Priority Report

Cancer Research

Interactions of Abiraterone, Eplerenone, and Prednisolone with Wild-type and Mutant Androgen Receptor: A Rationale for Increasing Abiraterone Exposure or Combining with MDV3100

Juliet Richards[1], Ai Chiin Lim[1], Colin W. Hay[3], Angela E. Taylor[4], Anna Wingate[1], Karolina Nowakowska[1], Carmel Pezaro[1,2], Suzanne Carreira[1], Jane Goodall[1], Wiebke Arlt[4], Iain J. McEwan[3], Johann S. de Bono[1,2], and Gerhardt Attard[1,2]

Abstract

Prostate cancer progression can be associated with androgen receptor (AR) mutations acquired following treatment with castration and/or an antiandrogen. Abiraterone, a rationally designed inhibitor of CYP17A1 recently approved for the treatment of docetaxel-treated castration-resistant prostate cancer (CRPC), is often effective, but requires coadministration with glucocorticoids to curtail side effects. Here, we hypothesized that progressive disease on abiraterone may occur secondary to glucocorticoid-induced activation of mutated AR. We found that prednisolone plasma levels in patients with CRPC were sufficiently high to activate mutant AR. Mineralocorticoid receptor antagonists, such as spironolactone and eplerenone that are used to treat side effects related to mineralocorticoid excess, can also bind to and activate signaling through wild-type or mutant AR. Abiraterone inhibited *in vitro* proliferation and AR-regulated gene expression of AR-positive prostate cancer cells, which could be explained by AR antagonism in addition to inhibition of steroidogenesis. In fact, activation of mutant AR by eplerenone was inhibited by MDV3100, bicalutamide, or greater concentrations of abiraterone. Therefore, an increase in abiraterone exposure could reverse resistance secondary to activation of AR by residual ligands or coadministered drugs. Together, our findings provide a strong rationale for clinical evaluation of combined CYP17A1 inhibition and AR antagonism. *Cancer Res; 72(9); 2176–82. ©2012 AACR.*

Introduction

The small-molecule CYP17A1 inhibitor, abiraterone acetate (Zytiga, Janssen), was recently approved for the treatment of men with castration-resistant prostate cancer (CRPC) progressing after docetaxel chemotherapy. Despite a significant survival advantage with 1,000 mg abiraterone daily and objective tumor responses in up to 60% of patients with CRPC, progressive disease on treatment invariably develops (1, 2). MDV3100 is a novel antiandrogen (3, 4) that has also recently been reported to confer a survival advantage in patients with CRPC progressing after docetaxel (5). As prostate-specific antigen (PSA) level often increases at progression on both these agents, we have hypothesized that resistance occurs secondary to reactivation of androgen receptor (AR) signaling. Inhibition of CYP17A1 results in significant suppression of androgens and estrogens but also of cortisol that is associated with a compensatory increase in adrenocorticotropic hormone level (2). Abiraterone acetate has therefore been developed in combination with exogenous glucocorticoids. However, up to 40% of patients on prednisone/prednisolone alone and 55% of patients on abiraterone acetate and prednisone/prednisolone develop a syndrome of secondary mineralocorticoid excess characterized by hypokalemia, hypertension, and fluid overload that can be controlled by increasing the dose of prednisone or adding a mineralocorticoid receptor antagonist (MRA) such as eplerenone (1). Eplerenone is currently recommended in preference to spironolactone as previous studies showed that eplerenone did not bind and activate wild-type (WT)-AR (2, 6). However, as eplerenone is not invariably available, spironolactone is also being used.

Point mutations of the AR, which appear to cluster in the ligand-binding domain, are rare in therapy naive patients but occur in 15% to 45% of castration-resistant disease and can increase AR affinity for a wide range of steroids (7, 8). Over 100

Authors' Affiliations: [1]Section of Medicine, The Institute of Cancer Research; [2]The Royal Marsden NHS Foundation Trust, Sutton, Surrey; [3]School of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen; and [4]Centre for Endocrinology, Diabetes and Metabolism, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, United Kingdom

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J.S. de Bono and G. Attard are joint senior authors.

