The Effect of F877L and T878A Mutations on Androgen Receptor Response to Enzalutamide

Stefan Prekovic1, Martin E. van Royen2, Arnout R.D. Voet3,4, Bart Geverts2, Rene Houtman5, Diana Melchers5, Kam Y.J. Zhang5, Thomas Van den Broeck1,6, Elien Smeets1, Lien Spans7, Adriaan B. Houtsmuller2,8, Steven Joniau6, Frank Claessens1, and Christine Helsen1

Abstract

Treatment-induced mutations in the ligand-binding domain of the androgen receptor (AR) are known to change antagonists into agonists. Recently, the F877L mutation has been described to convert enzalutamide into an agonist. This mutation was seen to co-occur in the endogenous AR allele of LNCaP cells, next to the T878A mutation. Here, we studied the effects of enzalutamide on the F877L and T878A mutants, as well as the double-mutant AR (F877L/T878A). Molecular modeling revealed favorable structural changes in the double-mutant AR that lead to a decrease in steric clashes for enzalutamide. Ligand-binding assays confirmed that the F877L mutation leads to an increase in relative binding affinity for enzalutamide, but only the combination with the T878A mutation resulted in a strong agonistic activity. This correlated with changes in coregulator recruitment and chromatin interactions. Our data show that enzalutamide is only a very weak partial agonist of the AR F877L, and a strong partial agonist of the double-mutant AR. Mol Cancer Ther; 15(7); 1702–12. ©2016 AACR.

Introduction

Globally, prostate cancer is the fourth most common cancer and the fifth leading cause of cancer-related death in men (1). As this cancer is hormone-dependent, the blockade of the androgen receptor (AR) signaling is an effective therapeutic strategy for men with advanced metastatic prostate cancer. The discovery of novel compounds that inhibit the AR and their clinical application has led to the improvement in survival time. One such compound is the potent antiandrogen enzalutamide, which has been approved under the name Xtandi for use in postchemotherapy patients with metastatic castration-resistant prostate cancer (mCRPC; refs. 2, 3). Enzalutamide acts as a direct competitor for dihydrotestosterone (DHT) binding to the ligand-binding pocket of the AR. In addition, it reduces the binding of the AR to DNA, and inhibits the recruitment of AR corepressors (4). Importantly, enzalutamide significantly prolonged the survival of men with mCRPC after chemotherapy by a median of 4.8 months in comparison with the placebo group (5). On the other hand, enzalutamide has no beneficial effect in a portion (~20%) of patients, while the majority of patients who initially respond will eventually develop resistance towards this therapy (6). It is imperative, therefore, to study the different mechanisms that can lead to enzalutamide resistance.

Several studies reported the co-occurrence of the F877L and the T878A mutations (F877L/T878A, hereinafter referred to as the double-mutant AR) in cell line models (10, 12, 13). Interestingly, this double-mutant AR was described in a patient who progressed on enzalutamide treatment (14). Here, we wanted to compare the agonistic activity of enzalutamide on the AR F877L, as well as on the double-mutant AR. We discovered that the LNCaP-specific T878A mutation modulates the F877L responses to enzalutamide.
Materials and Methods

Plasmid constructs

Expression vectors, pSG5 and pEGFP-C1, containing cDNA of wild-type flag- or EGFP-tagged human AR, respectively, (15) were used to generate the mutations in the ligand-binding domain (LBD) by two-step PCR site-directed mutagenesis. The resulting PCR fragments were ligated into pGEM-T Easy (Promega) and recloned as AsuII/BglII fragments into the pSG5-flag WT AR construct or the pEGFP-C1-WT AR construct. The WT DBD/LBD fragment of AR inserted into pSG5 backbone (16) was used to introduce mutations via two-step PCR site-directed mutagenesis. The same cloning strategy as described for the full-length AR constructs was used. The construct containing double-tagged (N-ter YFP and C-ter CFP) WT AR (17) has been used to generate mutations in the LBD using QuikChange II kit (Agilent Technologies). A plasmid containing WT AR (17) has been used to generate mutations in the LBD using the same strategy as described for the full-length AR constructs was used. The construct containing double-tagged (N-ter YFP and C-ter CFP) WT AR (17) has been used to generate mutations in the LBD using QuikChange II kit (Agilent Technologies).

