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## Galeterone Prevents Androgen Receptor Binding to Chromatin and Enhances Degradation of Mutant Androgen Receptor

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### Abstract

**Purpose**—Galeterone inhibits the enzyme CYP17A1 and is currently in phase 2 clinical trials for castration-resistant prostate cancer (CRPC). Galeterone is also a direct androgen receptor (AR) antagonist and may enhance AR degradation. This study was undertaken to determine the molecular basis for AR effects and their therapeutic potential.

**Experimental Design**—Effects of galeterone on AR expression and activities were examined in prostate cancer (PCa) cell lines.

**Results**—Similar to the AR antagonist enzalutamide, but in contrast to bicalutamide, galeterone did not induce binding of a constitutively active VP16-AR fusion protein to reporter genes and did not induce AR recruitment to endogenous androgen regulated genes based on chromatin immunoprecipitation. Galeterone at low micromolar concentrations that did not induce cellular stress responses enhanced AR protein degradation in LNCaP and C4-2 cells, which express a T878A mutant AR, but not in PCa cells expressing wildtype AR. Further transfection studies using stable LNCaP and PC3 cell lines ectopically expressing wildtype or T878A mutant ARs confirmed that galeterone selectively enhances degradation of the T878A mutant AR.

**Conclusions**—Similar to enzalutamide, galeterone may be effective as a direct AR antagonist in CRPC. It may be particularly effective against PCa cells with the T878A AR mutation, but may also enhance degradation of wildtype AR in vivo through a combination of direct and indirect mechanisms. Finally, these findings show that conformational changes in AR can markedly enhance its degradation, and thereby support efforts to develop further antagonists that enhance AR degradation.

### Introduction

Prostate cancer (PCa) is the second-leading cause of cancer death in men in the United States. The androgen receptor (AR) plays a central role in PCa, and the standard treatment for metastatic PCa is androgen deprivation therapy by medical or surgical castration. Although most patients initially respond, they invariably relapse despite castrate androgen

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levels (castration-resistant prostate cancer, CRPC). Previous studies have identified increased intratumoral androgen synthesis from precursor steroids generated by the adrenal glands, or possibly *de novo* androgen synthesis from cholesterol, as a mechanism of castration resistance (1-6). CYP17A1 is the critical enzyme required for the conversion of C21 steroids to C19 steroids such as DHEA that can be further reduced to the potent androgens testosterone and dihydrotestosterone (DHT). CYP17A1 inhibitors can thereby further markedly decrease the levels of residual androgens and precursor steroids that remain after castration, and the CYP17A1 inhibitor abiraterone is now approved by the FDA for treatment of CRPC (7, 8).

The direct AR antagonist enzalutamide has also recently been approved for treatment of CRPC (9, 10). Previous AR antagonists used for PCa (flutamide, nilutamide, and bicalutamide) do not effectively prevent AR binding to chromatin and may thereby have weak agonist properties that limit their efficacy in CRPC (11-13). In contrast, the enzalutamide liganded AR does not bind to chromatin, making this drug a purer AR antagonist with improved efficacy in CRPC (10). However, the survival advantages for abiraterone and enzalutamide therapy in CRPC postchemotherapy are only about 4 months (7, 9), and mechanisms of intrinsic or acquired resistance to these agents remain to be established (14).

Galeterone (previously known as VN/124-1 or TOK-001) was developed as a CYP17A1 inhibitor, but similar to related compounds it has AR antagonist activity and was also found to promote AR degradation (15-17). However, its effects on AR binding to chromatin have not been examined. Moreover, further studies indicated that galeterone at high concentrations could induce an endoplasmic reticulum (ER) stress response (18) and may decrease AR translation through direct or indirect effects on mTOR (19), suggesting that some of its effects on AR expression may be indirect. Galeterone is currently in phase II clinical trials for CRPC, and responses in these trials may be related to both its activities towards CYP17A1 and its direct effects on AR. Therefore, this study was undertaken to determine the molecular basis for galeterone actions as a direct AR antagonist and for its effects on AR protein expression.

## Materials and Methods

### Cell culture and immunoblot analyses

LNCaP, VCaP, LAPC4, CWR22RV1, PC3 and HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). LAPC4-CR and C4-2 cells were derived from castration resistant xenografts of LAPC4 and LNCaP, respectively. Cells were cultured in RPMI1640 or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Androgen-deprivation was conducted by culturing cells in RPMI1640 or DMEM supplemented with 5% charcoal-dextran stripped serum (CSS) for at least 48 hours. Whole cell lysates (WCL) were prepared using lysis buffer containing 2% SDS and subjected to immunoblotting. Nuclear and cytoplasmic fractions of the cell were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA) following manufacturer's instructions. The antibody against human AR (N20) and lamin A/C was obtained from Santa Cruz Biotechnology

(Santa Cruz, CA). The antibody against V5 epitope tag was from Invitrogen (Carlsbad, CA). Antibodies against  $\beta$ -actin (AC-15) and  $\beta$ -tubulin were from Millipore (Billerica, MA). Antibody against prostate specific antigen (PSA) was from Meridian Life Science (Memphis, TN). Antibodies against eIF2 $\alpha$  and phospho-eIF2 $\alpha$  were from Cell Signaling (Danvers, MA). The results from a minimum of two experiments were subjected to densitometry and normalized to  $\beta$ -actin or  $\beta$ -tubulin loading control and the mean values relative to vehicle control (set to 1.0) given.

