An F876L Mutation in Androgen Receptor Confers Genetic and Phenotypic Resistance to MDV3100 (Enzalutamide)

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ABSTRACT

Castration resistant prostate cancer (CRPC) is the most aggressive, incurable form of prostate cancer. MDV3100 (enzalutamide), an antagonist of the androgen receptor (AR), was approved for clinical use in men with metastatic CRPC. Although this compound showed clinical efficacy, many initial responders later developed resistance. To uncover relevant resistant mechanisms, we developed a model of spontaneous resistance to MDV3100 in LNCaP prostate cancer cells. Detailed characterization revealed that emergence of F876L mutation in AR correlated with blunted AR response to MDV3100 and sustained proliferation during treatment. Functional studies confirmed that AR-F876L confers an antagonist-to-agonist switch that drives phenotypic resistance. Lastly, treatment with distinct anti-androgens or CDK4/6 inhibitors effectively antagonized AR-F876L function. Together, these findings suggest that emergence of F876L may 1) serve as a novel biomarker for prediction of drug sensitivity, 2) predict a “withdrawal” response to MDV3100, and 3) be suitably targeted with other anti-androgens or CDK4/6 inhibitors.
SIGNIFICANCE: We uncovered a F876L agonist switch mutation in AR that confers genetic and phenotypic resistance to the novel anti-androgen drug MDV3100. Based on this finding, we propose new therapeutic strategies to treat prostate cancer patients presenting this AR mutation.
INTRODUCTION

Prostate cancer (PCa) is one of the most commonly diagnosed cancers among men worldwide (1, 2). Although localized tumors are often successfully treated, distant metastases emerge in a significant fraction of patients (3, 4). Androgen-deprivation therapy is initially effective; however, almost invariably resistance emerges and results in a much more aggressive form of tumor referred to as castration-resistant prostate cancer (CRPC). CRPC is the second most common cause of cancer-related deaths in American men (5) and is currently incurable. A conserved feature of CRPC is the sustained activity of AR-signaling (6), by virtue of mechanisms including AR gene overexpression/amplification and AR gene mutations (3). These data have suggested that PCa may largely remain dependent on AR signaling.

The continued reliance on AR signaling in CRPC has led to the development of CYP17 inhibitors (abiraterone) and improved antagonists that compete with androgens for binding to the ligand-binding pocket of AR. MDV3100 (enzalutamide) is a novel anti-androgen that was recently approved by the FDA for treatment of men with metastatic CRPC previously treated with docetaxel. Although MDV3100 has shown significant efficacy in clinical trials, many who initially responded favorably develop resistance to this second generation anti-androgen (7). The molecular mechanisms driving resistance, however, is currently unclear, and a deeper understanding is critical for the rational development of alternate therapeutics.

*In vitro* and *in vivo* experimental models of resistance serve as useful tools for expeditious discovery of mechanisms that allow drug escape, and in the evaluation of alternate therapies. We here present the derivation of a model of spontaneous
resistance in LNCaP cells, which led to the identification of a novel F876L mutation in AR that presents a potent driver of genetic/phenotypic resistance to MDV3100. This mutation allows AR-F876L to use MDV3100 as an agonist and consistent with this, F876L-bearing cells required the presence of MDV3100 for growth in vivo. These data continue to support the notion that despite improved AR antagonists, PCa cells remain dependent on AR signaling and hence evolve specific mutations to overcome anti-androgen therapies. Since we further demonstrate that the F876L variant retains sensitivity to bicalutamide, combination therapy with structurally distinct anti-androgens either in parallel or in series together with androgen deprivation may provide an appealing therapeutic strategy for combating AR resistance mechanisms in the clinic. Furthermore, in addition to targeting the hypermutable AR ligand-binding domain, we show that targeting downstream or interactive effectors of AR signaling such as CDK4, provides an alternative strategy for overcoming resistance mechanisms when AR-directed therapies become ineffective.

RESULTS

Development and Characterization of a Model of Spontaneous Resistance to MDV3100

To facilitate the discovery of resistance mechanisms, we generated a model of spontaneous resistance in LNCaP cells. Although short-term culture of LNCaP cells with MDV3100 (<1.5 months) induced population-wide stasis (Fig. 1A, FCS+MDV3100 relative to FCS), long-term culture with 1 μM MDV3100 led to the emergence of resistant clones (Fig. 1B). After isolation and expansion, resistant clones that displayed
similar growth properties as the control populations (C1-C3, Fig. 1B) were chosen for further characterization. Short-term (Fig. 1C) and long-term (Supplementary Fig. S1) proliferation assays confirmed the resistance phenotype. Specifically, four of seven resistant clones (#1, 15, 19, 24- strongly resistant, green curves and font) displayed significantly higher resistance to MDV3100 than weakly resistant clones (#9, 10, 14, red curves and font) or the control lines (C1-C3, brown curves and font) (Fig. 1C). In contrast, strongly resistant clones displayed sensitivity to bicalutamide (Fig. 1C), cumulatively suggesting that the mechanism(s) of resistance in strongly resistant clones may be fine-tuned to specifically promote resistance to MDV3100.

To elucidate mechanism(s) of resistance, we first analyzed expression levels of AR, as its overexpression has previously been shown to promote resistance to castrate levels of androgens (6), and anti-androgen bicalutamide (1). Here, we did not observe a significant change in AR expression (Fig. 1D), and global gene expression analysis of AR pathway activity showed a lack of significant modulation of the AR pathway at baseline (Fig. 1E, beige bars).