Cell culture

HEK 293T or PC-3 cells were seeded at a density of 15,000 cells per well or 10,000 cells per well, respectively, in 96-well plates. Cells were transfected with 10 ng of expression vector of the receptor (wild-type or mutant), 100 ng of reporter construct, and 10 ng of the pCMV-β-Gal expression vector per well. On the following day, medium was aspirated and fresh medium without or with compounds was added. Cells were stimulated with DHT (0.1 nmol/L and 1 nmol/L) as a positive control DHT and Hof were docked into the agonistic conformation of AR WT or T878A LBD, respectively. The top docking scores exhibited a similar docking mode within 0.4 Å RMSD deviation of the crystallographically determined structure.

Whole cell competition assay

HEK 293T cells were seeded at a density of 30,000 cells per well in 48-well plates. Cells were transfected with 375 ng of AR expression constructs, and 75 ng of pCMV-β-Gal expression vector. On the day after the transfections, medium was refreshed and cells were incubated overnight. Subsequently, cells were treated with a dilution series (0.1 nmol/L to 10 μmol/L) of compound with 1 nmol/L [3H]-labeled mibolerone (Perkin Elmer). After an incubation period of 90 minutes at 37°C, medium was aspirated, cells were washed three times with ice-cold PBS, and lysed in 100 μL of Passive Lysis buffer (Promega). Cell lysates (75 μL) were transferred to scintillation vials and 2 mL of LumaseSafe Plus (PerkinElmer) was added. Radioactivity measurements were done in a liquid scintillation counter (LK Wallac 1216 RackBeta, Wallace).

Western blot analysis

Total protein extracts (in Passive Lysis buffer) were run on a prestained NuPAGE Novex 4%–12% Bis-Tris Gel (Invitrogen). After electrophoresis, the proteins were blotted on a polyvinylidene difluoride transfer membrane (GE Healthcare). Antibodies against GAPDH (Santa Cruz Biotechnology) and AR (in-house antibody against N-terminus of AR as described in ref. 15), were used. The secondary antibodies anti-rabbit IgG (P0217 from Dako) and anti-Mouse IgG (P0260 from Dako) were conjugated to horseradish peroxidase, and visualized by immunodetection combined with chemiluminescence (Western Lightning Plus-ECL, PerkinElmer).

Double-hybrid assay

Table of contents

COS-7 cells were seeded at a density of 30,000 cells per well in 48-well plates. On the following day, cells were transfected with 25 ng of AR DBD/LBD expression vector with or without the mutations, 250 ng of the VP16 AD-NTD(WT) construct, 250 ng of the reporter construct, and 50 ng of pCMV-β-Gal construct. Cells were stimulated with either DHT (10 nmol/L) or enzalutamide (10 μmol/L), overnight. Cells were harvested in Passive Lysis buffer (Promega) and β-galactosidase activity was determined. As a reference for the correct torsional angle between the 3-fluoro-cyano-benzyl group and the thioximidazolidin, PDB entry 2NW4 was utilized (22). The top docking scores exhibited a similar docking mode within 0.4 Å RMSD deviation of the crystallographically determined structure.
buffer (Promega) and the luciferase and β-galactosidase measurements were done as indicated above.

Acceptor photobleaching fluorescence resonance energy transfer

Acceptor photobleaching fluorescence resonance energy transfer (FRET) was performed using a Zeiss LSM510 confocal laser scanning microscope equipped with a Plan-Neoﬂuar 40×/1.3 NA oil objective (Carl Zeiss) at a lateral resolution of 100 nm (17). For excitation of CFP and YFP, an Argon laser at 358 and 514 nm was used. Both the CFP and YFP images were taken prior to photobleaching of the acceptor fluorophore (514 nm at high laser power). Apparent FRET efﬁciency was estimated according to the instructions described in ref. (17). The abFRET datasets were tested for normality using the Kolmogorov–Smirnov test, and the datasets were compared using the one-tailed Student t test.

MARCoNI assay

To investigate the modulation of coregulator interaction with the WT AR and mutant receptors, we used the MARCoNI assay (Pamgene). HEK293T cells were seeded at a density of 20 million cells per T175 ﬂask. On the following day, cells were transfected with 85 μg of AR WT, AR F877L, AR T878A, or AR double-mutant expression constructs. After 24 hours, cells were incubated in serum-free medium without or with compounds (DHT, Hof, and enzalutamide) for 30 minutes. Subsequently, cells were scraped and pelleted. The MARCoNI assay was performed with cell lysates as described previously (24). Each sample was processed on three separate arrays. Subsequently, the binding of AR to each individual coregulator peptide was quantiﬁed. The FQNLF motif of the AR was discarded as the antibody to detect the AR was raised against a peptide that partly covered this motif. For each peptide, Student t test was applied to compare the receptor binding level to that of the nonstimulated control. All calculations and statistics were performed using R (version 3.1.2; ref. 25).