### Recombinant DNA and stable cell lines

The AR-WT, T878A and W742C cDNA were subcloned from previously described constructs (20, 21) to pCMV-3xFLAG vector and to pDONR223 entry vector. For AR-F877L mutant, site-directed mutagenesis using pDONR223-AR-WT as template was performed. The primer sets used were: sense 5'-cgagagagctgcatcagctcacttttgacctgcta-3', antisense 5'-tagcaggtcaaaagtgagctgatgcagctctctcg-3'. pLenti6.3 lentiviral expression vectors carrying AR-WT, T878A or F877L mutant sequences were then generated using the Gateway Cloning System (Invitrogen). For lentivirus production, pLenti6.3-AR-WT, T878A or F877L were cotransfected with pCMV-VSVG and pCMV-dR8.91 (Addgene, Cambridge, MA) into HEK293T (American Type Culture Collection, ATCC, Manassas, VA) using Lipofectamine 2000 (Invitrogen). LNCaP and PC3 cells were infected with lentivirus expressing AR-WT or AR-T878A in the presence of polybrene before subsequent selection for cells with stable ectopic gene expression with blasticidin (Invitrogen).

### Reporter gene assays and quantitative realtime PCR (qRT-PCR)

COS-7 cells were transfected with a luciferase reporter gene construct driven by 4 androgen responsive elements (ARE), *Renilla* luciferase gene construct and pCMV-FLAG-AR or pCMVVP16-AR in RPMI1640 with 5% CSS. Forty-eight hours later, transfected cells were treated with dihydrotestosterone (DHT) or vehicle (ethanol) in the presence of galeterone (Tokai Pharmaceuticals, Cambridge, MA), abiraterone, bicalutamide or enzalutamide. Transfection was done in triplicates. Luciferase and *Renilla* luciferase activity was measured using the Dual-luciferase Reporter Assay (Promega, Madison, WI). For qRT-PCR, RNA was isolated using RNeasy Mini Kit (Invitrogen). The TaqMan primer-probe sets for *PSA* were: Forward, 5'-gatgaaacaggctgtgccg-3', reverse, 5'-cctcacagctaccactgca-3', probe, 5'-FAM-caggaacaaaagcgtgatcttctgctggg-3'. The primer-probe sets for *FKBP5* and *GAPDH* were from Life Technologies (Grand Island, NY). The qRT-PCR was performed in an ABI7900 thermal cycler.

### Chromatin immunoprecipitation (ChIP)

For anti-AR ChIP, androgen-deprived VCaP cells were treated with DHT or antagonists for 4 hours. DNA-protein crosslinking was induced by formalin treatment and the cell lysates were subjected to sonication. Immunoprecipitation was done with anti-AR antibody or control IgG (Santa Cruz). The bound DNA was quantified by qRT-PCR using the following primer sets: PSA enhancer: Forward, 5'-tcgattgtccttgacagtaaaca-3', reverse, 5'-tctcatgccaggcttctt-3'; KLK2 enhancer: Forward, 5'-gcctttgctcagaagacaca-3', reverse, 5'-acaagagtgaaggctctgg-3'; TMPRSS2 enhancer: Forward, 5'-tggctctggatgataaaaaagttt-3',

reverse, 5'-gacatacgcgcccaacaga-3'. NKX3.1 enhancer: Forward, 5'-ctggcaaagagcatctaggg-3', reverse, 5'-ggcacttctgagcaaactt-3'.

## Results

### Galeterone functions directly as an AR antagonist

We first examined the effects of galeterone on AR signaling in PCa cells. Androgen-deprived LNCaP and VCaP cells were stimulated with dihydrotestosterone (DHT) at 0.1, 1 or 10 nM in the presence of 1 or 10  $\mu$ M galeterone, and AR activity was assessed by PSA protein expression. A dose-dependent repression of PSA expression by galeterone was observed in both LNCaP and VCaP cells (Fig. 1A). This repression could be partially rescued with higher concentrations of DHT, consistent with galeterone binding to the steroid binding pocket and acting as a competitive antagonist.

PSA mRNA levels were also examined in androgen-deprived LNCaP and VCaP cells treated with galeterone or vehicle control, in the presence or absence of DHT. The effect of galeterone was compared with those of abiraterone (CYP17A1 inhibitor) and of two other direct AR antagonists (enzalutamide and bicalutamide). Galeterone and enzalutamide decreased basal PSA mRNA in both cell lines in the absence of androgen (Fig. 1B). In contrast, abiraterone had minimal effects, consistent with galeterone directly suppressing basal AR activity in these cells independently of its CYP17A1 inhibitor activity. As expected, treatment with DHT was able to partially rescue PSA expression repressed by galeterone or enzalutamide. Bicalutamide more modestly decreased PSA expression in both cell lines treated with DHT, but did not decrease basal PSA levels in the absence of ligand, consistent with it being a less potent AR antagonist with weak partial agonist activity.

The antagonist activity of galeterone was further assessed using COS-7 cells cotransfected with an AR expression construct and a luciferase reporter driven by 4 consecutive androgen-responsive elements (ARE4-LUC) and treated with increasing concentration of DHT. As shown in Fig. 1C, galeterone potently blocked DHT induced AR transcription activity to a level comparable to that seen with bicalutamide. To directly evaluate the effect of galeterone on expression of an endogenous AR target gene, PC3 cells stably expressing AR-WT were treated with bicalutamide, enzalutamide, or galeterone. Expression of FKBP5 mRNA was tested as an indicator of AR transcription activity. Galeterone and enzalutamide both potently blocked FKBP5 mRNA expression induced by up to 10 nM DHT, while this inhibitory effect was partially reversed by the treatment with 100 nM of DHT. Overall these results confirm that galeterone can function as a direct AR antagonist.