**Strongly Resistant Clones are Resilient to AR Pathway Inhibition by MDV3100**

To further explore the molecular mechanisms that drive resistance, we performed gene-expression microarray analysis on RNA derived from three controls and seven resistant clones treated with DMSO or MDV3100 for 24 h. Unbiased hierarchical clustering of the transcriptional effects in response to MDV3100 treatment revealed that the strongly resistant clones clearly segregated from the weakly resistant and control lines (Fig. 2A, left), supporting the phenotypic differences described earlier (Fig. 1C).
Further interrogation of the data revealed four distinct gene expression patterns (labeled I-IV). Although pathway enrichment analysis (8) failed to reveal a significant enrichment of pathway signatures in classes I, II and IV, a strong enrichment of genes involved in AR pathway activation was observed in class III (Fig. 2A, middle and right, and Supplementary Table S1). These data suggested that strongly resistant cell lines could at least maintain AR pathway activity despite continuous exposure to MDV3100. In keeping with this notion, clustering (Supplementary Fig. S2A), pathway enrichment analysis (Fig. 2B and Supplementary Fig. S2B) and qPCR analysis of three AR target genes (Fig. 2C) confirmed that MDV3100 treatment down-regulated AR pathway activity in control and weakly resistant lines but not in the strongly resistant clones.

The mechanism by which MDV3100 inhibits AR signaling has been attributed partially to its ability to hinder nuclear translocation of AR (9). We therefore determined whether localization of AR was altered in the resistant clones in response to MDV3100. To this end, cytoplasmic and nuclear protein fractions from a control (C1) and strongly resistant (clone #1) line treated with DMSO or MDV3100 were isolated. In contrast to C1, where MDV3100 significantly reduced nuclear AR localization (nuclear:cytoplasmic ratio: DMSO=0.46, MDV=0.07), MDV3100 treatment did not significantly alter AR nuclear localization in the strongly resistant clone (nuclear:cytoplasmic ratio: DMSO=0.59, MDV=0.49) (Fig. 2D and Supplementary Fig. S3), supporting the notion that AR remains activated in the strongly resistant cell lines.

Whole-Transcriptome Sequencing Uncovers a Novel AR mutation Correlated with Resistance
In order to define the mechanism(s) underlying resistance to MDV3100, we next performed whole-transcriptome sequencing on nine of ten lines described above. Expressed protein-coding sequence variants were identified and compared to sequences in parental control lines. Somatic mutations found in more than one clone, but with discordant genotypes across clones, were used to cluster the samples using hierarchical clustering (Fig. 3A). While there were many recurrent mutations, only one candidate, a recurrent F876L mutation in the AR gene, was conserved among all four strongly resistant clones and not found in weakly resistant or control lines (Fig. 3A and Supplementary Table S2). Interestingly, clone #19 presented a very distinct mutational spectrum compared to other strongly resistant clones likely suggesting that AR-F876L occurred by independent mechanisms in these sub-clones (Fig.3A).

The F876L mutation is localized to the ligand-binding domain of AR and is adjacent to the homozygous T877A mutation in LNCaP cells (Fig. 3B), a mutation previously shown to confer resistance to hydroxyflutamide (10-12). In all cases, the mutation was heterozygous with a mutant allelic mRNA frequency of ~40% with the exception of clone #15, which presented the lowest frequency at 20% (Fig. 3B, right). The presence of the F876L mutation in genomic DNA was confirmed by Sanger sequencing (Supplementary Fig. S4).

Having shown that AR-F876L spontaneously emerges in majority of the MDV3100-resistant clones, we next aimed to assess whether AR-F876L is also detectable in MDV3100-resistant tumors using an in vivo xenograft model (Fig. 3C). Strikingly, whole-transcriptome sequencing revealed that 3 of 4 resistant tumors harbored the F876L mutation (Fig. 3D), with frequencies ranging from 46-52% of the total AR transcript.
reads (Fig. 3E). Interestingly, there were two unique nucleotide substitutions observed promoting F876L, implying that these mutations may have been acquired during the course of treatment (Fig. 3D and E). Furthermore, the MDV3100-resistant tumor lacking AR-F876L showed the slowest growth kinetics in the presence of MDV3100 (Fig. 3C), and lowest expression of AR target genes *KLK3* and *NKX3-1* relative to those harboring the mutation (Fig. 3F). Cumulatively, these data strongly suggest that emergence of AR-F876L may represent a dominant tumor-autonomous mechanism of resistance to MDV3100.

**AR-F876L Confers an Antagonist-to-Agonist Switch for MDV3100**

To assess the potential impact of the F876L mutation on MDV3100 response, we computationally modeled the binding of MDV3100 to the wild-type (WT) and mutant receptors. We found that in the docking modes of the antagonistic state of WT AR, MDV3100 only displayed a weak interaction with F876 (Fig. 4A, left). However, in the presence of the F876L mutation, the benzamide motif of MDV3100 can extend into the access channel created by the smaller leucine residue. This would potentially prevent the compound from clashing with helix-12 of the AR ligand-binding domain in the agonistic mode (Fig. 4A, right). Thus, computational modeling suggested that F876L mutation in AR may abolish the antagonistic activity of MDV3100 and could potentially allow agonist activity.

To functionally test the impact of the F876L mutation, we next assessed the transcriptional responses of the wild-type; single mutants T877A, W741C, W741L and F876L; and the double mutant F876L/T877A AR to an agonistic synthetic androgen...
R1881 and anti-androgens bicalutamide and MDV3100 in transactivation reporter assays using HEK293T cells. W741C/L mutant ARs have previously been shown to confer an agonist switch specific for bicalutamide and were used as controls (13). Notably, we found that only AR-F876L and AR-F876L/T877A had a substantial transactivation response of an ARE-driven reporter gene (14) to MDV3100—comparable to 0.1 nM R1881—suggesting an antagonist-to-agonist switch exquisitely specific for MDV3100 (Fig. 4B and Supplementary Fig. S5A and B). These findings were further validated in AR-PC3 (Supplementary Fig. S6) and AR+ VCaP PCa lines (Fig. 4B).

In support of the transactivation data, a similar agonist switch was also observed in spontaneous resistant clones that harbored the F876L mutation when KLK3 expression was analyzed in response to MDV3100 in media stripped of endogenous androgens—a condition that minimized basal AR pathway activity and the response of endogenous WT AR to MDV3100 (Fig. 4C). Notably, whereas most strongly resistant clones (#1, #19 and #24) expressed significantly higher levels of KLK3 in the presence of MDV3100 relative to vehicle, clone #15 showed similar sensitivity to MDV3100 as the control (C1) and weakly resistant clones, likely attributed to the low mutant allelic mRNA frequency (Fig. 3B, right). These data in aggregate strongly suggest that the F876L mutation in AR is a driver of an antagonist-to-agonist switch highly specific for MDV3100.