Generation of stable cell lines

Stable Hep3B cells were generated to constitutively express the EGFP-tagged AR with either the F877L mutation or the F877L/T878A double mutation. The expression of EGFP-fusions was visualized by ﬂuorescence microscopy and functional properties of the receptor was conﬁrmed by transfection of an ARE-driven luciferase reporter construct. Clones with moderate induction factor (F877L clone: 13.3 ± 5.5; F877L/T878A clone: 20.4 ± 11.8) after DHT stimulation were selected (Supplementary Fig. S1). Hep3B cell lines expressing EGFP-fused AR WT, AR T878A, or AR A574D were reported previously (26).

Fluorescence recovery after photobleaching

Cells were seeded in 6-well plates on a cover glass, ligands were added, and cells were incubated overnight. For each experiment, a cover glass with stably transfected Hep3B cells was placed in a preheated ring. Cells were kept at 37°C and used for no longer than 90 minutes. A Zeiss LSM510 META confocal laser scanning microscope equipped with a 40×/1.3NA oil immersion objective, an argon laser (30 mW) and an acousto-optic tunable filter was used. Fluorescence recovery after photobleaching (FRAP) analysis was performed according to the instructions by van Royen and colleagues (27). For each treatment group, 30 cells were measured by FRAP in two independent experiments. All curves were normalized to ﬂuorescent intensity before bleaching. The FRAP data were quantitatively analyzed by comparing the experimental data to curves generated using a previously described Monte Carlo approach (28). In short, a large set of computer-simulated FRAP curves with a 3-population model was generated, containing a diffusing fraction (ranging from 1 to 3 μm²/s) and two bound (immobile) fractions (ranging from 0.65 to 0.8 and 3 to 15 s, respectively). Ranges in the simulation are based on quantitative FRAP analysis of AR curves in previous work (29). The top 10 simulated curves that are ﬁtting best to the experimental curve (by ordinary least squares) were averaged and provide the properties of the experimental data.

Results

T878A enhances the partial agonistic effects of enzalutamide on AR F877L

To unravel possible interferences between the F877L and the T878A mutations, we ﬁrst compared the response of the AR WT, AR F877L, AR T878A, and the AR double-mutant F877L/T878A to several compounds on a transactivation assay. Reporter assays were performed in HEK293T and PC-3 cells (Fig. 1 and Supplementary Fig. S1). The mutations had some effect on the maximal DHT induction: 1 nmol/L DHT resulted in induction factors of approximately 82, 71, 28, and 22 respectively for AR WT, AR T878A, AR F877L, and the double mutant (Supplementary Fig. S2). To allow comparisons, the luciferase response to 1 nmol/L DHT was put at 100. As expected, the DHT-induced transactivation of AR WT was inhibited by the antagonists, enzalutamide, ARN, bicalutamide, and Hof, to at least 25%. These effects were independent of the receptor levels (Supplementary Fig. S3). As expected, none of the antagonists had robust agonistic activity on WT AR. The induction of WT AR by 10 nmol/L Hof rose to ~6% of the 1 nmol/L DHT response; (Fig. 1A) which was comparable to the signal observed for castrate levels of DHT (0.1 nmol/L). Thus, for AR WT, enzalutamide, ARN, and bicalutamide are full antagonists, while hydroxyﬂutamide is a weak partial agonist.

For the LNCaP AR T878A mutant, hydroxyﬂutamide gained full agonistic activity (~98% of DHT 1 nmol/L signal) and lost its antagonistic properties (Fig. 1B), as reported in earlier studies (30). The other compounds (enzalutamide, ARN, and bicalutamide) efﬁciently antagonize AR T878A. When it comes to the AR F877L mutant, enzalutamide and ARN led to an increase in luciferase signal to approximately 10% and 14% of the signal induced by 1 nmol/L DHT, respectively (Fig. 1C and Supplementary Fig. S4). It should be noted that the level of transactivation induced by these compounds was also similar to that of castrate levels of DHT (0.1 nmol/L). Importantly, both enzalutamide and ARN retain their antagonistic effects on DHT-activated AR F877L. In our assays, enzalutamide and ARN are only weak partial agonists on AR F877L (Fig. 1C and Supplementary Fig. S4), comparable with the report by Joseph and colleagues (12). In case of the double mutant, however, enzalutamide and ARN were able to activate the receptor to a substantial extent (33% and 34% of the response to 1 nmol/L DHT, respectively) and exhibited weaker antagonistic properties than on AR WT, AR F877L, and AR T878A (Fig. 1D and Supplementary Fig. S4). In other words, enzalutamide and ARN are considerably stronger agonists of the double-mutant AR than of the AR F877L. Surprisingly, the combination of the
F877L mutation with the T878A mutation impaired the full agonist activity of hydroxyflutamide (compare Fig. 1D with B). These effects are independent of AR levels and of the cell line used (Fig. 1E and Supplementary Fig. S1).

