Glucocorticoid Receptor Confers Resistance to Antiandrogens by Bypassing Androgen Receptor Blockade

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SUMMARY

The treatment of advanced prostate cancer has been transformed by novel antiandrogen therapies such as enzalutamide. Here, we identify induction of glucocorticoid receptor (GR) expression as a common feature of drug-resistant tumors in a credentialed preclinical model, a finding also confirmed in patient samples. GR substituted for the androgen receptor (AR) to activate a similar but distinguishable set of target genes and was necessary for maintenance of the resistant phenotype. The GR agonist dexamethasone was sufficient to confer enzalutamide resistance, whereas a GR antagonist restored sensitivity. Acute AR inhibition resulted in GR upregulation in a subset of prostate cancer cells due to relief of AR-mediated feedback repression of GR expression. These findings establish a mechanism of escape from AR blockade through expansion of cells primed to drive AR target genes via an alternative nuclear receptor upon drug exposure.

INTRODUCTION

Recently approved drugs that target androgen receptor (AR) signaling such as abiraterone and enzalutamide have rapidly become standard therapies for advanced-stage prostate cancer (Scher et al., 2012; de Bono et al., 2011). Despite their success, sustained response with these agents is limited by acquired resistance, which typically develops within ~6–12 months. Clinical success of kinase inhibitors in other tumors such as melanoma, lung cancer, leukemia, and sarcoma is similarly transient (Sawyers et al., 2002; Chapman et al., 2011; Demetri et al., 2002; Maemondo et al., 2010), resulting in numerous efforts to define mechanisms of acquired resistance. One strategy that has proven particularly useful is prolonged treatment of drug-sensitive preclinical models to derive drug-resistant sublines, followed by genome-wide profiling studies to ascertain differences that may play a causal role in conferring drug resistance. A common mechanism that has emerged from these kinase inhibitor studies is reactivation of the signaling pathway targeted by the drug, directly by mutation of the kinase target or indirectly by bypassing pathway inhibitor blockade through amplification of an alternative kinase (Glickman and Sawyers, 2012). Both scenarios have been validated in clinical specimens and are guiding efforts to discover next-generation inhibitors and to develop rational drug combinations.

Clinically relevant mechanisms of resistance to hormone therapy in prostate cancer have also been elucidated using preclinical models. Hormone therapy, through the use of drugs that lower serum testosterone or competitively block the binding of androgens to AR, has been the mainstay of treatment for metastatic prostate cancer for decades but is not curative. The late stage of disease, which is refractory to hormone therapy, is termed castration-resistant prostate cancer (CRPC). We previously examined the molecular basis of progression to CRPC in mouse models and discovered that increased AR expression was the primary mechanism (Chen et al., 2004). We then used this observation to screen for antiandrogens that restore AR inhibition in the setting of increased AR levels. These efforts yielded three second-generation antiandrogens: enzalutamide, ARN-509, and RD162 (Tran et al., 2009; Clegg et al., 2012). Enzalutamide and ARN-509 were further developed for clinical use, culminating in US Food and Drug Administration (FDA) approval of enzalutamide in 2012 based on increased survival (Scher et al., 2012).

Now, with widespread use, resistance to enzalutamide is a major clinical problem. We and others have recently identified an AR point mutation as one resistance mechanism by derivation of drug-resistant sublines following prolonged exposure to enzalutamide or ARN-509 (Balbas et al., 2013; Joseph et al., 2013; Korpal et al., 2013). This AR mutation has also been recovered from patients with resistance to ARN-509 but only in a minority of cases (Joseph et al., 2013). Here, we define a
potentially more prevalent mechanism of resistance by which tumors bypass AR blockade through upregulation of the glucocorticoid receptor (GR).

RESULTS

GR Is Expressed in Antiandrogen-Resistant Tumors

We previously showed that LNCaP/AR xenograft tumors regress during the first 28 days of treatment with ARN-509 (Clegg et al., 2012), enzalutamide, or RD162 (Tran et al., 2009). In a pilot study to explore mechanisms of acquired resistance to these drugs, we treated mice continually and harvested tumors after progression (mean 163 days, Table S1A available online). Tissue from 15 resistant tumors obtained from long-term antiandrogen-treated mice (n = 6 ARN-509, n = 9 RD162) and from 3 control tumors from vehicle-treated mice were analyzed by expression array. Aggregated data from resistant and control tumors in this pilot cohort were compared to identify expression changes commonly associated with resistance (Figure 1A). Among the most upregulated genes in the resistant tumors was the GR (gene symbol NR3C1), which shares overlapping target specificity with AR (Mangelsdorf et al., 1995). Of note, several of the most differentially expressed genes were known androgen-regulated genes (confirmed by transcriptome analysis of short-term dihydrotestosterone (DHT)-treated LNCaP/AR cells in vitro [Table S1B]), but they were altered in directions that did not reflect restored AR signaling. On the one hand, SGK1 (Serum Glucocorticoid Induced Kinase 1), a known AR- and GR-induced target gene, was among the most upregulated, but several other androgen-induced genes (PMEPA1, SNAI2, KCNN2, LONRF1, and SPOCK1) were among the most repressed. Conversely, several androgen-repressed genes (UGT2B15, PMP22, CAMK2N1, and UGT2B17) were among the most upregulated (Figure 1A). These findings indicated that resistance in this model system is unlikely to be mediated by simple restoration of AR activity and raised the possibility that GR may play a role.