### Galeterone impairs AR binding to chromatin

An important distinction between bicalutamide and enzalutamide is that the bicalutamide liganded AR can associate with chromatin, which may contribute to weak partial agonist activity (10, 12). To test whether galeterone stimulates AR binding to DNA, we used a plasmid encoding the full-length AR fused to the VP16 transactivation domain (VP16-AR), which can stimulate transcription independently of coactivator recruitment by AR. COS-7 cells were transfected with this plasmid and with the ARE4-LUC reporter construct, and

then stimulated with a series of ligands (Fig. 2A). As a positive control, DHT induced ARE-LUC activity in a dose-dependent fashion, indicating the binding of VP16-AR to the ARE4-LUC construct (Fig. 2A). As we showed previously, the VP16-AR similarly could be stimulated by bicalutamide, indicative of AR binding to the reporter gene (12). In contrast, also as shown previously, enzalutamide did not stimulate the VP16-AR (10). Significantly, galeterone similarly failed to activate the VP16-AR, indicating that it does not support DNA binding.

We next used chromatin-immunoprecipitation (ChIP) to test whether galeterone can induce recruitment of endogenous AR to its target genes. When androgen-deprived VCaP cells were treated with galeterone or enzalutamide, no increases in AR recruitment to the ARE sites on the *PSA*, *KLK2*, *NKX3.1* and *TMPRSS2* genes were observed compared to vehicle-treated cells (Fig. 2B). As a positive control, DHT treatment led to substantial binding of AR at these sites. Consistent with previous studies, bicalutamide stimulated AR binding to the *PSA* gene (Fig. 2C). These data together indicate that galeterone, similar to enzalutamide, does not stimulate AR binding to chromatin.

To test whether galeterone has an effect on nuclear AR levels, VCaP and LNCaP cells were treated overnight with DHT, galeterone, enzalutamide or vehicle. Nuclear and cytoplasmic fractions were separated and AR protein expression in these fractions was tested. As a positive control, DHT treatment led to substantial increases in nuclear AR protein expression in both VCaP and LNCaP cells (Fig. 2D). Consistent with previous findings, enzalutamide somewhat decreased nuclear AR expression in VCaP or LNCaP cells, without substantial effects on cytoplasmic AR (10). The effects of galeterone in VCaP cells were similar to enzalutamide. In contrast, while galeterone markedly decreased nuclear AR in LNCaP cells, there was also a decrease in cytoplasmic AR. These data together show that galeterone does not induce AR accumulation in the nucleus or binding to chromatin, and may also decrease overall AR expression.

### **Galeterone does not directly enhance degradation of wildtype AR**

The unliganded AR is degraded by a proteasome dependent pathway, and its stability is increased by androgen binding (22-24). Galeterone has been shown previously to decrease AR protein expression in PCa cell lines (LNCaP and LAPC4) and in LAPC4 xenografts (16, 17). However, these decreases in AR protein and increases in AR degradation may in part be due to competitive displacement of androgen from the AR ligand domain by galeterone. Moreover, galeterone at higher concentrations (generally over 10  $\mu$ M) has been reported to activate cellular stress pathways that may indirectly decrease AR protein expression (18, 19). Therefore, to determine whether galeterone binding to the AR could directly enhance AR degradation, we assessed the effects of galeterone at up to 10  $\mu$ M in androgen-deprived cells. Importantly, based on the results in figure 1, we anticipate that most of the AR would be liganded to galeterone at 10  $\mu$ M, so that effects at higher concentration would be indirect and mediated by off-target mechanisms.

In one set of experiments we used LAPC4 cells and its subline LAPC4-CR cells, which both express wildtype AR. The LAPC4-CR line was established from a relapsed castration-resistant LAPC4 xenograft. The cells were cultured in androgen-depleted medium for 48

hours and then treated with galeterone at 0.5-10  $\mu\text{M}$  for 24 hours. In LAPC4 cells, galeterone at concentrations of 0.5-5  $\mu\text{M}$  did not lead to decreased AR expression, but did lead to a 48% decrease at 10  $\mu\text{M}$  (Fig. 3A). To more directly assess AR protein stability, we then treated the androgen-deprived and vehicle or galeterone stimulated LAPC4 cells with cycloheximide (CHX) to block new protein synthesis. Significantly, the rate at which AR protein decayed in these cells was not altered by the treatment with galeterone compared to vehicle control (Fig. 3B). In LAPC4-CR cells, galeterone had no clear effect on overall AR protein levels or stability (Fig. 3C and D).

We next similarly examined VCaP cells, which express an amplified wildtype AR. In androgen-deprived VCaP cells, galeterone led to a moderate decrease of AR protein levels (Fig. 3E). However, as there is substantial *de novo* androgen synthesis in VCaP cells (2), any decrease in AR protein may reflect decreased intracellular androgen levels due to the inhibition of CYP17A1 by galeterone. To clarify whether any decrease in AR expression is a direct effect of galeterone on AR rather than an indirect effect via its CYP17A1 activity, we treated VCaP cells with abiraterone at up to 5  $\mu\text{M}$ , which effectively blocks CYP17A1 activity in these cells (2). Abiraterone treatment alone did not have a clear effect on AR protein, but addition of galeterone to the abiraterone did modestly decrease AR protein, suggesting a direct effect of galeterone on AR (Fig. 3F). The addition of galeterone at 10  $\mu\text{M}$  to the abiraterone similarly decreased AR protein in VCS2 cells, which were derived from a castration-resistant VCaP xenograft (2). However, when we next used cycloheximide treatment to directly assess for effects of galeterone on AR stability in VCaP cells, we found that galeterone at 10  $\mu\text{M}$  did not increase the rate of AR degradation (Fig. 3G). Based on these results, we conclude that galeterone at concentrations up to 10  $\mu\text{M}$  does not substantially enhance AR degradation in VCaP cells, but may decrease AR protein by indirect mechanisms at higher concentrations or in combination with other agents.