Corresponding Author: Gerhardt Attard, Section of Medicine, The Institute of Cancer Research and the Royal Marsden NHS Foundation Trust, Downs Road, Sutton, Surrey SM2 5PT, United Kingdom. Phone: 0044-7793077493; Fax: 0044-2086427979; E-mail: Gerhardt.attard@icr.ac.uk

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mutations have been described and many have been shown to give a functional advantage to maintain AR signaling. We hypothesized that progressive disease on abiraterone acetate could occur secondary to activation of mutated "promiscuous" AR by steroidal agents administered to patients to prevent or treat side effects of mineralocorticoid excess.

Materials and Methods

Materials

FBS and charcoal-stripped serum (CSS) were purchased from Gibco. Bicalutamide, dexamethasone, prednisone, and dihydrotestosterone (DHT; Sigma-Aldrich), titrated [^{3}H]-R1881 (Perkin-Elmer), R1881 (Steraloids), eplerenone and spironolactone (Tocris-Bioscience) were obtained from commercial sources. Abiraterone and MDV3100 were synthesized using the publicly available chemical structures and checked by mass spectrometry. Drugs were dissolved in dimethyl sulfoxide (DMSO) and then diluted to a maximum DMSO concentration of 0.2%. LNCaP, VCaP, PC-3, DU145, and COS-7 cells were obtained from American Type Culture Collection (ATCC; LGC Standards), grown according to ATCC recommendations, used less than 6 months from receipt and freeze down and confirmed mycoplasma free.

Luciferase reporter assays

We constructed a PSA-ARE3-luc luciferase reporter plasmid that was cotransfected with a human AR expression plasmid, F527-AR [wild-type or mutant as stated; mutations confirmed by sequencing (Beckman Coulter Genomics)] into PC-3 cells. These were seeded in white opaque 384-well plates and grown in 10% CSS-supplemented phenol red–free RPMI-1640 for 30 hours. Cells were then treated with the indicated concentration of compound and R1881 for 16 hours. Luciferase activity was determined by adding ONE Glo (Promega) and measuring luminescence on a TopCount plate reader (Perkin-Elmer). Transfection efficiency and protein expression are shown in Supplementary Fig. S1.

Cell viability

LNCaP and VCaP cells were seeded in 96-well plates and grown in CSS-supplemented phenol red–free or FBS-supplemented media for 7 days. Cells were treated with compound at 24 and 96 hours after plating and cell viability was determined on day 7 by adding CellTiter Glo (Promega) and measuring luminescence.

Ligand-binding assay

PC-3 cells transfected with wild-type or T877A mutant AR or LNCaP cells were seeded in 24-well plates and grown in CSS-supplemented phenol red–free media for 24 hours. To determine the kinetics of [^{3}H]-R1881 binding to the wild-type and T877A AR, cells were treated with 0.25 to 25 nmol/L [^{3}H]-R1881 for 2 hours, then washed, lysed, and radioactivity was measured (1900CA analyzer, Perkin-Elmer). The K_d and B_{max} were determined by nonlinear regression using GraphPad Prism software. When the concentration of [^{3}H]-R1881 required to almost saturate AR in both wild-type and T877A AR mutant transfections was established (5 nmol/l), displacement of [^{3}H]-R1881 by test compound was determined. The concentration at which 50% of [^{3}H]-R1881 was displaced (EC_{50}) was established using nonlinear regression (GraphPad Prism).

Quantitative real time-PCR

LNCaP and VCaP cells were seeded in 6-well plates and grown in CSS-supplemented phenol red–free media for 24 hours and then treated for 5 hours as indicated. Following RNA extraction and cDNA synthesis, quantitative PCR (qPCR) was carried out on the Mx3000P QPCR System (Agilent) using the RT2 SYBR Green ROX qPCR Mastermix (SABiosciences). Every sample was run in duplicate and each reaction contained 50 ng of cDNA in a total volume of 20 μL. ΔC_t for each gene was determined after normalization to actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and $\Delta\Delta C_t$ was calculated relative to the designated reference sample. Gene expression values were set equal to $2^{-\Delta\Delta C_t}$(Applied Biosystems). Primers were purchased from SABiosciences.

Measurement of plasma prednisolone

Plasma was collected from patients with CRPC after 48 days of continuous daily abiraterone acetate and prednisolone. All patients provided written, informed consent to blood withdrawal for research purposes, and this study was approved by the Royal Marsden Hospital ethics review committees. Prednisolone was quantified by comparison to a calibration series ranging from 5 to 500 ng/mL prepared in 50:50 methanol:water. A Waters Xevo mass spectrometer with Acquity uPLC system was used, fitted with a HSS T3, 1.8 μm, 1.2 × 50 mm^2 column (Waters). The column temperature was maintained at 60°C and the settings used were an electrospray source in positive ionization mode; capillary voltage 4.0 kV; source temperature, 150°C; and desolvation temperature, 500°C.