The F876L Mutation is Sufficient to Induce Genetic and Phenotypic Resistance to MDV3100 in Genetically Engineered LNCaPs
Given that strongly resistant clones harbor several sequence variants in addition to the F876L mutation, we wanted to formally test whether the AR-F876L variant is sufficient to promote an MDV3100-resistant growth phenotype. To this end, LNCaP cells were engineered to inducibly express T877A, F876L/T877A and W741C/T877A mutant ARs (Supplementary Fig. S7). In support of previous reports, expression of all T877A-bearing mutant alleles and the W741C/T877A allele promoted an antagonist-to-agonist switch specific for hydroxyflutamide (10-12) and bicalutamide (13), respectively, based on the regulation of the AR target gene KLK3 (PSA) (Supplementary Fig. S8). Despite the slightly lower expression of AR-F876L/T877A, a significant rescue of AR pathway activity was observed under 10% FCS culture conditions (Fig. 5A and Supplementary Fig. S8) and a significant agonist effect was observed under 10% CSS culture conditions (Supplementary Fig. S9A). The influence of AR-F876L/T877A on pathway activity closely paralleled changes in localization of AR as AR-F876L/T877A showed greater nuclear influx (MDV3100 vs. DMSO) relative to the T877A line, under both 10% FCS (Supplementary Fig. S10A and B) and 10% CSS (Fig. 5B, Supplementary Figs. S9B, S10B) culture conditions. Unexpectedly, we also observed a significant rescue of pathway activity in the W741C/T877A line, likely due to the constitutive active function conferred by the double mutation as reported previously (13) (Fig. 5A and B).

Having established that AR-F876L/T877A can utilize MDV3100 as an agonist, we next tested whether ectopic expression of AR-F876L/T877A was sufficient to confer phenotypic resistance. Although only a modest rescue in growth was observed in a short-term proliferation assay (Supplementary Fig. S11A and B), a robust rescue was
observed in a long-term colony formation assay in response to MDV3100, but not bicalutamide (Fig. 5C). Notably, the W741C/T877A line also showed significant growth in the presence of MDV3100 (Fig. 5C), consistent with the rescue of pathway activity observed earlier (Fig. 5A and B).

To assess whether the T877A mutation is necessary for the resistance phenotype, LNCaP cells were engineered to stably express the single mutant AR-F876L. Consistent with the transactivation data (Fig. 4B and Supplementary Fig. S6), AR-F876L conferred genetic and phenotypic resistance to MDV3100, suggesting that F876L—alone or in conjunction with T877A—is equally as capable of conferring an agonist switch (Supplementary Fig. S12A and B).

The Resistance Conferring Role of AR-F876L is Broadly Applicable to Several Prostate Cancer Lines

Given that the F876L mutation in AR can promote resistance to MDV3100 in PTEN-null LNCaP cells, we next questioned whether this mutation is sufficient to broadly confer resistance in PCa lines of various genetic backgrounds. To this end, human VCaP (harboring TMPRSS2-ERG fusion and AR amplification) and murine Myc-CaP (overexpressing Myc) lines were transduced to stably express the mutant AR. Consistent with the resistance phenotype observed in LNCaP cells, both lines also presented partial resistance to MDV3100 (Fig. 5D and Supplementary Fig. S13), suggesting that F876L mutation in AR has the potential for conferring resistance to MDV3100 under various genetic contexts.
Taken together, our data implicates a direct role for AR-F876L, AR-F876L/T877A and potentially constitutively active AR variants, such as W741C/T877A, as drivers of resistance to MDV3100. Whereas constitutively active variants may promiscuously promote resistance to various classes of anti-androgens, we show that F876L mutation in AR selectively promotes resistance to MDV3100.

**AR-F876L-Bearing Cells are Resistant to MDV3100 in Castrate Setting In Vivo**

Having shown that the F876L mutation in AR confers an agonist switch for MDV3100 that can promote resistance, we next asked whether F876L-bearing cells become dependent on this switch for cellular growth under androgen-deprivation conditions, a dependence that has been observed in the clinic for other anti-androgens such as flutamide (15). Interestingly, although the switch did not drive proliferation of F876L-bearing clones *in vitro* (Supplementary Fig. S14), we observed a marked dependence on MDV3100 for growth *in vivo* when the F876L-bearing cells were implanted into castrated male mice (Fig. 5E). Whereas control (C1) tumors showed immediate stasis upon MDV3100 treatment, resistant tumors (clone #1) failed to grow until stimulated by MDV3100 (Fig. 5E). Specifically, after a prolonged period of stasis, 10 of 15 mice treated with MDV3100 developed rapidly growing tumors compared to 2 of 15 in the vehicle group ($P = 0.0078$, Fishers exact t-test, two-tailed), while the tumor take rates of control cells (C1) were indistinguishable (9 of 15 vs. 10 of 15) (Fig. 5F). Furthermore, resistant tumors continued to grow in the presence of MDV3100, in agreement with earlier *in vitro* observations (Fig. 1C), cumulatively suggesting that AR-F876L-bearing cells require MDV3100 for growth *in vivo*. No significant body weight loss was observed.
during the treatment period in all four groups (Supplementary Fig. S15A and B). End point molecular analysis confirmed an agonist switch of MDV3100 associated with the F876L mutation \textit{in vivo} as resistant tumors treated with MDV3100 showed modestly higher AR pathway activity, as shown by AR target gene expression, relative to vehicle-treated resistant tumors, and significantly higher activity relative to vehicle-treated control tumors (Fig. 5G). These data cumulatively highlight the dependence of F876L-bearing cells to MDV3100 for \textit{in vivo} growth under androgen-deprivation conditions.