Similar results were obtained when we examined CWR22Rv1 cells. The AR in the parental CWR22 cells has a mutation in codon 875 that broadens its ligand specificity, and the CWR22Rv1 cells have in addition a duplication of exon 3 encoding the DNA binding domain (25). These cells also express an alternatively spliced AR isoform that deletes the ligand binding domain (26). As observed in VCaP cells, AR protein was slightly decreased at 10  $\mu\text{M}$  galeterone, while bicalutamide, enzalutamide or abiraterone treatment did not change AR levels (Fig. 3H). As expected, the full length AR protein was slightly increased by DHT, with no clear effect on the AR splice variant (AR-V). Overall, these results indicate that galeterone does not have marked direct effects on AR protein stability, but may decrease AR protein by indirect mechanisms at concentrations at or above 10  $\mu\text{M}$ .

### **Galeterone markedly enhances AR protein degradation in LNCaP and C4-2 cells**

In contrast to the results above, galeterone at concentrations well below 10  $\mu\text{M}$  in androgen-deprived C4-2 cells markedly decreased AR protein (Fig. 4A). This decrease was from 36-87% at 0.5-10  $\mu\text{M}$ , while there was no decrease in AR protein with abiraterone at 2.5 or 5  $\mu\text{M}$  (Fig. 4A). Using cycloheximide to block new protein synthesis, we found that galeterone decreased the half-life of endogenous AR in androgen-deprived C4-2 cells from ~6 hours to ~2 hours (Fig. 4B). The parental line of C4-2, LNCaP, was also tested.

Galeterone at 5 or 10  $\mu\text{M}$  similarly dramatically decreased AR protein expression in androgen-deprived LNCaP cells (Fig. 4C), and treatment with cycloheximide showed that galeterone was increasing AR degradation in these cells (Fig. 4D).

To confirm that the decrease in AR protein was through proteasome mediated degradation, we used a proteasome inhibitor, MG132. Treatment of LNCaP cells with MG132 prevented the decrease in AR protein expression caused by galeterone (Fig. 4E). To determine whether the effects of galeterone were direct and mediated by AR binding, we also assessed whether DHT could prevent the decline in AR protein. As shown in figure 4F, DHT could partially block the galeterone mediated decrease in AR protein.

Finally, as galeterone has been reported to induce an ER stress response that may indirectly affect AR expression (18), we addressed whether galeterone treatment in these cells was inducing an ER stress response. The ER stress response was assessed based on phosphorylation of eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ). Consistent with a previous report (18), an ER stress response could be induced by galeterone, which we observed at 20  $\mu\text{M}$  (Fig. 4G). However, galeterone at 10  $\mu\text{M}$ , which still markedly reduced AR protein, did not induce this stress response. These results together indicate that galeterone is directly enhancing AR protein degradation in LNCaP and C4-2 cells.

### Galeterone enhances degradation of the T878A mutant AR

The marked effects of galeterone on AR protein expression in the C4-2 and LNCaP cells could reflect their T878A mutant AR or other distinct features of these cell lines such as their PTEN deficiency. To initially address the role of the T878A mutation, LNCaP cells were transfected with a construct coding for FLAG-tagged wildtype AR and geneticin resistance, and short-term geneticin resistant cells were selected. These cells were then androgen-deprived for 48 hours and treated with galeterone for 24 hours at increasing doses. Expression of ectopic wildtype AR was probed using anti-FLAG antibody. No decrease in the expression of ectopic wildtype AR was observed at up to 10  $\mu\text{M}$  galeterone, while the endogenous T878A AR protein in the parental LNCaP cells was decreased (Fig. 5A).

To rule out the possible confounding effect of the ectopic expression vector versus the endogenous AR, we established PC3 stable cells using lentiviral vectors expressing AR WT or AR T878A. Treatment of these stable PC3 cells with galeterone led to a ~30% decrease in AR WT, but the decrease in AR T878A was substantially greater (~70%) (Fig. 5B). To further validate this result, LNCaP stable cells expressing epitope-tagged AR WT or T878A were also established. Similar to the PC3 stable cells, galeterone substantially decreased AR T878A expression, with minimal effect on AR WT levels (Fig. 5C).

The T878A AR can be strongly stimulated by the AR antagonists hydroxyflutamide and nilutamide. Therefore, we also tested whether galeterone enhances degradation of AR with other known mutations that can mediate resistance to AR antagonists such as enzalutamide or bicalutamide. Recent reports have found that enzalutamide can activate a F877L mutant AR (27-29). However, in contrast to the transfected T878A AR, galeterone did not decrease expression of the F877L mutant AR (Fig. 5D). Similarly, we found that expression of the W742C mutant AR, which is stimulated by bicalutamide, was not decreased by galeterone

(Figure 5E). Taken together, these results show that galeterone markedly and directly enhances degradation of the T878A mutant AR, while having only modest effects on expression of wildtype AR that may be cell line dependent and through direct or indirect mechanisms.

## Discussion

Galeterone suppresses AR activity both by functioning as a CYP17A1 inhibitor and by binding directly to AR (15, 16). It has clear activity against AR dependent PCa cells in vitro and in xenograft models, and its efficacy in patients is currently being assessed in phase 2 clinical trials. However, the molecular basis for its direct effects on AR remains to be fully established. In this study we confirmed that galeterone functions as a direct AR competitive antagonist. Moreover we found that galeterone, in contrast to bicalutamide but similar to enzalutamide, does not stimulate AR binding to chromatin. Previous studies have shown that bicalutamide functions as an AR antagonist because it does not effectively recruit coactivator proteins and may instead recruit corepressors (12, 30). However, this antagonist activity may be circumvented in CRPC through several mechanisms, such as increased AR or alterations in transcriptional coactivator or corepressor proteins, which may enhance the weak partial agonist activity of bicalutamide (14). In contrast, enzalutamide has a distinct mechanism of action as it does not stimulate AR binding to chromatin, which makes it a purer antagonist and likely contributes to its efficacy in CRPC (10). Therefore, our findings suggest that galeterone will function similar to enzalutamide in CRPC as a relatively pure direct AR antagonist, which in conjunction with its activity as a CYP17A1 inhibitor may enhance the ability of galeterone to suppress AR function in CRPC.

