR protein while endogenous variant expression was similar to untreated cells (Fig. 4D).

4. Discussion

AR splice variants lacking the AR ligand-binding domain have been identified in many CRPC tumors. Although expression of many of the variants has been limited to the detection of variant mRNA, antibodies that recognize the unique 16 amino acid sequence in the V7 variant have been developed. Using these, investigators have identified V7 protein in CRPC [11, 13]. Limited western blotting studies show that V7 typically is expressed at lower levels than full-length receptor in the same tumor [11]. However, levels of variants and full-length AR can be comparable in bone metastases [14]. Assuming the mRNA level is a reflection of the protein level, some investigators have concluded that the levels of variants must be very low. However, Plymate's group, using amino and carboxyl terminal antibodies, showed that while relative staining by the two antibodies was consistent with expression of full-length AR in primary tumors [27], about a quarter of CRPC tumors showed at least a 50% reduction in C-terminal signal relative to N-terminal suggesting high levels of variant expression. Thus, understanding the actions of these variants and developing means to inhibit their actions is important in developing treatments for CRPC.

Conclusions regarding the activities of these variants have been inconsistent, but various investigators have used models with widely differing expression levels. Our inducible

LNCaP-V7 and LNCaP-NTD cell models provide a unique system in which we are able to tightly control expression level of the variants to better mimic CRPC conditions. To detect inhibition of variant specific activity, we expressed the variants at a level similar to or somewhat less than the full-length AR protein in the hormone-dependent LNCaP cells (Fig. 1B). This provides a good V7 dependent level of target gene expression. The absolute level of V7 may be similar to that in many CRPC tissue samples because full-length AR often is overexpressed and V7 can reach the level of AR in the hormone dependent tumors [11]. The ratio of full-length to variant in our studies reflects that seen in CRPC bone metastases [14]. V7 and NTD both induced the classical AR target genes, *TMPRSS2* and *PSA* (Fig. 1C). Induction was not as robust as that induced by R1881. This was due, in part, to treatment conditions. Dox and R1881 were given at the same time. Whereas R1881 immediately activates full-length AR, Dox takes up to 12 hours to reach final expression levels of V7. In a more direct comparison (24 hours Dox versus 16 hours R1881), these genes still are not as robustly induced by V7 although there are many other genes that are induced by V7, but weakly induced (if at all) by R1881 treatment suggesting gene specific actions (Krause, Weigel, et al. in preparation).

Because they lack hormone-binding domains, variants do not require Hsp90 complexes to maintain the protein in a conformation capable of binding hormone. However, whether they require Hsp90 during the initial folding process or for subsequent function as has been shown for GR [17] was unknown. Similarly, the role, if any, of FKBP52 in variant activity had not been investigated. Previous studies had shown that this co-chaperone was required for nuclear localization and optimal transcriptional activity of AR, but not for all steroid receptors [21]. We found, as expected, that GA reduced AR expression and activity (Fig. 2). However, GA had no effect on variant expression or induction of *TMPRSS2* or *FKBP5* mRNA. There was a slight decrease in *PSA* mRNA in cells treated with GA, but GA also decreased *PSA* mRNA levels of vehicle treated samples suggesting that one or more proteins required for basal expression of *PSA* requires Hsp90 for optimal activity. The overall fold difference in V7 induction was not changed with GA treatment. The dramatic inhibition of AR activity was due in large part to decreased AR protein expression presumably due to the expected decrease in protein stability [28] since AR mRNA levels were unaltered by GA (Fig. 2C). V7 and NTD protein levels were unaltered (Fig. 2B). It was formally possible that the apparent resistance of V7 to destabilization by GA was due to V7 being an intrinsically less stable protein whose turnover rate was not altered by treatment as we observed for an AR A748T mutation originally detected in a tumor from a prostate cancer patient [28]. Interactions with heat shock proteins and agonist-dependent N/C terminal interactions both stabilize AR. Variants lack both of these interactions. However, the cycloheximide treatment studies in Fig. 3C–D show that V7 is at least as stable as unliganded AR and that GA does not reduce V7 stability (Fig. 3A–B).