**Targeting CDK4/6 as a Therapeutic Strategy for Overcoming MDV3100 Resistance**

Having established that AR-F876L can promote resistance to MDV3100 under various genetic contexts \textit{in vitro} and \textit{in vivo}, we next aimed to develop rational strategies to antagonize the mutant allele. As an alternative to developing novel anti-androgens that continue to target the hypermutable ligand-binding pocket of AR (16), we aimed to identify therapeutic strategies that may be more sustainable in the clinic. To this end, we observed a significant enrichment for genes belonging to ‘cell cycle’ and ‘E2F1 activation’ gene sets in addition to ‘AR activation’ in strongly resistant clones treated with MDV3100 (Fig. 2A), suggesting that these clones may potentially maintain proliferation under MDV3100-treatment conditions through continued expression of E2F1 target genes. This is an appealing hypothesis as androgen signaling, a critical regulator of G1-S transition, is known to promote active CDK4/cyclin D1 assembly and hence activation of E2F1 function (17). Consistent with this notion, we observed that MDV3100-treatment suppressed expression of E2F1 target genes \textit{DHFR} and \textit{TK1} in a control line, confirming AR signaling as a regulator of E2F1 function. In contrast, F876L-
bearing cells were capable of maintaining higher expression of DHFR and TK1 under MDV3100-treatment conditions relative to a control line (Fig. 6A). Based on these data, we reasoned that E2F1 activity may serve as a downstream effector of AR signaling, and as such, regulators of E2F1 function may serve as critical therapeutic nodes when AR-directed therapies become ineffective. In agreement with this hypothesis, we found that growth of strongly resistant clones (Fig. 6B) and LNCaP lines engineered to express AR-F876L/T877A (Fig. 6C) were as sensitive to CDK4/6 inhibitors LEE011 (18) and PD033299, as bicalutamide. Interestingly, response to CDK4/6 inhibition was not only limited to F876L-bearing cells as androgen-independent 22Rv1 cells—a PCa line expressing WT AR and capable of maintaining AR signaling and expression of DHFR and TK1 under MDV3100 treatment conditions (Fig. 6D)—also showed strong sensitivity to CDK4/6 inhibition (Fig. 6E). These data cumulatively suggest that targeting CDK4/6 function may serve as an effective strategy for treatment of multiple mechanisms of resistance to MDV3100 and likely androgen-independence in general.

**DISCUSSION**

In this study, we functionally confirmed an agonist switch mutation (F876L) in AR that drives phenotypic resistance to MDV3100. Furthermore, we highlight the therapeutic potential of targeting downstream effectors of AR signaling, such as CDK4/6, when AR-directed therapies become ineffective. Collectively, these data imply 1) that patients bearing a pre-existing AR-F876L mutation might show clinical progression of the disease in response to MDV3100 treatment, 2) that patients developing the F876L mutation upon MDV3100 treatment might benefit from drug withdrawal and 3) all F876L-
bearing patients (pre-existing or acquired) might respond favorably to existing anti-androgens and CDK4/6 inhibitors (Fig. 7). Therefore, screening patients to identify this mutation will aid in predicting drug sensitivity and guide alternate clinical management strategies.

**F876L: Hotspot Mutation in AR in Response to MDV3100 Treatment**

The relatively quick emergence of the F876L mutation during *in vitro* selection (Fig. 3A, B) initially suggested that this mutation was likely pre-existing at a low frequency. However, the fact that two independent nucleotide substitutions led to the F876L mutation during *in vivo* selection (Fig. 3D and E) implies that the mutation may have been acquired during treatment. Since LNCaP cells possess defects in the mismatch repair (MMR) mechanism, deficiencies found to also exist in a subset of PCa (19), it is conceivable that they may more easily acquire this mutation (either with or without MDV3100 treatment). However, the fact that Balbas and colleagues (2013) also reported the spontaneous emergence of F876L upon prolonged treatment of CWR22PC cells (16), a line that is believed to possess an intact MMR mechanism, suggests that F876L may truly be a hotspot mutation that may possess the capacity to confer resistance in prostate tumors irrespective of the MMR status.

**Agonist Switch Suggests a Treatment Withdrawal Effect for MDV3100**

In some patients that become refractory to anti-androgen therapy, discontinuation of treatment often leads to clinical improvement. This “anti-androgen withdrawal syndrome (AAWS)” has been reported for flutamide (20), bicalutamide (21) and other non-steroidal
and steroidal anti-androgens (22-24). In a subset of patients presenting ‘addiction’ to anti-androgen therapy, mutations in AR that promote an agonist switch have been identified and considered to be one potential mechanism driving growth during treatment (15). In the current study, we also observed an ‘addiction’ phenomenon as the growth of an F876L-bearing cell line (clone #1) became dependent on MDV3100 for growth in vivo (Fig. 5E). These parallels imply that refractory patients bearing the AR-F876L variant may, at least temporarily benefit from withdrawal of MDV3100 in the clinic.

**Alternative Therapeutic Strategies**

The frequent emergence of an agonist switch as a resistance mechanism to anti-androgens necessitates that 1) more potent compounds with better therapeutic indices be developed, 2) alternate methods to target AR be sought or 3) non-AR targeting therapeutic strategies be explored. To address the first point, Clegg and colleagues (2012) recently developed ARN-509, an anti-androgen that is more potent, has a higher therapeutic index and longer half-life than MDV3100, all of which might translate to less frequent resistance (4). Interestingly, two independent groups recently reported the spontaneous emergence of the F876L mutation in AR as an agonist switch specific for both MDV3100 and ARN-509 (16, 25). To overcome this mutation, Balbas and colleagues (16) coupled modeling with focused chemical screens to discover novel anti-androgens that effectively antagonized AR-WT and AR-F876L. Although too early to predict, if agonist switch conferring mutations develop to these novel compounds, then applying strategies that may be less amenable to resistance by mutations in the AR ligand-binding pocket should be explored. Strategies such as combining two anti-
androgens (in combination or sequentially), blocking activation function 1 (AF1) (26) or AF2 (26, 27) functions of AR, or development of compounds that destabilize/degrade AR or hinder nuclear translocation might be also considered. Lastly, non-AR targeting therapies may also serve as a viable strategy. To this end, we found that inhibiting CDK4/6 potently targeted both strongly resistant clones (Fig. 6B) and LNCaPs engineered to overexpress AR-F876L/T877A (Fig. 6C), implying that targeting this arm may serve as a viable strategy for overcoming this mutation-based resistance mechanism. Even more generally, inhibition of CDK4/6 also targeted weakly resistant LNCaP clones (Fig. 6B) and MDV3100-resistant 22Rv1 cells (Fig. 6E)—PCa lines lacking AR-F876L—implying that this therapy may be applied to overcome multiple mechanisms of resistance to MDV3100 and androgen-independence in general. In support of our findings, several reports have suggested that RB loss plays a critical role in PCa development and resistance to AR-directed therapies (28). More relevantly, consistent with our findings, a recent study has reported the use of CDK4/6 inhibitors as an effective therapeutic to target early and late stage PCa that is mechanistically dependent on RB (29). Since a significant fraction of CRPCs maintain functional RB, we speculate that inhibition of CDK4/6 may serve as a viable approach for treating resistance to AR-directed therapies in a large subset of patients.