Previous studies have also shown that galeterone treatment can decrease AR protein levels and may increase AR protein degradation (16). However, it has not been clear whether this is due to direct effects on AR, as galeterone at higher concentrations (~20  $\mu$ M) has been found to induce an ER stress response (18), and to decrease phosphorylation of 4EBP1, possibly reflecting decreased TORC1 activity (19). Using galeterone at lower concentrations (up to 10  $\mu$ M) that could effectively suppress DHT stimulated AR activity, but did not stimulate a stress response, we found that galeterone did not substantially enhance degradation of wild-type AR in androgen starved LAPC4 or VCaP PCa cells. In contrast, degradation of the T878A mutant AR, which is expressed by LNCaP and C4-2 cells, was markedly increased by a proteasome-dependent mechanism in response to galeterone binding.

These findings are consistent with previous data showing that galeterone at 5-10  $\mu$ M could decrease AR protein expression in LNCaP cells in vitro (16). While galeterone has also been found previously to decrease AR protein in LAPC4 cells in vitro, this effect was observed at higher galeterone concentrations (15-20  $\mu$ M) that may have decreased AR expression by indirect mechanisms (16). Significantly, in vivo studies using LAPC4 xenografts have shown marked decreases in AR protein in response to galeterone (16, 17). Our in vitro data certainly do not rule out the possibility that galeterone binding may directly enhance AR degradation in vivo due to displacement of an endogenous ligand and/or subtle differences in mechanisms regulating AR degradation in vivo versus in vitro. Alternatively, the



decreased AR protein in vivo may be mediated by indirect mechanisms, or possibly synergy between direct and indirect mechanisms. Finally, a recent study found that galeterone and related compounds could enhance degradation of the full-length AR and truncated AR (ligand-binding domain deleted) in CWR22Rv1 cells, further establishing that galeterone has a mechanism of action that is independent of binding to the AR ligand-binding domain (31).

Structural studies have shown that T878 in helix 9 of the AR ligand-binding domain sits in the steroid-binding pocket and that the T878A mutation provides more room in this pocket (32, 33). As a result, binding of the AR antagonist hydroxyflutamide to the T878A mutant AR does not distort the ligand-binding pocket and this drug thereby functions as potent agonist of the T878A AR(34). Galeterone is a steroidal compound modified with a bulky benzimidazole group at the C17 position, which in DHT has an hydroxyl group that contacts T878. However, as galeterone is still an antagonist for the T878A AR, it is apparent that the benzimidazole group must still distort the ligand binding pocket. Nonetheless, we propose that the T878A mutation alters the positioning of helix 9 in the galeterone liganded AR, and may thereby enhance the ubiquitylation of one or more helix 9 lysines by an E3 ubiquitin ligase that normally mediates degradation of the unliganded AR, or that mediates turnover of the agonist-liganded AR. Alternatively, the galeterone-liganded T878A mutant AR may be degraded through a distinct mechanism that does not normally mediate AR degradation. In either case, these findings demonstrate that certain conformations of AR can markedly enhance its degradation, and support efforts to identify novel antagonists that can similarly alter the structure of wild-type AR and thereby enhance its degradation.

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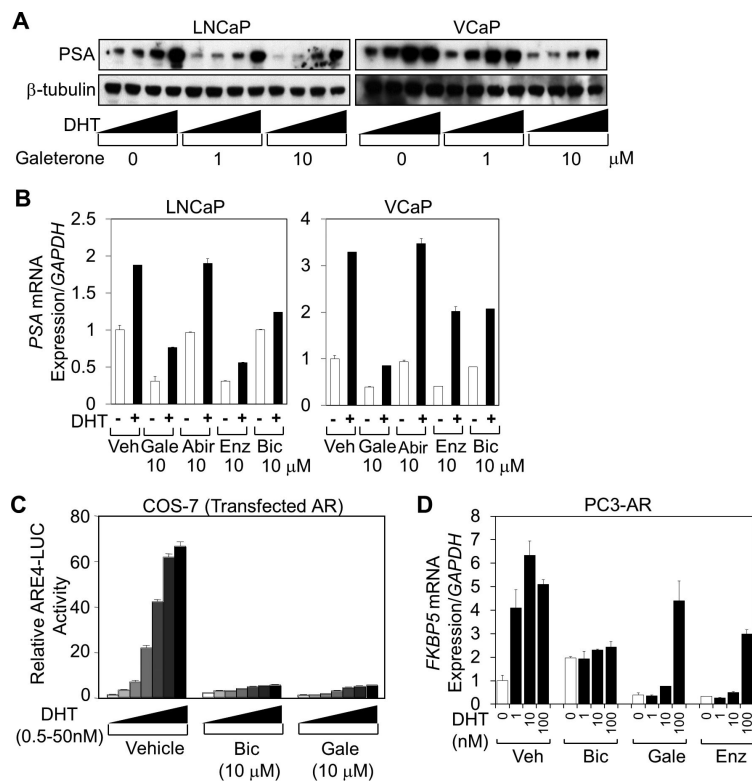
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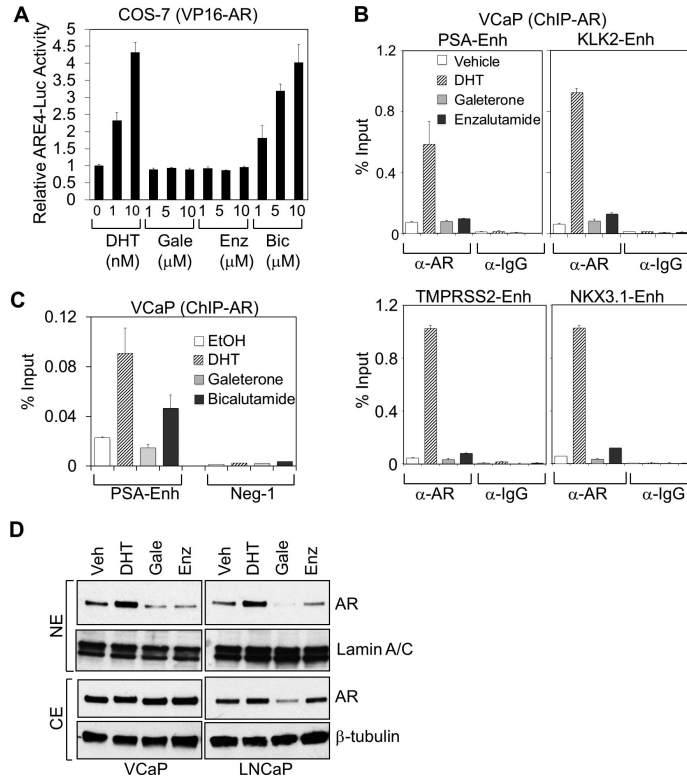
### Statement of Translational Relevance