Hsp90 interacts with a broad range of proteins, including some kinases (RB, EF2, and HER2) and steroid receptors (GR and AR) [29]. Hsp90 inhibitors can have broad transcriptional effects since Hsp90 complexes have numerous client proteins. Because FKBP52 is also required for AR function, but only for a subset of Hsp90 actions, we tested a previously identified FKBP52-Hsp90-AR specific inhibitor: MJC13 [21]. MJC13 strongly blocked full-length AR transcriptional activity at low levels of hormone as previously reported [21] but we found that it had no effect on AR variant activity measured using an AR responsive reporter or on variant dependent expression of endogenous target genes (Fig. 4A–B). As reported previously, MJC13 increased AR protein expression [21] (Fig. 4C–D); however, it did not have the same effect on V7 protein expression. Thus, although these chaperone inhibitors will be useful in blocking activity of full-length AR and other client proteins, V7 and presumably other variants will remain active in CRPC. Although we show here that inhibitors of the Hsp90-AR heterocomplex are ineffective against AR splice

variants that bypass the final step in the receptor folding and activation process, it remains possible that the variants still require early chaperone-assisted folding mediated by heat shock protein 70 (Hsp70) and the Hsp70-associated co-chaperones Hsp40, Hip and Hop. Thus, the inhibition of 'early' Hsp70 complexes remains a potential therapeutic strategy for the inhibition of AR splice variant activity and CRPC treatment.

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Abbreviations

PCa	prostate cancer
AR	androgen receptor
CRPC	castration-resistant prostate cancer
Hsp	heat shock proteins
DMSO	dimethyl sulfoxide
EtOH	ethanol
GA	geldanamycin

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Highlights

- LNCaP cells with regulated expression of AR splice variants were generated.
- The AR-V7 variant is resistant to treatment with the Hsp90 inhibitor, Geldanamycin.
- AR-V7 is resistant to MJC13, a FKBP52-Hsp90-AR specific inhibitor.

Figure 1. Characterization of AR and Variants (V7 and NTD)

(A) Schematic of full-length androgen receptor (AR) composed of distinct functional domains: amino-terminal transactivation domain (encoded by exon 1), DNA-binding domain (DBD encoded by exon 2 and 3), a hinge region (H encoded by the 5' portion of exon 4), and a ligand-binding domain (LBD encoded by the remainder of exon 4 through exon 8) [6, 7]. The naturally-occurring V7 splice variant is truncated at the exon 3-exon 4 boundary (amino acids 1–627) followed by 16 unique amino acids and the artificial NTD is composed of amino acids 1–660 containing a portion of the hinge. (B) Inducible LNCaP-V7 and LNCaP-NTD cells were treated with ethanol (Veh), 10 nM R1881, or Doxycycline (Dox) for 24 hrs and protein detected by western blot. Dox treatments were chosen to induce protein expression of AR variants (V7 and NTD) to a level similar to full-length AR. (C) LNCaP, LNCaP-V7, and LNCaP-NTD cells were treated with ethanol (Veh), 10 nM R1881, or Dox for 24 hrs and harvested for RNA. AR target gene (*TMPPRSS2* and *PSA*) mRNAs were measured by q-PCR and normalized to 18S mRNA. V7 and NTD both significantly increased AR target gene expression indicating that the AR variants can induce these genes. * $p < 0.05$ compared to respective vehicle.

Figure 2. Geldanamycin Inhibits Full-Length AR Activity and Expression, while AR Variants are Resistant