METHODS

Cell Culture

LNCaP-FGC and PC3 cells were maintained in RPMI 1640 media with 10% FCS (Hyclone) or 10% CSS (Omega, cat# fb-04) as indicated. 22Rv1 cells were maintained
in RPMI 1640 media with 10% FCS. VCaP cells were maintained in DMEM media with 10% FCS or 10% CSS. Myc-CaP cells were maintained in DMEM media with 10% FCS. 293FT cells were maintained in DMEM supplemented with 10% FCS and 1xNEAA. All lines, except Myc-CaP cells, were obtained from the American Type Culture Collection and cells were tested and authenticated by single-nucleotide polymorphism fingerprinting. Myc-CaP cells were kindly provided by Dr. Charles Sawyers and were not further tested or authenticated.

**Generation of Spontaneous MDV3100 Resistant LNCaP Clones In Vitro**

2 million LNCaP cells were seeded into four 150 cm² tissue culture dishes in phenol red-free RPMI 1640 media supplemented with 10% FCS. The next day, DMSO and ethanol (control 1, C1), DMSO and 1.0 nM R1881 (control 2, C2), 1 μM MDV + 1.0 nM R1881 (control 3, C3), and 1 μM MDV3100 were added to dishes 1 to 4 respectively. Cells were re-seeded into 150 cm² dishes supplemented with the appropriate treatments once each week. Media and compound was replaced for dish 4 once every week. Once resistant clones emerged, media was aspirated from dish 4. Trypsin soaked 3 mm sterile cloning discs (Scienceware, cat# 17-2.X) were used to overlay the resistant clones and following a 1-2 min incubation, cloning discs were transferred to 24-well plates with phenol red-free RPMI 1640 media supplemented with 10% FCS and MDV3100. Cells from control dishes 1-3 were maintained until resistant clones were cryopreserved to maintain a constant culture time for control and experimental lines.

**Generation of Stable Transduced Lines**
Codon-optimized cDNAs encoding WT or mutant ARs were synthesized (DNA2.0) and subcloned into the pLKO-TREX-HA-Neo (Invitrogen). Lentiviruses were produced by transfecting 293FT cells with VSVG:deltaR8.9:cDNA constructs at a ratio of 1:2.5:1.25. Virus was harvested 2–3 d after transfection, filtered, and used to infect LNCaP, MyC-CaP and VCaP cell cultures in the presence of 8 μg ml-1 polybrene. Infected cells were maintained in neomycin for 3 weeks. In all cases, at least 1000 independent clones were pooled to generate stable cell lines to avoid clonal variations. Stable cell lines infected with control vectors were generated in parallel for use as experimental controls.

Microarray

3x10^5 cells were seeded in 6-well plates 2-3 d prior to treatment with DMSO or 10 μM MDV3100 for 24 h. Total RNA was isolated from cells using the Qiagen RNeasy Kit. RNA integrity and purity were assessed with the RNA 6000 Nano LabChip system on a Bioanalyzer 2100 (Agilent Technologies). Generation of labeled cDNA and hybridization to Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix Inc.) was performed using standard protocols as previously described (8).

Expression Analysis

Probesets from the Affymetrix gene expression datasets were normalized using MAS5 with a trimmed-mean target of 150, and log2-transformed. Probesets were then filtered for inclusion only if their maximum value over different samples was at least 5. Ordinary least squares was performed using 0-1 indicator variables as the covariates; one
indicator was used to represent the baseline (untreated sample) of each clone, and three indicator variables represented treatment with MDV3100 for each of the classes of samples (control, weakly resistant, and strongly resistant clones). This regression was used to generate nominal p-values and regression coefficients (i.e. fold-changes). Individual probesets were considered significantly differentially expressed if their fold-change was >= 1.5, with a nominal p-value <= 0.05.

**Gene Set Enrichment Analysis**

Correlation between an AR gene signature (30) was compared to the top-ranked genes upon treatment with MDV-3100. Blue line represents expressed probeset position and is ranked by average fold-change; only those probesets expressed at a MAS5-150 level of at least 32 in at least 1 sample are included. The red lines indicate where the probesets mapping to genes in the AR gene signatures appear in our dataset; gray lines for probesets that do not pass nominal significance, and the taller red line represents probesets with a fold-change of at least 1.5 and a nominal p-value less than 0.05. The black curve shows the cumulative sum of the probesets in the AR gene signature, and the dotted line represents the hypothetical cumulative sum for a random list of genes which are unenriched.

**Pathway Enrichment Scores**

For the candidate signature (30), a two-tailed fisher’s exact test was used to determine if probesets representing genes in those signatures were under- or over-represented in the set of probesets that were up- or down-regulated at least 1.5-fold
compared to expressed but non-differentially-expressed probesets, with a nominal p-value of 0.05 or less. For an unbiased approach, pathways derived from GO terms and transcription-factor networks were analyzed for overrepresentation via a one-tailed interpolated fisher's exact test, using genes that varied 1.5-fold or more with a nominal p-value of 0.05 or less compared to all genes represented on the array; Benjamini-Hochberg (BH) correction was then applied to these p-values (8).