Results

The selective mineralocorticoid receptor antagonist, eplerenone, activates mutant AR

We first cotransfected PC-3 AR-negative prostate cancer cells with PSA-ARE2-luc and either wild-type (WT)-AR or 3 mutations previously described in CRPC (T877A-AR, D879G-AR, and W741C-AR). The T877A mutation has been identified in several studies in patients treated with flutamide (8, 9) and has been extensively studied as it is found in the LNCaP prostate cancer cell line (Supplementary Table S1). D879G and W741C mutations have been identified in patients previously treated with bicalutamide (8, 9). We then compared activation of wild-type or mutant AR by synthetic androgen (R1881) to activation by the MRAs, eplerenone, and spironolactone. In keeping with previous reports, spironolactone activates WT-AR (7) and also T877A-AR, D879G-AR, and W741C-AR only 2-log less potently than R1881 does (Fig. 1A and B and Supplementary Fig. S2). Eplerenone does not activate WT-AR, D879G-AR, or W741C-AR but importantly can activate T877A-AR with a dose-proportional response and an EC_{50} value of 5.2 μmol/L [95% confidence interval (CI),

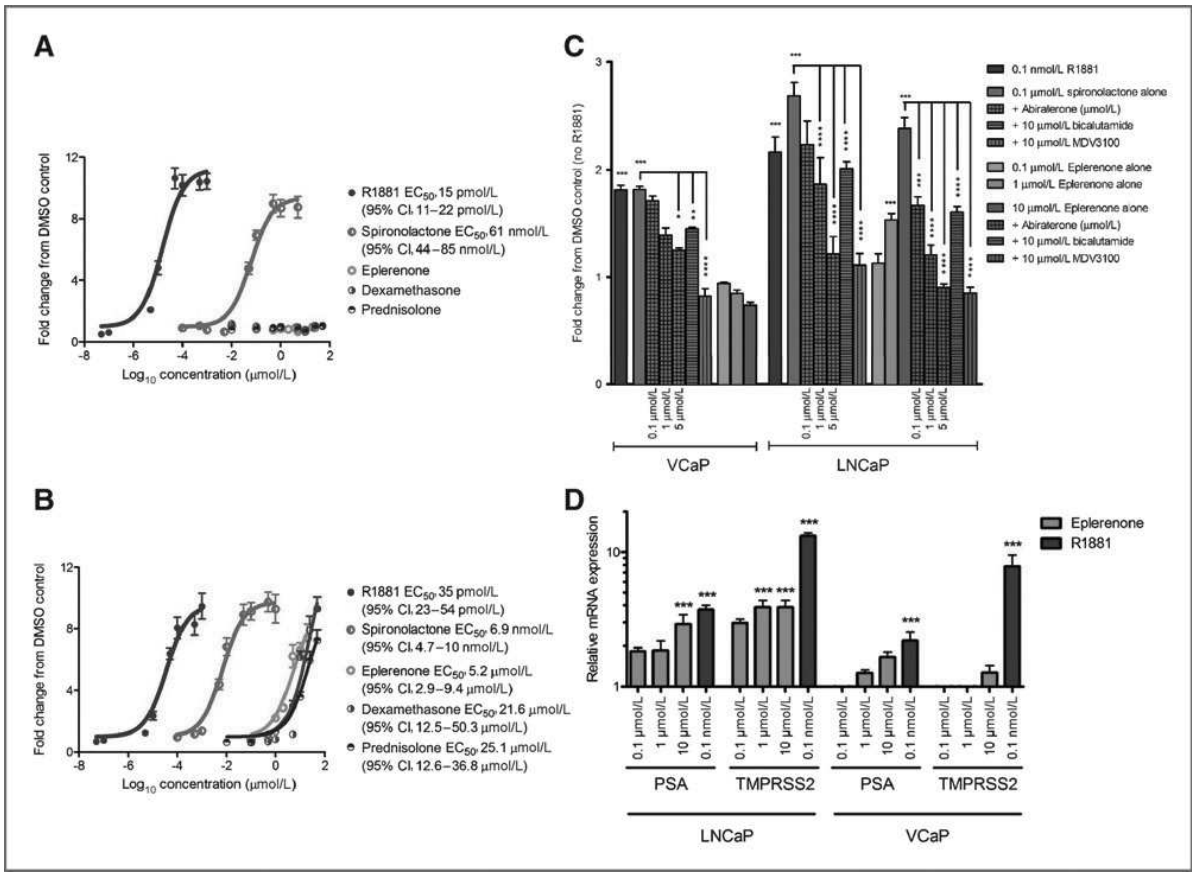

Figure 1. Eplerenone activates T877A-AR and spironolactone activates both T877A-AR and wild-type (WT)-AR. Sigmoidal dose–response curves show activation of WT-AR by R1881 and spironolactone (A) and T877A-AR by R1881, spironolactone, eplerenone, prednisolone, dexamethasone. (B) Fold change from the DMSO control was plotted and EC_{50} values calculated using nonlinear regression (GraphPad). EC_{50} values and 95% CIs are given. C, LNCaP and VCaP prostate cancer cells in CSS were treated with eplerenone or spironolactone alone or in combination with 0.1, 1, or 5 μmol/L abiraterone, 10 μmol/L bicalutamide, or 10 μmol/L MDV3100 for 7 days and then analyzed for cell viability. Fold change from the DMSO control was then calculated and plotted. Significance is shown for stimulation by eplerenone or spironolactone compared with DMSO control (*, horizontal) and for inhibition by bicalutamide, MDV3100, or abiraterone when compared with stimulated levels (*, vertical). D, LNCaP and VCaP cells were treated with 0.1 nmol/L R1881 or 0.1 to 10 μmol/L eplerenone for 5 hours. RNA was