Galeterone may be efficacious in castration-resistant prostate cancer (CRPC) through its dual activities as a CYP17A1 inhibitor and androgen receptor (AR) antagonist, but many AR antagonists may be ineffective in CRPC as they can stimulate AR binding to chromatin and may thereby have partial agonist activity. This study shows that galeterone, in contrast to bicalutamide but similar to enzalutamide, blocks AR binding to chromatin. Galeterone also directly enhanced degradation of a mutant AR (T878A), but wildtype AR degradation required higher galeterone concentrations that induce cellular stress responses. These findings indicate that galeterone, by functioning similar to enzalutamide, may be effective as a direct AR antagonist in CRPC, and may also enhance AR degradation by direct or indirect mechanisms in a subset of tumors. Finally, the enhanced degradation of the galeterone-liganded T878A AR supports efforts to identify novel antagonists that similarly alter wildtype AR structure and thereby enhance its degradation.



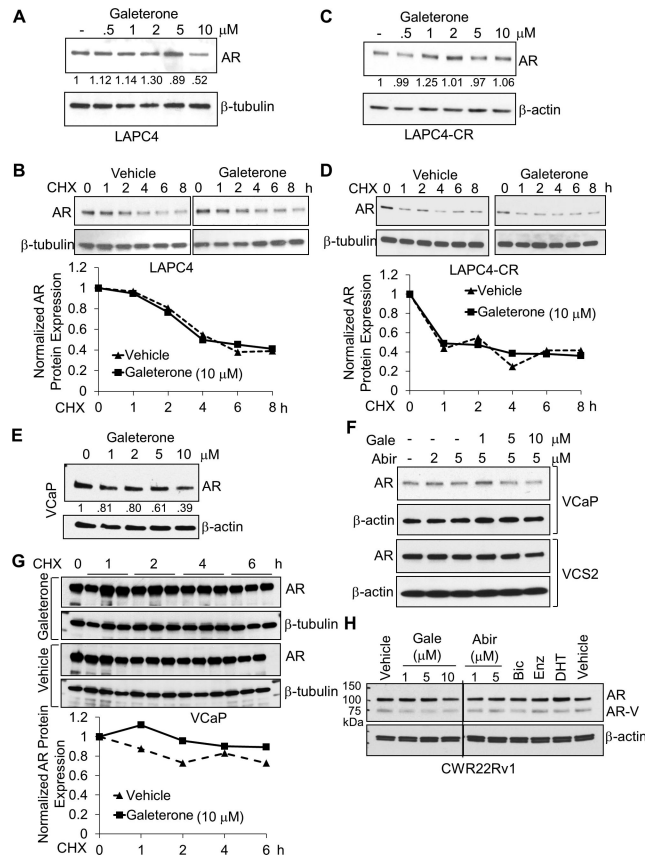
**Figure 1. Galeterone is a competitive antagonist of AR transcriptional activity**

A, Androgen deprived LNCaP (left) or VCaP (right) cells were treated with DHT (0, 0.1, 1 or 10 nM) in the presence of 1 or 10 μM galeterone or vehicle control (DMSO). Twenty-four hours later, samples were immunoblotted for PSA protein expression. β-tubulin was probed as control for loading. B, Androgen deprived LNCaP (left) or VCaP (right) cells were treated with galeterone (Gale), abiraterone (Abir), enzalutamide (Enz), bicalutamide (Bic), all at 10 μM, or DMSO (veh), in the presence or absence of DHT (10 nM) for overnight. PSA mRNA expression was assessed using qRT-PCR and normalized to GAPDH message level. C, COS-7 cells were transfected with pCMV-AR, Renilla luciferase and ARE4-Luc constructs in medium with 5% CSS. Thirty-two hours after transfection, cells were treated with DHT (1 or 10 nM) in the presence of bicalutamide (10 μM) or galeterone (10 μM) for overnight. ARE4-driven luciferase activity was measured and normalized to that of Renilla luciferase as a transfection control. Data presented are average of two experiments with biological triplicates. D, PC3 cells stably expressing ARWT (PC3-AR) were overnight treated with 0-100 nM of DHT in the presence of bicalutamide (Bic), galeterone (Gale), enzalutamide (Enz), all at 10 μM, or DMSO (Veh). RNA was isolated and subjected to qRT-PCR in triplicate using primer/probe sets for FKBP5 and GAPDH mRNA. Data shown is an average of biological duplicates.