**Sanger Sequencing**

Genomic DNA was isolated from various 1 μM MDV resistant clones and appropriate control lines using the Blood and Tissue DNAeasy kit (Qiagen, cat# 69581) according to the manufacturer's directions. Primers were used to amplify exon 8 (the site of the F876L mutation) (Forward: 5'-ATTGCGAGAGAGCTGCATCA-3'; Reverse: 5'-TTCTCGTCACTATTGGCCTC-3') and the amplified cDNA was sequenced by Genewiz using the same primers. Trace files were analyzed to confirm the presence of the F876L mutation.

**Quantitative Real-Time PCR**

For spontaneous clones: 200k cells were seeded in 6 well plates in RPMI 1640 supplemented with 10% FCS for 2 d prior to treatment with various compounds for 24 h. Alternatively, for experiments involving androgen-depletion, 200k cells were seeded and grown in 6 well plates in phenol-free RPMI 1640 supplemented with 10% FCS for 2 d. Media was aspirated and replaced with phenol red-free RPMI 1640 supplemented with 10% CSS for 3-4 d prior to treatment with various compounds for 24 h. Similar protocols
were applied for genetically engineered lines except that cells were pretreated with Dox for at least 2 d prior to treatment with various compounds to allow sufficient transgene expression. Total RNA was extracted using the RNeasy plus mini kit (Qiagen, cat# 74136) according to the manufacturer's instructions. 1-2 μg of total RNA was used for cDNA synthesis using a high capacity cDNA reverse transcription kit (Applied Biosystems, cat# 4368813). cDNA from each sample was diluted 15–20 fold and real-time was performed in triplicates using gene-specific primers and FastStart Universal Probe Master Mix (Rox) (Roche Applied Science) on an ABI 7900HT series PCR machine. Expression levels were normalized to TBP expression. All analysis was performed using the SDS2.3 software. The following gene-specific primers (Invitrogen) were used: AR (Hs00171172_m1), KLK3 (Hs02576345_m1), NKX3-1 (Hs00171834_m1), TMPRSS2 (Hs01120965_m1), SLC45A3 (Hs00263832_m1), TBP (Hs00427620_m1), DHFR (Hs00758822_s1) and TK1 (Hs01062125_m1).

**In Vivo Tumorigenesis Assays**

10 million LNCaP cells (C1, clone #1) mixed 1:1 with matrigel were injected subcutaneously into the flank of castrated 7-8 week male nu/nu mice. Tumor-bearing mice (tumor volume=150-300 mm³) were treated with vehicle or 30 mg/kg MDV3100 daily. Tumors were measured using calipers and tumor volumes were calculated using length x width²/2. Data is expressed as mean ± SEM. All animal experiments were performed in compliance with the guidelines of Novartis Biomedical Research Animal Care and Use Committee protocols and regulations.
Statistical Analysis

Results were reported as mean ± SEM (standard error of the mean). Two-sided independent student’s t-test without equal variance assumption was performed to analyze gene expression levels and end-points of in vitro luciferase assays. For gene-set enrichment analysis, p-values shown are based on a two-tailed Fisher’s exact test comparing probesets in the gene signature that pass significance to those that do not, versus the significance / insignificance of all other probesets. For animal experiments, a two-tailed Fishers exact test was performed to determine the significance in percentage of mice growing palpable tumors in each group.

Accession Number

Gene expression microarray data used to analyze differential gene expression upon treatment with MDV3100 vs. DMSO in spontaneous and engineered lines has been deposited at the NCBI Gene Expression Omnibus with the accession GSE44924 and GSE44927 respectively.

Additional experimental procedures are listed in the SUPPLEMENTARY METHODS.
Authors' Contributions

Conception and design: M. Korpal, P. Zhu

Development of methodology: M. Korpal, X. Gao, P. Zhu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Korpal, X. Gao, D. P. Rakiec, D. A. Ruddy, J. Yuan, S. G. Kovats, V. G. Cooke, J. Monahan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Korpal, J. M. Korn, X. Gao, S. Doshi

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REFERENCES


FIGURE LEGENDS

Figure 1. Characterization of MDV3100-resistant clones derived from LNCaP cells. A, phase contrast images of LNCaP cells grown in RPMI media supplemented with 10% FCS (upper left), 10% CSS (upper right), 10% FCS + 1 μM MDV3100 (lower left) and 10% FCS + 1 μM MDV3100 + 1 nM R1881 (lower right). Scale bar, 50 μM. B, schematic representation of the experimental paradigm applied (see main text for details). C, individual (left) and average (right) growth inhibition curves for the three controls (brown line) and seven resistant clones (green and red) treated with MDV3100 (top) and bicalutamide (bottom). Data presented as percent growth relative to DMSO treatment condition as measured by CellTiter-Glo assay. D, western blot analysis of AR expression in control and resistant lines. β-actin used as loading control. E, AR pathway activity scores for C1-C3 and seven resistant clones grown in 10% FCS at baseline (following drug withdrawal for 2 weeks) (beige bars). Scores also calculated for C1-C3 treated with 10 μM MDV3100 for 24 h as reference (green bars). Genes up-regulated by androgens were used for analysis.