extracted and cDNA synthesized for analysis by qPCR to determine relative levels of PSA and TMPRSS2 mRNA expression. Significance compared with DMSO controls is shown. Data shown for all experiments are the mean (error bars, SEM) of 3 independent experiments of 16 replicates (A and B) or in duplicate (C and D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, one-way ANOVA with the Bonferroni correction.

2.89–9.37 μmol/L; Fig. 1A and B and Supplementary Fig. S2). Pharmacokinetic studies with eplerenone report a C_{max} of 1.72 ± 0.28 μg/mL (equivalent to 4.2 ± 0.7 μmol/L) and a half-life of 3 hours with 100 mg eplerenone (6); doses of eplerenone between 50 and 200 mg are used to treat toxicities secondary to mineralocorticoid excess from abiraterone in patients with CRPC (Supplementary Table S2). We proceeded to confirm that both spironolactone and eplerenone (1 and 10 μmol/L) increased proliferation of hormone-stripped LNCaP (T877A-AR) but only spironolactone increased the proliferation of VCaP (WT-AR; Fig. 1C). The increase in proliferation was inhibited by AR antagonism, suggesting this effect was secondary to binding to and activation of the AR (Fig. 1C). Similarly, eplerenone significantly increased expression of the androgen-regulated and clinically important genes PSA and TMPRSS2 in LNCaP but not in VCaP (Fig. 1D).

Exogenous glucocorticoids can activate mutant AR at clinically relevant doses observed in CRPC patients treated with abiraterone acetate

Prednisolone or its precursor prednisone are commonly administered in combination with abiraterone acetate although 2 phase II studies combined abiraterone acetate with dexamethasone (2, 10). Prednisone and dexamethasone do not activate WT-AR but activate T877A-AR with EC_{50} values of 25.1 μmol/L (95% CI, 12.64–36.83 μmol/L) and 21.6 μmol/L (95% CI, 12.53–50.26 μmol/L), respectively (Fig. 1A and B). Previous reports have shown that other AR mutations such as T877A in combination with L701H are highly sensitive to glucocorticoids with activation by concentrations as low as 10 nmol/L (11). We therefore proceeded to measure plasma levels of prednisolone in 15 patients with CRPC on continuous daily treatment with 1,000 mg abiraterone acetate and 10 mg prednisolone.