**Figure 2. Galeterone impairs AR DNA binding**

A, COS-7 cells were transfected with VP16-AR, Renilla luciferase and ARE4-Luc constructs in medium with 5% CSS. Thirty-two hours after transfection, cells were treated with DHT (1 or 10 nM), galeterone (Gale, 1, 5 or 10  $\mu$ M), enzalutamide (Enz, 1, 5 or 10  $\mu$ M) or bicalutamide (Bic, 1, 5 or 10  $\mu$ M) for overnight. ARE4-luciferase activity was then measured and normalized to that of Renilla luciferase as a transfection control. Data are average of two experiments with biological triplicates. B, androgen-deprived VCaP cells were treated with vehicle (ethanol), DHT (10 nM), galeterone (10  $\mu$ M), or enzalutamide (10  $\mu$ M) for 4 hours. After crosslinking and fragmentation, DNA fragments were coprecipitated using an anti-AR antibody and enrichment of the indicated AR binding sites (all in enhancer regions, Enh) were assessed by qRT-PCR. DNA samples co-precipitated using normal IgG were employed as negative controls. C, androgen-deprived VCaP cells were treated with vehicle, DHT (10 nM), galeterone (10  $\mu$ M), or bicalutamide (10  $\mu$ M) as indicated for 4 hours. ChIP was performed as in B and enrichment of AR binding sites in PSA enhancer (PSA-Enh) was assessed by qRT-PCR. A chromatin-region containing no known ARE consensus sequences was employed as negative control (Neg-1). D, androgen-deprived VCaP and LNCaP cells were treated with DHT (10 nM), galeterone (Gale, 10  $\mu$ M), enzalutamide (Enz, 10  $\mu$ M) or DMSO (Veh) for overnight. Nuclear (NE) and cytoplasmic (CE) extracts were separated using an NE-PER kit (Thermo Scientific) and subjected to Western blot analysis for AR protein expression. Lamin A/C was used as a control for loading of NE and  $\beta$ -tubulin ( $\beta$ -tub) was a loading control for CE.