Figure 2. Resistant clones segregate based on AR pathway sensitivity to MDV3100. A, left, heat map showing differential expression of genes in control (brown font), weakly (red) and strongly (green) resistant lines upon treatment with 10 μM MDV3100 for 24 h. Probesets with an average fold change of at least 2 (MDV vs. DMSO) for at least one sample set (controls, weakly resistant samples, and strongly resistant samples) are shown. Roman numerals listed on right represent broad classes of differentially expressed genes. Middle, table showing results from GO pathway enrichment analysis.
of genes from class III. Blue shade highlights significant enrichment of genes from the “AR activation” gene set. Right, zoomed image of class III showing relative location of AR regulated genes (blue arrowheads) that add to significance score from pathway analysis. All data presented as log2 ratio of MDV vs. DMSO treatment. B, correlation between an AR gene signature in comparison to the top-ranked genes upon treatment with 10 μM MDV3100 for 24h in controls (left), weakly (middle) and strongly (right) resistant lines. Green line represents level of pathway activity— stronger deviation from black diagonal represents greater inhibition of pathway activity by MDV3100 treatment. C, qPCR analysis of expression of canonical AR target genes in ten lines treated with DMSO or MDV3100 for 24 h. Data is presented as percentage of expression in MDV3100-treated sample relative to DMSO (arbitrarily set at 100%). D, DMSO; M, 10 μM MDV3100. TBP was used to normalize expression. Data represent mean ± SEM; n = 3. * P < 0.05 (Student’s t-test). D, quantitation of nuclear to cytoplasmic ratio of endogenous AR expression in control (C1, brown) and resistant clone (#1, green) treated with DMSO, 10 μM MDV3100 (MDV) or 0.1 nM R1881 (R1881) for 24 h. Western blot data presented in Supplementary Fig. S3 was analyzed by ImageJ to determine ratios.

**Figure 3.** Whole-transcriptome sequencing analysis identifies a novel F876L mutation in AR in strongly resistant lines and xenograft tumors. A, upper, unbiased hierarchical clustering of somatic mutations detected in controls (brown font), weakly resistant (red font), and strongly resistant clones (green font). Grey line represents lack of mutation; orange line represents single recurrent heterozygous mutation; red line represents
recurrent homozygous mutation; green line represents recurrent mutation in AR, as indicated by green arrowhead. Lower, schematic of human androgen receptor. NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain. Site of F876L mutation indicated by green line and green font. B, left, IGV plot showing heterozygous nature of F876L mutation in exon 8 of AR in strongly resistant clones (indicated by green arrowheads). F876L mutation is adjacent to a preexisting homozygous T877A mutation (orange shade) in LNCaP. Right, raw counts of wild-type (WT, red shade) and mutant (Mut, green shade) AR alleles in strongly resistant clones. C, tumor growth kinetics of two vehicle-treated (red curves) and four MDV3100-treated (green curves) LNCaP tumors. D, IGV plot showing the relative position and frequency of nucleotide substitutions promoting F876L in MDV3100 resistant tumors. C1-C2, vehicle-treated tumors; R1-R4, MDV3100-resistant tumors. E, table summarizing data from IGV plot. Red shade represents tumors bearing WT AR whereas green shade highlights tumors bearing F876L mutant AR. F, bar graphs showing relative expression of AR target genes KLK3 and NKX3-1 in LNCaP tumors. Red bars, expression data from tumors expressing AR-WT; green bars, expression data from tumors harboring AR-F876L.

**Figure 4.** F876L mutation functionally confers an antagonist-to-agonist switch. A, comparison of the docking modes of MDV3100 in an inactivated state of wild-type (WT) AR (left) with a speculative model of MDV3100 bound to an activated state of mutant AR (right). B, bar graphs showing normalized AR reporter activity following transfection of 293T (top) and VCaP (bottom) cells with various AR expression constructs (AR\textsuperscript{WT}, AR\textsuperscript{T877A}, AR\textsuperscript{F876L}, AR\textsuperscript{F876L/T877A}) and subsequent treatment with vehicle (Veh), 0.1 nM...
R1881 (R), 10 μM MDV3100 (MDV), 10 μM bicalutamide (Bic), 10 μM MDV3100 + 0.1 nM R1881 (M/R) or 10 μM bicalutamide + 0.1 nM R1881 (B/R) for 24 h. All data is normalized to Renilla luciferase (RLUC) expression. Data represent mean ± SEM; n = 3. * \( P < 0.001 \) (Student’s t-test). C, qPCR analysis of KLK3 expression in a control line (C1), weakly resistant (red font) and strongly resistant (green font) clones following treatment with vehicle (V), 0.1 nM R1881, 0.1 nM R1881 + 10 μM MDV3100 (MDV) and 0.1 nM R1881 + 10 μM bicalutamide (Bic) in 10% CSS for 24 h. TBP used to normalize expression. Data represent mean ± SEM; n = 3. * \( P < 0.05 \), ** \( P < 0.01 \) (Student’s t-test).

**Figure 5.** PCa cells engineered to overexpress AR-F876L/T877A are genetically and phenotypically resistant to MDV3100. A, qPCR analysis of KLK3 (top) and NKX3-1 (bottom) expression in various engineered lines (vector controls (Vec); T877A; F876L/T877A, F876L; W741C/T877A, W741C). All lines were pre-treated with vehicle (-) or doxycycline (+) for 3 d prior to treatment with DMSO (left) or 10 μM MDV3100 (right) for 24 h in 10% FCS. TBP used to normalize expression. Data represent mean ± SEM; n = 3. * \( P < 0.05 \), ** \( P < 0.01 \) (Student’s t-test). B, western analysis of exogenous AR (HA) expression in cytoplasmic and nuclear fractions isolated from various engineered lines treated with DMSO (D) or 10 μM MDV3100 (M) in media supplemented with 10% CSS. All lines were pretreated with Dox for 3 d prior to treatment with DMSO or MDV3100 for 24 h. F876L, F876L/T877A; W741C, W741C/T877A. β-tubulin (β-tub) and histone H3 (H3) were probed to validate purity of cytoplasmic and nuclear fractions respectively. C, colony formation assays of various engineered lines (Dox-/+ ) treated with DMSO, 1 μM MDV3100 (MDV) or 1 μM bicalutamide (BIC) for 3-4 weeks.
Representative experiment from three independent experiments is shown. D, top, colony formation assays of Myc-CaP cells engineered to express AR-T877A (left) or AR-F876L/T877A (right) (Dox-/+) treated with DMSO (left wells) or 10 \( \mu \)M MDV3100 (right wells, MDV) for \( \sim \)14d. Bottom, quantitation of colony formation data by ImageJ. E, tumor volume measurements of control (C1, red) and resistant clone #1 (#1, green) tumors treated with vehicle (Veh) or 30 mg/kg MDV3100 (MDV). Solid red and green arrows indicate the day daily administration of MDV3100 was initiated in control (C1) and clone #1 (#1) mice, respectively. Dashed green arrow highlights two consecutive days of treatment with MDV3100 prior to a drug holiday in 15 of 30 mice injected with clone #1 (#1, dark green). \( n = 7-8 \) tumors for all groups except #1 veh (\( n = 2 \)). Data represent mean ± SEM. F, bar graphs showing percentage of mice growing palpable tumors (numbers above bars represent number of mice presenting tumors). G, qPCR analysis of \( \text{TMPRSS2} \) and \( \text{SLC45A3} \) expression in control (C1) and resistant (#1) tumors treated with vehicle (Veh) or MDV3100 (MDV). \( \text{TBP} \) used to normalize expression. Data represent mean ± SEM; \( n = 3 \) for all groups except C1 vehicle treated, \( n = 2 \). * \( P < 0.05 \), ** \( P < 0.01 \) (Student’s t-test).