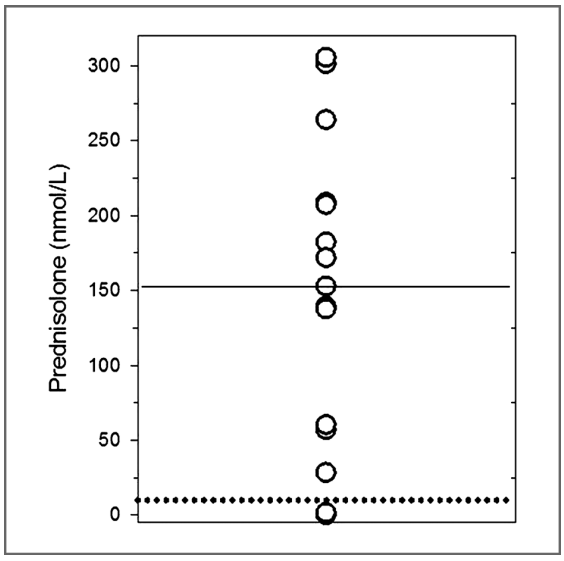

Figure 2. Plasma concentrations (nmol/L) of prednisolone in 15 patients with CRPC treated with abiraterone acetate measured with liquid chromatography/tandem mass spectrometry. The median concentration of 152 nmol/L (SD, 100 nmol/L) is marked by the solid line. The 10 nmol/L limit above which activation of T877A-L701H-AR has been previously reported to occur is shown by the dashed line.

Prednisolone levels were less than 4 nmol/L in 2 patients but more than 30 nmol/L in the other 13 patients. The median concentration was 153 nmol/L (range, <4–305 nmol/L; Fig. 2 and Supplementary Table S2).

Abiraterone binds and inhibits wild-type and mutant AR

Following the observation of activation of T877A-AR by eplerenone, we proceeded to evaluate the effect of abiraterone on wild-type and mutant AR (T877A, D879G, R629Q, W741C, and M749L). We did not observe an increase in reporter luciferase activity with doses of abiraterone up to 25 μmol/L with WT-AR or any mutation tested (Supplementary Fig. S3) but observed dose-proportional inhibition of stimulated wild-type and mutant AR activity (Fig. 3A) with significant inhibition observed at doses $\leq$10 μmol/L. Inhibition was however not as potent as for same concentrations of MDV3100. We then proceeded to confirm our findings by comparing inhibition of AR activation using abiraterone or MDV3100 in a different model system (COS-7 cells cotransfected with AR and a GRE_2-TATA-luc reporter gene and activated by 10 nmol/L DHT for 24 hours). Similarly we observed dose-proportional inhibition of WT-AR, T877A-AR, G142V-AR, P533S-AR, T575A-AR, and H874Y-AR by abiraterone (Fig. 3B). Higher concentrations of abiraterone were required for inhibition of R629Q-AR in this system than was observed in PC-3 cells transfected with an ARE3-luc assay (Fig. 3A). We also confirmed significant inhibition of proliferation of the AR-positive prostate cancer cell lines LNCaP and VCaP with doses of abiraterone $\geq$1 μmol/L (Fig. 3C). No inhibitory effect was observed with the AR-negative prostate cancer cell lines, PC-3, and DU145 (Supplementary Fig. S4). We proceeded to confirm downregulation by qPCR of PSA and TMPRSS2 in LNCaP cells treated with abiraterone (Fig. 3D).

Binding of abiraterone or eplerenone to the AR is confirmed by competitive displacement of [^{3}H]-R1881

To confirm that AR antagonism by abiraterone and agonism by eplerenone (both previously undescribed) occurred secondary to binding to the AR ligand–binding domain, we used a competitive radiolabeled assay to show displacement of R1881 from PC-3 cells transfected with either WT-AR or T877A-AR. The EC_{50} value of eplerenone for WT-AR was 6-fold higher than T877A-AR (EC_{50}, 2.4 μmol/l; 95% CI, 2.0–2.9 μmol/L; Fig. 4A and B). In keeping with the inhibitory activity of abiraterone observed in our reporter luciferase studies, abiraterone displaced ligand from both WT-AR (EC_{50}, 13.4 μmol/L; 95% CI, 10.3–17.4 μmol/L) and T877A (EC_{50}, 7.9 μmol/L; 95% CI, 6.7–9.3 μmol/L; Fig. 4A and B). We also confirmed displacement of radiolabeled R1881 from LNCaP with abiraterone (EC_{50}, 2.6 μmol/L; 95% CI, 1.0–6.8 μmol/L) and eplerenone (EC_{50}, 4.3 μmol/L; 95% CI, 2.4–7.8 μmol/L; Supplementary Fig. S5).