Modeling the effect of the F877L and T878A mutations on the AR-enzalutamide interactions
To get an insight into the mechanisms how the T878A and the F877L mutations would convert the antagonist enzalutamide into
an agonist, we modeled the latter into the agonistic conformation of the AR ligand-binding pocket (Fig. 2A). In this agonistic conformation, the interactions of testosterone (T) or DHT with the R753 side chain are crucial (31, 32). However, if a similar interaction between R753 and enzalutamide is preserved, the latter cannot be accommodated in the agonistic conformation of AR WT or AR T878A. The top scoring orientation for enzalutamide in the WT structure significantly deviated from the binding mode observed in the mutant or protein in the modeled antagonistic conformation (33) with an 8 Å RMSD deviation and a non-negative predicted binding energy of 54 kcal/mol. Similarly, oriented and bad scoring binding modes were observed in the top solutions for both ARN and RD162.

Indeed, there is a strong steric hindrance of enzalutamide with the phenylalanine at position 877, as well as with the threonine residue at position 878 (Fig. 2B). The F877L mutation resolves most of this, as leucine has a more flexible side chain compared with phenylalanine. This leucine can adopt a rotamer position without steric hindrance for enzalutamide, thus allowing the AR LBD to assume an agonistic conformation (Fig. 2C). This mechanism clearly differs from the mechanisms of agonism of the flexible bicalutamide, which fits as an agonist into a nearby cavity of the AR W741C/L mutants, thus stabilizing the agonistic conformation of the AR LBD in an alternative way (34). Moreover, modeling enzalutamide into the ligand-binding pocket of the double-mutant AR predicted even fewer steric clashes, as well as a moderate improvement in docking scores compared with the single F877L mutant (Table 1; Supplementary Fig. S5). Similar results were obtained for ARN and RD162 (Table 1).

Modeling of hydroxyflutamide into the agonistic conformation of the ligand-binding pocket of the double-mutant AR suggested less favorable contacts between Leu877 and the dimethyl group of hydroxyflutamide (Supplementary Fig. S6; Supplementary Table S1), predicting a less efficient AR activation.

Modeling of DHT into the WT and mutant AR also indicated slightly reduced docking scores for the F877L and the double-mutant AR compared with the WT AR (Supplementary Fig. S7) and the T878A mutant (data not shown).

The F877L mutation leads to an increase in the affinity of the receptor for enzalutamide

Whole cell competition assays with 1 nmol/L 3H-labeled mibolerone, were performed to investigate whether binding affinities of the mutated receptors for DHT and enzalutamide have changed. A decrease was observed in relative binding affinities (RBA) of DHT for both mutant receptors tested when compared with the WT receptor [RBA(DHT)wt = 100, RBA(DHT)877 = 75.81, RBA(DHT)877/878 = 77.51; Fig. 3A; Table 2]. On the other hand, there was a drastic increase in RBA of enzalutamide for both AR F877L and AR F877L/T878A when compared with the AR WT [RBA(Enza)wt = 1.38, RBA(Enza)877 = 12.77, RBA(Enza)877/878 = 19.28; Fig. 3A; Table 2].

Effect of mutations on the ligand-dependent N/C interactions

Upon binding of an agonist, the C-terminal LBD of the AR is known to interact with the FQNLF motif in the N-terminal domain (35). To measure these N/C interactions, FRET and mammalian double-hybrid assay were performed (Fig. 3B and Supplementary Fig. S8). DHT induced N/C interactions in both the FRET and the mammalian double-hybrid assays (35), whereas no AR N/C interactions were observed for enzalutamide-bound AR WT. While there was only a trend towards N/C interactions for

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The double mutant enables an optimal agonistic conformation for binding of enzalutamide (Enza). A, in silico models for enzalutamide interaction with the LBD; enzalutamide (contacting the side chain of R752) was docked into the agonistic conformation of the ligand binding pocket of WT (B), F877L (C), and the double mutant (D).