(A) LNCaP-V7 cells were changed to charcoal stripped serum and then treated with vehicle (DMSO), 0.25 ng/mL Dox, and/or 1 μM GA. R1881 (10 nM) or corresponding vehicle (EtOH) were added 8 hours after addition of the first series of treatments and RNA was isolated 24 hours after the initial treatments. TMPRSS2, PSA, and FKBP5 mRNA levels were measured using q-PCR and normalized with 18S mRNA. GA greatly reduced full-length AR activity while having little effect on V7 activity. (B) Cells were treated with vehicle (DMSO), 1 μM GA, and/or Dox in stripped serum, 1 nM R1881 or vehicle (EtOH) was added after 8 hours, and all cells harvested after another 16 hours. LNCaP-V7 and LNCaP-NTD cells were treated with 0.25 ng/mL and 0.5 ng/mL Dox respectively. Cells were harvested for protein analysis and proteins analyzed using western blot. GA diminished full-length AR protein expression while V7 and NTD protein levels were unaffected by GA treatment. (C) LNCaP cells were treated as described in panel A and RNA was isolated after a total of 24 hours. AR mRNA levels were measured using q-PCR and normalized to 18S mRNA. GA does not affect AR mRNA levels. One-way ANOVA followed by a Tukey post-hoc statistical analysis was performed. * $p < 0.05$ and *** $p < 0.001$ relative to their corresponding Dox or hormone-treated samples.

Figure 3. Stability of AR and V7 Protein

(A–D) LNCaP-V7 cells were transferred to stripped serum and treated with vehicle (EtOH), 10 nM R1881, or 0.25 ng/mL Dox overnight followed by treatment of 10 μ g/mL Cycloheximide and 1 μ M GA for the indicated time points. Cells were harvested and AR, V7, and Tubulin protein expression was detected by western blot. In panels A and C, AR and V7 were analyzed from the same blot in each case, but slightly different exposures were used to optimize detection of changes in protein expression. Protein expression was quantified relative to each respective Tubulin. To calculate the ratios below each AR and V7 band, those values were normalized to the “0 hr” lane for each blot. GA accelerated AR protein degradation but not that of V7 (compare A versus C). Densitometry analysis using Image J software was performed to show average fold change across three independent experiments (B and D). (E) To examine the effect of Hsp90 inhibition on endogenous V7 protein, 22Rv1 cells were transferred to charcoal stripped serum and treated with vehicle (DMSO) or 1 μ M GA for 24 hours. Cells were harvested, protein was isolated, and AR, V7, and Tubulin expression were analyzed by western blotting. GA decreased AR protein while endogenous V7 protein was unaffected.

Figure 4. FKB52 Co-Chaperone Inhibitor, MJC13, Stabilizes AR Protein and Decreases AR Activity but does not alter V7 Expression or Activity

(A) To determine the effect of FKB52 inhibition on AR and variant activity, HeLa cells were transiently transfected with 250 ng of ARE-luciferase reporter, 30 ng of pCR3.1-AR, or 0.03 ng of pCR3.1-V7 (chosen to provide a level of activity similar to full-length AR) and treated immediately with vehicle (EtOH and DMSO), 100 pM R1881, and/or 30 μM MJC13 for 24 hours. Luciferase and β-galactosidase activities were measured and luciferase levels were normalized to β-galactosidase levels. MJC13 decreased hormone dependent activity but did not affect V7 activity. (B–C) To determine the effect of FKB52 inhibitor on AR dependent gene regulation and receptor protein expression, LNCaP-V7 cells were transferred to charcoal stripped serum and treated with vehicle (EtOH and DMSO), 10 or 100 pM R1881, 30 μM MJC13, and/or 0.25 ng/mL Dox for 24 hours. Cells were harvested, RNA isolated, and gene expression was measured. TMPRSS2 and PSA mRNA values were normalized to 18S mRNA values (B). Protein was isolated and AR, V7, and Actin expression were analyzed by western blotting. Both a lighter and darker exposures are shown to better see both receptors (C). MJC13 increased AR protein, but significantly decreased endogenous full-length AR activity while V7 activity and protein were unaffected. (D) To examine the effect of FKB52 inhibition on endogenous V7 protein, 22Rv1 cells were transferred to charcoal stripped serum and treated with vehicle (DMSO) or 30 μM MJC13 for 24 hours. Cells were harvested, protein was isolated, and AR, V7, and Tubulin expression were analyzed by western blotting. MJC13 increased AR protein while endogenous V7 protein was unaffected. One-way ANOVA followed by a Tukey post-hoc statistical analysis was performed. **p<0.01 and ***p<0.001 relative to their corresponding hormone-treated samples.