Mutant AR activation by eplerenone can be inhibited by abiraterone or bicalutamide but most effectively by MDV3100

We observed dose-proportional growth inhibition with abiraterone of LNCaP cells stimulated by eplerenone and of LNCaP and VCaP cells stimulated by spironolactone (Fig. 1C). Similar levels of inhibition were observed with bicalutamide, with more profound inhibition by MDV3100 (Fig. 1C). Abiraterone, MDV3100, and bicalutamide achieved similar levels of inhibition of upregulation of PSA by eplerenone but MDV3100 inhibited induction of TMPRSS2 expression more significantly than bicalutamide or abiraterone (Fig. 3D). Similarly, MDV3100 showed more significant inhibition of spironolactone-stimulated PSA and TMPRSS2 expressions than abiraterone or bicalutamide (Supplementary Fig. S6). Also, abiraterone (5 μmol/L) significantly inhibited activation of T877A-AR (in transfected PC-3) by 1 μmol/L eplerenone but not by 10 μmol/L eplerenone; stimulation by 10 μmol/L eplerenone was significantly inhibited by both bicalutamide and MDV3100 (Fig. 4C).

Increased hormone levels reduce AR inhibition by MDV3100

Recent studies have suggested that intratumoral testosterone levels increase in patients treated with MDV3100 (12). We found that $\geq$1μmol/L and $\geq$10 μmol/L MDV3100 significantly inhibited WT-AR luciferase activity stimulated by 0.1 nmol/L R1881 or 1 nmol/L DHT, respectively, but $\geq$50μmol/L MDV3100 was required to significantly inhibit AR stimulated by 1 nmol/L R1881 (Fig. 4D) or 10 nmol/L DHT (Supplementary Fig. S7).

Discussion

Abiraterone was developed as a specific CYP17A1 inhibitor (13). Previous studies have failed to identify binding of