Table 1. Docking scores of enzalutamide (Enza), ARN-509, and RD162

<table>
<thead>
<tr>
<th>Docking scores (kcal/mol)</th>
<th>WT</th>
<th>F877L</th>
<th>F877L/T878A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enza</td>
<td>/</td>
<td>-10.4</td>
<td>-15.1</td>
</tr>
<tr>
<td>ARN</td>
<td>/</td>
<td>-10.8</td>
<td>-15.9</td>
</tr>
<tr>
<td>RD</td>
<td>/</td>
<td>-10.1</td>
<td>-14.8</td>
</tr>
</tbody>
</table>

**NOTE:** Docking simulations were used to predict the affinity of the compounds (kcal/mol) according to the top solutions for the different AR mutants. Decrease of the docking score is in relation increased stability of the receptor–ligand pair.
Comparison of enzalutamide-induced coregulator recruitment by WT and mutant receptors

To explore whether DHT, enzalutamide, and hydroxyflutamide could induce a modulation of the interaction of WT and mutant AR with coregulators, we performed a MARCoNI assay for the full-length receptors. The natural ligand (DHT) induced strong interactions of coactivator peptides with both WT and all mutant receptors, while corepressor interactions were downregulated (Fig. 4A and Supplementary Fig. S9). Unsupervised clustering showed that a very similar binding pattern was observed for the hydroxyflutamide-bound AR T878A (Supplementary Figs. S10 and S11) and the enzalutamide-bound double-mutant AR. In fact, out of all enzalutamide-stimulated ARs, the double mutant resembled most closely to the pattern observed for DHT-stimulated receptors (Fig. 4A and B and Supplementary Fig. S11). In contrast, no strong effects on coregulator interactions could be observed for enzalutamide-bound AR WT or AR T878A (Fig. 4A and B and Supplementary Fig. S11).

Comparison of the intranuclear mobility of AR WT and mutant receptors

Nuclear mobility of a transcription factor can be studied by FRAP. The extent of immobilization is strongly correlated with chromatin-binding characteristics (27). For all receptors tested in our FRAP experiments, DHT induced nearly overlapping redistribution curves from which very similar immobile fractions can be calculated (Fig. 5A and D).

Hydroxyflutamide-stimulated AR F877L reacted like the inactive DNA-binding defective mutant, while the curves of the hydroxyflutamide-bound AR T878A and the double mutant were nearly superimposable on that of the DHT-bound AR WT (Fig. 5B). The calculated immobilized fraction size and free diffusion time reflected these similarities in mobility behavior (Fig. 5D; Supplementary Table S2).

We also compared the effect of enzalutamide on the mobility of the AR mutants. The FRAP curves of the WT AR, AR T878A were superimposable with that of the inactive DNA-binding defective AR A574D. The double-mutant AR showed a slower redistribution pattern after photobleaching when compared with AR WT, AR T878A, and AR F877L (Fig. 5C). Clearly, the AR double mutant approaches the FRAP profile of the DHT-stimulated AR WT, while the curve for the enzalutamide-bound AR F877L mutant is more similar to that of the enzalutamide-bound AR WT (Fig. 5C). Comparable results were observed for ARN-stimulated AR WT and mutant receptors (Supplementary Fig. S13).

Computational calculations of the fraction sizes of receptors that have an apparent long immobilization time revealed that the AR F877L/T878A double mutant, when stimulated with enzalutamide, behaved more like the antagonist-bound AR WT (Supplementary Table S2).

The double AR mutant is targetable by other AR antagonists

We explored whether the double-mutant AR would be resistant to other antagonists unrelated to enzalutamide or ARN. For this, we used the well-known AR axis inhibitors abiraterone acetate (primarily discovered as CYP17 inhibitors, however, it can also directly inhibit the AR; refs. 36, 37), galaterone, Epi (this compound targets the NTD of the AR; ref. 38), AZD, and ASC, as well as some experimental ARAN compounds which we described earlier for their full antagonistic activity on AR W742C and AR T878A (33). All of the tested compounds exhibited no agonistic and
strong to moderate antagonistic activity on the AR WT, AR T878A, AR F877L, and AR F877L/T878A (Fig. 6 and Supplementary Fig. S14) when tested on an androgen-responsive luciferase reporter. The only exception being ARAN-4, which slightly activated the double-mutant AR, although not to the same extent as enzalutamide (Supplementary Fig. S14).

Proliferation studies in LAPC4 cells (Supplementary Methods) also show that cells transfected with the double-mutant AR were rescued from the growth-suppressing effect of enzalutamide when compared with WT AR–transfected cells (Supplementary Fig. S15A). Furthermore, the same rescue was observed for T878A-transfected cells treated with hydroxyflutamide (Supplementary Fig. S15B). Abiraterone acetate, however, had a similar effect on proliferation of all the cells regardless of the receptor transfected (Supplementary Fig. S15C).