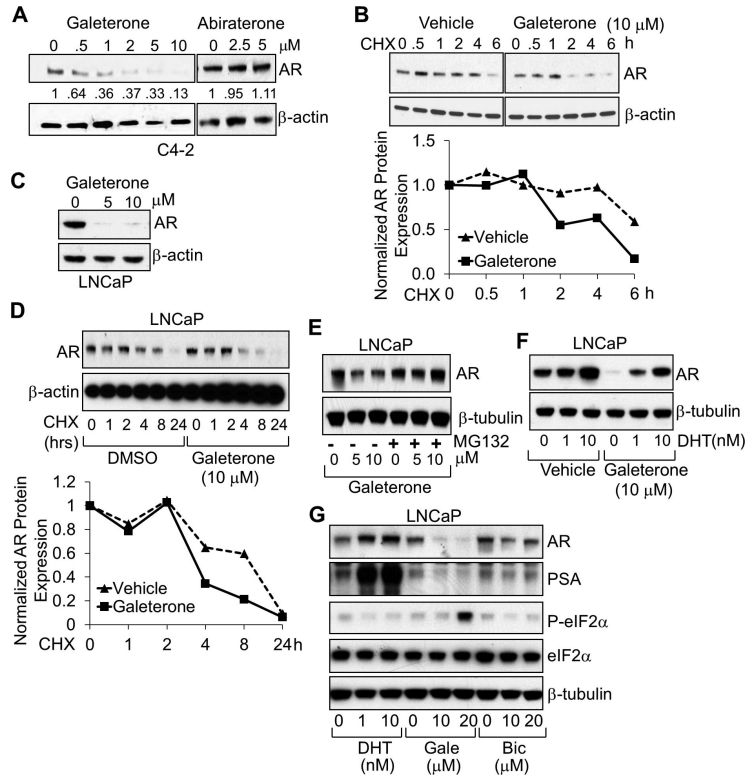


**Figure 3. Galeterone does not directly enhance degradation of wildtype AR**

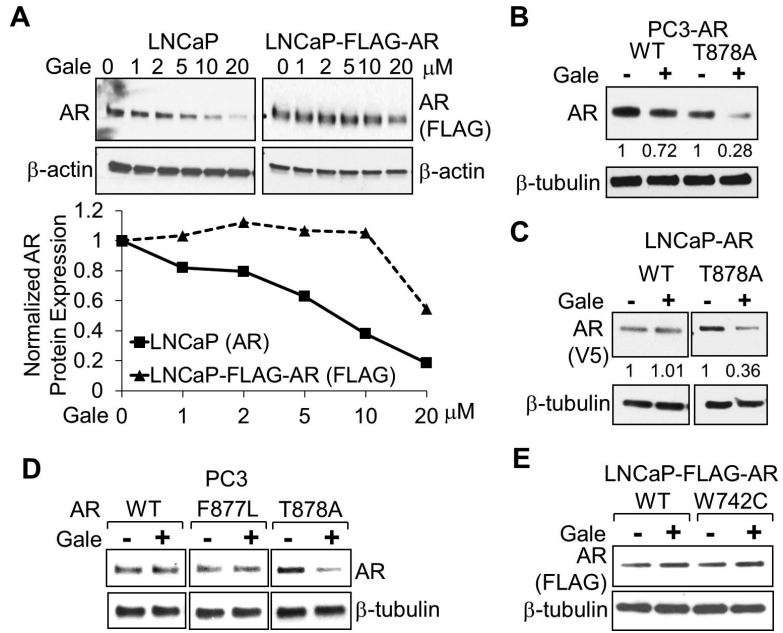
A and C, LAPC4 (A) and LAPC4-CR (C) cells were hormone-starved for 48 hours and then treated with galeterone at the indicated concentrations for overnight. Cell lysates were subjected to immunoblotting for AR and  $\beta$ -actin or  $\beta$ -tubulin was probed as loading control. Gel images from two independent experiments were quantitated using NIH Image J software. The intensity of AR bands was normalized to that of loading controls and the value of relative AR expression in vehicle-treated cells was set to 1. B and D, LAPC4 (B) and LAPC4-CR (D) cells were hormone-starved for 48 hours and then treated with cycloheximide (CHX) in the presence of either galeterone or vehicle. Samples were harvested at the indicated time points and subjected to immunoblotting. Relative AR protein expression was quantified and plotted. E, androgen-deprived VCaP cells were treated with galeterone at the indicated concentrations for overnight. Relative AR protein expression was determined as in A. F, VCaP and VCS2 cells were hormone-starved for 48 hours and then treated with abiraterone (Abir), galeterone (Gale) or abiraterone and galeterone combined at the indicated concentrations for overnight and their lysates were immunoblotted to assess AR protein expression. G, androgen-deprived VCaP cells were treated with cycloheximide (CHX) in the presence of either galeterone or vehicle. Samples were harvested at the indicated time points and subjected to immunoblotting. Relative AR protein expression was quantified and plotted. Data is presented as an average of biological triplicates. H, androgen-deprived CWR22Rv1 cells were treated with galeterone (Gale), abiraterone (Abir), bicalutamide (Bic), enzalutamide (Enz) or DHT at the indicated concentrations for

overnight, and lysates were then immunoblotted for AR protein expression and  $\beta$ -actin for equal loading.





**Figure 4. Galeterone markedly enhances AR protein degradation in LNCaP and C4-2 cells**  
 A, androgen-deprived C4-2 cells were treated for 24 hours with vehicle, galeterone (0.5-10  $\mu$ M, left panels) or abiraterone (2.5 or 5  $\mu$ M, right panels). AR protein expression was examined and normalized to that of  $\beta$ -actin. B, androgen-deprived C4-2 cells were treated with cycloheximide (CHX) in the presence of either galeterone (10  $\mu$ M) or vehicle. Samples were subjected to immunoblotting at the indicated times, and relative AR protein expression was quantified and plotted (average of biological duplicates). C, androgen-deprived LNCaP cells were treated with galeterone (5 or 10  $\mu$ M) or vehicle. AR protein expression was examined and  $\beta$ -actin was the loading control. D, androgen-deprived LNCaP cells were treated with cycloheximide (CHX) in the presence of either galeterone (10  $\mu$ M) or vehicle. Samples from the indicated time points were immunoblotted and relative AR protein expression quantified and plotted. E, androgen-deprived LNCaP cells were treated with vehicle or galeterone (5 or 10  $\mu$ M) for overnight. Cells were then incubated in the presence or absence of MG132 (10  $\mu$ M) for 3 hours before being lysed and immunoblotted for AR. F, androgen-deprived LNCaP cells were treated with vehicle or DHT (1 or 10 nM) in the presence or absence of galeterone (10  $\mu$ M). AR and  $\beta$ -tubulin was examined using immunoblot analysis. G, androgen-deprived LNCaP cells were treated with DHT (1 or 10 nM), galeterone (Gale, 10 or 20  $\mu$ M), bicalutamide (Bic, 10 or 20  $\mu$ M) or vehicle for overnight. The protein expression of AR, PSA, phospho-eIF2 $\alpha$  (P-eIF2 $\alpha$ ), eIF2 $\alpha$  and  $\beta$ -tubulin were examined.



**Figure 5. Galeterone enhances degradation of AR T878A mutant**

A, LNCaP cells were transiently transfected with pCMV-3xFLAG-AR vector and selected for ectopic AR expression. The resulting LNCaP-FLAG-AR cells were treated with galeterone (Gale, 1-20 μM) or vehicle alongside the parental LNCaP cells in hormone-depleted medium. Twenty-four hours later, cell lysates were subjected to immunoblotting. The endogenous AR T878A mutant in LNCaP cells was probed for with anti-AR antibody and the ectopic AR (wildtype) in LNCaP-FLAG-AR cells was detected with anti-FLAG antibody. AR expression was quantified and normalized to β-actin. B and C, PC3 and LNCaP stably expressing AR-WT or T878A were treated with galeterone (10 μM) or vehicle for 24 h in hormone-depleted medium and cell lysates were prepared. Anti-AR antibody was used to detect ectopic AR in PC3 cells and anti-V5-epitope antibody was used for ectopic AR in LNCaP cells. The expression of AR was quantified and normalized to that of β-tubulin. Data are representative of two independent experiments. D, PC3 cells were transfected with constructs expressing AR-WT, F877L or T878A and allowed to grow for 30 h in medium containing 5% CSS. Cells were then treated with galeterone (10 μM) or vehicle for 24 h and lysates were subjected to immunoblotting for AR and β-tubulin (as control). Data are representative of two independent experiments. E, LNCaP cells were transfected with pCMV-3xFLAG-AR-W742C and selected with geneticin. These AR-W742C-expressing LNCaP cells were androgen-starved and treated with galeterone (10 μM) or vehicle alongside the LNCaP-FLAG-AR-WT cells as described in A. Twenty-four hours later, cell lysates were subjected to immunoblotting for ectopic AR using FLAG antibody. β-tubulin expression was used as loading control. Data are representative of two independent experiments.