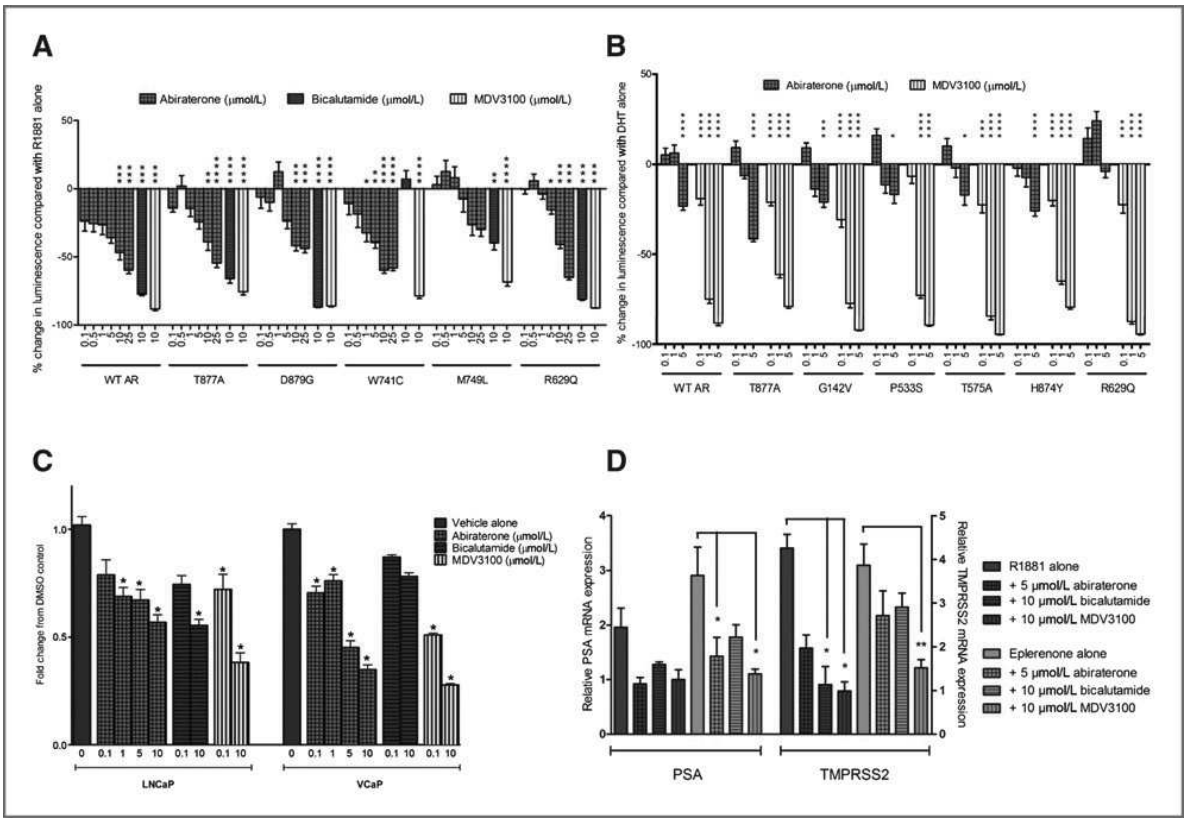

Figure 3. Inhibition of wild-type and mutant stimulated AR activity by abiraterone, bicalutamide, and MDV3100. A, PC-3 cells were cotransfected with ARE3-luc and wild-type or mutant AR (T877A, D879G, W741C, M749L, and R629Q). Cells were treated with 0.1 to 25 μmol/L abiraterone, 10 μmol/L bicalutamide, or 10 μmol/L MDV3100 in CSS medium containing 0.1 nmol/L R1881 for 16 hours and then analyzed for luciferase activity. Fold change from the DMSO control was calculated and then percentage change relative to the R1881-stimulated DMSO control was determined. Data shown are representative of 3 independent experiments and represent mean and SEM of 8 replicates. B, COS-7 cells were cotransfected with GRE_2-TATA-luc and the wild-type or mutant human expression plasmid pSVARo (T877A, G142V, P533S, T575A, H874Y, R629Q). Cells were treated with 0.1 to 5 μmol/L abiraterone or MDV3100 in CSS medium containing 10 nmol/L DHT for 24 hours. The luciferase activities were assayed in duplicate and normalized for the amounts of expressed AR determined immunologically by dot blot analysis and normalized for protein concentration. The change in normalized luciferase activity relative to cells incubated without any compound for each AR variant was determined. Data shown represent 2 or 3 independent experiments carried out in quadruplicate. C, dose-proportional inhibition of proliferation of LNCaP and VCaP cells by abiraterone, MDV3100, and bicalutamide. LNCaP and VCaP prostate cancer cells in FBS were treated with 0.1, 1, 5 or 10 μmol/L abiraterone, 0.1 or 10 μmol/L bicalutamide, or 0.1 or 10 μmol/L MDV3100 for 7 days and then analyzed for cell viability. Fold change from the DMSO control was then calculated and plotted. Data shown are the mean (error bars, SEM) of 3 independent experiments in quadruplicate. D, LNCaP cells were treated with 0.1 nmol/L R1881 or 10 μmol/L eplerenone in combination with DMSO, 10 μmol/L bicalutamide, 10 μmol/L MDV3100, or 5 μmol/L abiraterone for 5 hours. RNA was extracted and cDNA synthesized for analysis by qPCR to determine relative levels of PSA and TMPRSS2 mRNA expression. Data shown are the mean and SEM of 3 independent experiments in duplicate. Significance is shown for *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ relative to DMSO control (one-way ANOVA with the Bonferroni correction).

abiraterone to the AR (14). However, in this study we used both reporter luciferase and competitive radiolabeled assays to show that abiraterone binds and inhibits WT-AR. Another study published while our article was under review reported supporting evidence that abiraterone binds the AR and produces a dose-dependent decrease in AR levels (15). This study failed to identify the EC_{50} value with wild-type or mutant AR but predicted it as over $\geq$3 μmol/L. We also tested 8 AR mutations selected from a screen of 42 mutations for causing a differential response to various hormones. We included mutations in the amino terminal (G142V, P533S), DNA-binding (T575A), and ligand-binding (W741C, M749L, T877A, D879G, and H874Y) domains and the hinge region (R629Q; Supplementary Table S1). As previously described, bicalutamide activated W741C (4, 16) but no agonistic activity was observed with any mutation and abiraterone. Similarly MDV3100 potently inhibited WT-AR and all mutant AR tested. However, these mutations were mostly identified in patients progressing on bicalutamide or flutamide and different, new mutations may develop in patients progressing on abiraterone or MDV3100.

Abiraterone is an active treatment for CRPC due to CYP17A1 inhibition and significant suppression of hormones (2). However, we observed up to 32% AR inhibition with 1 μmol/L abiraterone, with significantly greater inhibition at 5 and 10 μmol/L. Pharmacokinetic studies have reported maximum

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