Discussion
It is well documented that mutations in the AR can lead to clinical resistance against antiandrogens, and can even turn the latter into agonists. The AR T878A mutation of the AR for instance converts hydroxyflutamide to an agonist in both preclinical and clinical settings (8, 30). Recently, it has been proposed that the AR F877L mutation leads to enzalutamide resistance, as it might convert enzalutamide into an agonist (9, 10, 12, 39). Here, we compared the effect of these mutations and their combination on the biochemical properties of the AR.

Transactivation properties
Enzalutamide is only a weak partial agonist for the AR F877L mutation, while when this mutation co-occurs with T878A, there is a dramatic increase in agonistic effect of enzalutamide. A similar observation was made for the combination of L701H and T878A mutations, which resulted in greater responsiveness to cortisol compared with the single L701H mutant (40). Possibly, in both cases, the T878A mutation leads to a stabilization of the agonistic conformation of the receptor. This might be attained by the reduction in steric hindrance (discussed below) or by repositioning of helix (H) 11 and H12 towards the ligand-binding pocket, facilitating the transition into the agonistic mode of the AR as argued in refs. 30, 41.

In silico modeling
The antagonists that are currently used in the clinic all act via the ligand-binding pocket, preventing the correct realignment of H12 which is recognized by coactivators. However, the details of their mechanisms of action are different (reviewed in refs. 41, 42). It has been proposed that bicalutamide binds an additional binding pocket (B-site) in the proximity of the androgen-binding pocket.
In contrast, for enzalutamide, it has been suggested that when it binds to the AR it contacts residues on H11, thus disrupting H11–H12 interactions which prevents H12 from adopting the correct agonistic alignment with the LBD (9). Our in silico model indicates that the F877L mutation expands the ligand-binding pocket, allowing for enzalutamide to fit inside and interact with R753 like the agonists DHT or T. Similarly, the molecular dynamics simulation by Balbas and colleagues proposed that F877L allows repositioning of enzalutamide from an antagonistic binding mode in such a way that steric clashes resulting in dislocation of H12 in WT AR are eliminated (9). The modeling further proposes that the additional exchange of threonine at site 878 to alanine leads to extra reduction of steric hindrance for enzalutamide. This is corroborated by crystallographic data of the bicalutamide-bound T878A mutant (34). Furthermore, according to our modeling, the F877L mutation leads to a reduced hydrophobic contact and putative solvent exposure for DHT. This might be the explanation why the mutant AR is less responsive to DHT.

Our modeling may provide some insight valuable for design of next generation compounds. Putative future antiandrogens could for instance harbor a group incompatible with the F877L due to steric hindrance (rigid bulky modifications) or polar incompatibility with the hydrophobic contact surface of either the phenylalanine or the mutant leucine.

**Ligand binding**

In concordance with the computational modeling, we observed a decrease in affinity of DHT for the receptors containing the F877L mutation, while the affinity of enzalutamide was increased. Although co-occurrence of F877L and T878A led to a strong increase in enzalutamide agonism, only a modest further increase in affinity for the double-mutant AR was observed. Therefore, we propose that F877L is crucial for the increase in affinity of enzalutamide and ARN, while the co-occurrence with T878A (through mechanisms discussed above) is pivotal in strongly enhancing the agonistic activity of enzalutamide.

**N/C interactions and binding to coregulator peptides**

When the AR is activated by agonists it undergoes a conformational change that leads to close positioning of the NTD and LBD and thus interaction between these two domains (27, 43). The enzalutamide-bound AR WT or F877L did not show any N/C interactions. However, strong interaction was observed for the double-mutant AR bound by enzalutamide, or any mutant or WT receptor stimulated by DHT. As we detected transactivation and chromatin binding (discussed below) for the enzalutamide-bound F877L receptor, it might be that the absence of N/C interaction poises the F877L receptor only for specific promoters, as it was observed that deletions/mutations of FQNLF selectively diminish the activity of the AR at specific promoter elements (44).

To investigate the effect of DHT and antiandrogens on AR–coregulator interactions, we employed the MARCoNI assay (24). The modulation of interactions by DHT was similar, but clearly distinct from the one recorded for the ER stimulated with estradiol (45). As expected, we observed strong interactions of the agonist-bound F877L receptor, it might be that the absence of N/C interaction poises the F877L receptor only for specific promoters, as it was observed that deletions/mutations of FQNLF selectively diminish the activity of the AR at specific promoter elements (44).