**Figure 6.** Targeting CDK4/6 reduces proliferation of MDV3100-resistant prostate cancer cell lines. A, qPCR analysis of E2F1 targets \( \text{DHFR} \) and \( \text{TK1} \) in C1 and clone #1 lines upon treatment with DMSO (D), 10 \( \mu \)M MDV3100 (M), 10 \( \mu \)M LEE011 (L) or 10 \( \mu \)M MDV3100 and 10 \( \mu \)M LEE011 (M+L) for 24h. B, average growth inhibition curves for the three controls (brown line) and seven resistant clones (green and red) treated with PD033299 (top) and LEE011 (bottom). Data presented as percent growth relative to
DMSO treatment condition as measured by CellTiter-Glo assay. **C,** relative quantitation of data from colony formation assays of AR-T877A and AR-F876L/T877A lines treated with DMSO, 10 \( \mu \text{M} \) MDV3100 (MDV), 10 \( \mu \text{M} \) bicalutamide (Bic) or 10 \( \mu \text{M} \) LEE011 (LEE) for 28 d (quantitated by Image J). **D,** qPCR analysis of E2F1 targets DHFR and TK1 in androgen-independent 22Rv1 line treated with DMSO, 10 \( \mu \text{M} \) MDV3100 (MDV), 10 \( \mu \text{M} \) LEE011 (LEE) or 10 \( \mu \text{M} \) MDV3100 and 10 \( \mu \text{M} \) LEE011 (MDV+LEE) for 48h. **E,** colony formation assays of 22Rv1 cells treated with various indicated compounds (DMSO, bicalutamide (Bic), MDV3100 (MDV) or LEE011 (LEE)) for 14 d.

**Figure 7.** Schematic representation of the therapeutic strategies that can be applied to target AR-F876L. Left, MDV3100 (brown circles) suppresses expression of E2F1 and canonical AR target genes upon binding to AR-WT (red ovals). Middle, AR-F876L (green ovals) utilizes MDV3100 as an agonist and thus maintains expression of E2F1 and canonical AR target genes. Right, targeting CDK4/6 or AR with structurally distinct anti-androgens can hinder proliferation through suppression of E2F1 and canonical AR target genes respectively. Length of grey arrow represents relative level of pathway activity. Red and blue circles represent CDK4/6 inhibitor and bicalutamide respectively. EBS, E2F1-binding sites; ARE, androgen-responsive elements.
Korpal et al., Figure 1
Korpal et al., Figure 2
Korpal et al., Figure 3

A

Recurrent mutations

- No mutation
- Het
- Homo
- AR mutation

F87L

N
NTD
DBD
LBD
C

B

In vitro-selection

AR Exon 8

#1
#15
#19
#24

Allelic counts

0
100
200
T
C
A
G
T
C
G
C
T
T
T
G

C1
C2

Q
F
A
F

C

In vivo-selection

AR Exon 8

#1
#15
#19
#24

Allelic counts

0
100
200
WT
Mut

C

C1 (vehicle)
C2 (vehicle)
R1 (MDV)
R2 (MDV)
R3 (MDV)
R4 (MDV)

E

Tumor

AA

Codon

Frequency of
F876L

Control 1 (C1)
F
TTC
0

Control 2 (C2)
F
TTC
0

Resistant 1 (R1)
L
CTC
52%

Resistant 2 (R2)
L
TTA
50%

Resistant 3 (R3)
L
CTC
46%

Resistant 4 (R4)
F
TTC
0%

F

Relative expression normalized to TBP

KLK3

NKX3-1

C1
C2
R1
R2
R3
R4

Days post-xenograft

Tumor volume (mm³)

0
500
1000
1500
2000

0
50
100
150
200

Days post-xenograft
A

F876

L876

Normalized FLUC activity (FLUC:RLUC) ARF876LARWT ART877A ARF876L/T877A

0 2 4 6 8

123456

* *

0 5 10 15 20

123456

* *

B

ARWT

AR\textsuperscript{T877A}

AR\textsuperscript{F876L}

AR\textsuperscript{F876L/T877A}

Normalized FLUC activity (FLUC:RLUC) ARF876LARWT ART877A ARF876L/T877A

0 5 10

123456

* *

0 20 40 60

123456

* *

Fold change in FLUC:RLUC (relative to DMSO)

Veh R MDV Bic M/R B/R

Veh R MDV Bic M/R B/R

Veh R MDV Bic M/R B/R

Veh R MDV Bic M/R B/R

Bic

C

Sensitivity

Weakly resistant

Strongly resistant

C1

#9

#10

#14

#1

#15

#19

#24
Korpála et al., Figure 5
MDV 'resistant'

MDV 'sensitive'

MDV

MDV

Bicalutamide, CDK4/6 inhibition

MDV3100

AR

E2F1

EBS

ARE

Stasis

Growth

Stasis

Korpal et al., Figure 7
An F876L Mutation in Androgen Receptor Confers Genetic and Phenotypic Resistance to MDV3100 (Enzalutamide)

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