The modulation of interactions by DHT was similar, but clearly distinct from the one recorded for the ER stimulated with estradiol (45). As expected, we observed strong interactions of the agonist-bound F877L receptor, it might be that the absence of N/C interaction poises the F877L receptor only for specific promoters, as it was observed that deletions/mutations of FQNLF selectively diminish the activity of the AR at specific promoter elements (44).

To investigate the effect of DHT and antiandrogens on AR–coregulator interactions, we employed the MARCoNI assay (24). The modulation of interactions by DHT was similar, but clearly distinct from the one recorded for the ER stimulated with estradiol (45). As expected, we observed strong interactions of the agonist-bound F877L receptor, it might be that the absence of N/C interaction poises the F877L receptor only for specific promoters, as it was observed that deletions/mutations of FQNLF selectively diminish the activity of the AR at specific promoter elements (44).

To investigate the effect of DHT and antiandrogens on AR–coregulator interactions, we employed the MARCoNI assay (24). The modulation of interactions by DHT was similar, but clearly distinct from the one recorded for the ER stimulated with estradiol (45). As expected, we observed strong interactions of the agonist-bound F877L receptor, it might be that the absence of N/C interaction poises the F877L receptor only for specific promoters, as it was observed that deletions/mutations of FQNLF selectively diminish the activity of the AR at specific promoter elements (44).

To investigate the effect of DHT and antiandrogens on AR–coregulator interactions, we employed the MARCoNI assay (24). The modulation of interactions by DHT was similar, but clearly distinct from the one recorded for the ER stimulated with estradiol (45). As expected, we observed strong interactions of the agonist-bound F877L receptor, it might be that the absence of N/C interaction poises the F877L receptor only for specific promoters, as it was observed that deletions/mutations of FQNLF selectively diminish the activity of the AR at specific promoter elements (44).
single mutant receptors by enzalutamide cluster more closely to the antagonistic enzalutamide-treated WT AR. In contrast, the enzalutamide stimulated double-mutant AR clusters together with the agonistic hydroxyflutamide-bound WT and double-mutant AR, further supporting the fact that enzalutamide acts as a potent partial agonist of the double-mutant AR. Clearly, all these data validate this assay for the simultaneous comparison of relative effects of agonists and antagonists on the in vitro binding to a series of coregulator-derived peptides. In conclusion, the MARCoNI data show that ligands with different activity can be separated according to their modulation of AR interactions with coregulator peptides into full agonists, partial agonists, and antagonists.

**Ligand-induced intranuclear behavior of mutant receptors**

It is believed that the immobile fraction of AR measured in FRAP assays corresponds to the chromatin-bound receptor during the transcription activation process, because of the overlap of AR locations with the sites of RNA synthesis (17). There is also a strong correlation between AR activity and the immobile fraction, as observed for AR WT and AR T878A stimulated with agonists and antagonists (43). Antagonist-bound ARs behave differently, having smaller immobile fractions, resembling that of DNA-binding defective receptors (26).

Using FRAP, we observed that while the AR F877L mutant had a smaller enzalutamide-induced immobile fraction, the chromatin-bound fraction of enzalutamide-stimulated double-mutant AR was similar to the fraction of the DHT-bound WT AR.

**Inhibiting the F877L and T878A mutant ARs**

Both enzalutamide and ARN share a 4-cyano-3-(trifluoromethyl)phenyl group with Bic and hydroxyflutamide required for specific binding to the AR. Because of the structural similarities between enzalutamide and ARN, the F877L mutation is likely to be enzalutamide/ARN structure-specific. We were able to show that structurally diverse AR inhibitors (abiraterone acetate, galasterone, and Epi) can completely abolish the DHT-activation of the F877L and double-mutant AR (38, 46, 47). Furthermore, we have shown that abiraterone acetate effectively inhibited proliferation of cells harboring transfected double-mutant AR, while presence

**Figure 6.**
The AR double-mutant is targetable by other AR antagonists. HEK293T cells were used to compare the activity of several antiandrogens on transactivation of AR. Transcriptional activity of none of the AR mutants was induced by abiraterone acetate (Abi), galasterone (Gal), Epi, AZD, or ASC. Moreover, these compounds successfully reduced the DHT-induced transactivation of AR for both the WT and the mutant receptors. The mean and SEM of three independent experiments are shown. AR WT (A), AR T878A (B), F877L (C), and AR F877L/T878A (D).
of the double mutant led to sustained survival under enzalutamide treatment. These results illustrate that targeting AR with compounds that bind differently to the LBD or with compounds that target other domains rather than the LBD, might be favorable and potential candidates for future treatment of enzalutamide-resistant patients harboring F877L mutation.

Clinical implications
Mutations such as T878A have been found recurrently in mCRPC patients, in hydroxyflutamide-treated patients, but also in patients that were never treated with hydroxyflutamide (14, 48). Moreover, the antiandrogen withdrawal syndrome, which is known to occur in patients after cessation of antiandrogens such as hydroxyflutamide, bicalutamide, or nilutamide treatment is known to be partly explained by the occurrence of mutations in the AR that convert the antagonists into agonists (49). Similar mechanisms have been anticipated for enzalutamide.

For now, only a limited number of genomic studies on post-enzalutamide patients have been reported. However, none of these identified the single F877L mutation in this population. Only one patient was found to have the F877L mutation, but it was co-occurring in the same allele with the T878A mutation (14, 39). In addition, withdrawal syndrome seems to be either extremely rare or nonexistent after enzalutamide progression (50, 51). All this suggests that in the clinic, the frequency of point mutations that convert enzalutamide to an agonist is likely low. Possibly, other events like AR overexpression or activation of other signaling pathways are more relevant (14, 48).

Conclusion
In conclusion, the molecular modeling and ligand-binding data, the transactivation data, the N/C interactions, the MAR-CONIdata, and the FRAP data all converge to the same hypothesis: the F877L mutation enhances enzalutamide binding, but has a limited effect on the activation potential of this antagonist. Only when F877L is combined with T878A, the receptor becomes strongly activated by enzalutamide. In addition, these mutant ARs can be targeted with antiandrogens such as abiraterone acetate or galaterone, opening the possibility for mutant ARs can be targeted with antiandrogens such as abiraterone acetate or galaterone, opening the possibility for

agonist and antagonist activity of enzalutamide, hydroxyflutamide, bicalutamide, and experimental BF3 binding antagonists on a series of AR single or combined mutations which they found in castration-resistant prostate cancer (39). The data presented here focus on the effect of the F877L/T878A mutation on the (ant)-agonist binding, the intramolecular N/C interactions, the coregulator interactions and the intranuclear mobility. Together with Lallou and colleagues, we conclude a full molecular characterization of AR mutations that occur in CRPC could provide recommendations for future personalized therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Prekovic, R. Houtman, F. Claessens, C. Helsen Development of methodology: M.E. van Royen, D. Melchers, A.B. Houtsmuller, F. Claessens, C. Helsen Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Prekovic, M.E. van Royen, R. Houtman, D. Melchers, T. Van den Broeck, E. Smeets, S. Joniau, F. Claessens, C. Helsen Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Prekovic, M.E. van Royen, A.R.D. Voet, R. Houtman, K.Y.J. Zhang, F. Claessens Writing, review, and/or revision of the manuscript: S. Prekovic, M.E. van Royen, A.R.D. Voet, R. Houtman, K.Y.J. Zhang, T. Van den Broeck, E. Smeets, S. Joniau, F. Claessens, C. Helsen Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Geverts Study supervision: S. Joniau, F. Claessens, C. Helsen

Acknowledgments
The authors thank H. Deheyns, R. Bollen, and D. Schollaert for their excellent technical assistance and the colleagues from the Molecular Endocrinology Laboratory for the helpful discussions.

Grant Support
This work supported by research grants from the FWO-Vlaanderen (G.0830.13N), KU Leuven (GOA/15/017), and Kom op tegen Kanker. F. Claessens is the principal investigator for all the grants previously mentioned. A.R.D. Voet was supported by RIKEN FPR fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 3, 2015; revised April 18, 2016; accepted April 26, 2016; published OnlineFirst May 16, 2016.

References
3. Ha Y, Goodin S, DiPaola R, Kim I. Enzalutamide for the treatment of mCRPC patients, in hydroxyflutamide-treated patients, but also in patients that were never treated with hydroxyflutamide (14, 48). Moreover, the antiandrogen withdrawal syndrome, which is known to occur in patients after cessation of antiandrogens such as hydroxyflutamide, bicalutamide, or nilutamide treatment is known to be partly explained by the occurrence of mutations in the AR that convert the antagonists into agonists (49). Similar mechanisms have been anticipated for enzalutamide.


The Effect of F877L and T878A Mutations on Androgen Receptor Response to Enzalutamide

Stefan Prekovic, Martin E. van Royen, Arnout R.D. Voet, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0892

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2016/05/14/1535-7163.MCT-15-0892.DC1

Cited articles
This article cites 50 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/15/7/1702.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/15/7/1702.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/15/7/1702.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.