Figure 1. GR mRNA and Protein Are Expressed in Resistant Tissues

(A) Most differentially expressed genes in a pilot cohort of LNCaP/AR xenograft tumors with acquired resistance to ARN-509 (n = 6) or RD162 (n = 9) compared to control (n = 3) determined by microarray (Affymetrix Ex1.0). Mice with resistant tissues were continued on drug treatment through time of harvest. (B) Mean tumor volumes ± SEM of LNCaP/AR xenografts in validation cohort. Days tumors that were harvested are annotated on x axis (long hash mark).

(C) RT-qPCR analysis of GR and AR mRNA expression in a validation cohort of LNCaP/AR xenograft tumors from mice treated with vehicle (control, n = 10), 4 days of antiandrogen (n = 8), or with acquired resistance to 10 mg/kg enzalutamide (n = 8) or 10 mg/kg ARN-509 (n = 8). See also Table S1B.

(D) Western blot analysis of GR and AR protein expression in a subset of tissues also analyzed in (C). Control (n = 6), 4 day (n = 5), and resistant (n = 13). Resistant samples were loaded for protein analysis from highest to lowest GR levels based on corresponding mRNA analysis (see also Table S1C).

(E) Intracellular GR flow cytometric analysis of LNCaP/AR, CS1, and LREX0, cells passaged in vitro under standard passage conditions (see Experimental Procedures). See also Figure S1.
To explore this question further, we generated an independent set of drug-resistant tumors (the validation cohort), focusing on the two second-generation antiandrogens in clinical use, enzalutamide and ARN-509 (Figure 1B). GR messenger RNA (mRNA) levels in resistant tumors were substantially higher compared to control (median 26.9-fold increase) or 4 day treated tumors (Figure 1C). Of the tissues analyzed by RT-qPCR, most were also analyzed for GR expression by western blot, based on availability of protein lysates (control, n = 6; 4 day, n = 5; resistant, n = 13). No GR was detected in control samples, minimal expression was noted in 4 day treated samples, and substantial expression was found in most resistant tumors in a pattern that tended to correlate with GR mRNA levels (Figure 1D). There was no correlation between GR expression and the specific antiandrogen treatment used (Table S1C). In contrast to GR, AR RNA or proteins levels were not consistently different across the treatment groups (Figures 1C and 1D).

To explore AR and GR signaling in more detail, we established cells lines from control and drug-resistant tumors by adaptation to growth in vitro. LREX’ (LnCaP/AR Resistant to Enzalutamide Xenograft derived) was derived from an enzalutamide-resistant tumor with high GR expression, and CS1 was derived from a vehicle-treated tumor. We also developed a flow cytometry-based assay to measure GR expression on a cell-by-cell basis. In both LnCaP/AR and CS1, most cells showed no evidence of GR expression, with the exception of a small subpopulation (black arrow, discussed later) (Figure 1E). In contrast, essentially all LREX’ cells expressed GR. Intracellular AR staining confirmed that AR levels in LREX’ did not notably differ from control cells (Figure S1A).

**LREX’ Tumors Are Dependent on GR for Enzalutamide-Resistant Growth**

Having established the LREX’ model as representative of high GR expression, we next confirmed that these cells maintain a resistant phenotype in vivo. LREX’ or control cells were injected into castrated mice that were then immediately initiated on antiandrogen treatment. LREX’ showed robust growth, whereas LnCaP/AR or CS1 lines were unable to establish tumors in the presence of antiandrogen (Figures 2A and 2B). Strong expression of GR was confirmed in multiple LREX’ xenograft tissues by western blot and by IHC (Figures S2 and S1B). As expected, untreated LnCaP/AR tumors were negative for GR expression with the exception of rare GR-positive cells (Figure 2C). Although many of these GR-positive cells had morphologic features of
GR Expression Is Associated with Clinical Resistance to Enzalutamide

To determine whether GR expression is a feature of clinical antiandrogen resistance, we evaluated GR expression in bone metastases from patients receiving enzalutamide. Bone marrow samples were obtained prior to enzalutamide treatment (baseline) and again after 8 weeks of treatment, as previously reported in a cohort of abiraterone-treated patients (Efstathiou et al., 2012). Using a GR immunohistochemistry (IHC) assay optimized for use in bone marrow samples, we quantified the percentage of GR-positive tumor cells and dichotomized the data based on clinical response. Patients who continued to benefit from therapy for greater than 6 months were defined as good responders, whereas those in whom therapy was discontinued earlier than 6 months due to a lack of clinical benefit were classified as poor responders (Figure 3A). Consistent with the designation of good versus poor clinical response based on treatment status at 6 months, 11 of 13 good responders but only 1 of 14 poor responders had a maximal PSA decline greater than 50% (Figure 3B). Akin to the findings in the preclinical model, GR positivity at baseline was low—3% of tumor cells in good responders and 8% in poor responders. Of note, 3 of 22 tumors had evidence of high GR expression at baseline (≥20% of tumor cells), and all three had a poor clinical response (Figures 3C and 3D). At 8 weeks, the mean percentage of GR-positive cells was higher than baseline levels in both response groups but was more significantly elevated in poor responders (29% versus 8%, p = 0.009). In addition, the percentage of GR-positive cells at 8 weeks was significantly higher in poor compared to good responders (29% versus 10%, p = 0.02) (Figures 3C and 3D), and similar results were obtained when the analysis was limited to patients from whom matched baseline and 8 week samples were available for analysis (Figure 3E). Furthermore, when GR IHC data were dichotomized based on PSA decline instead of clinical response, GR induction was also associated with a limited PSA decline (Figure S2). These findings establish a correlation between GR expression and clinical response to enzalutamide and raise the possibility that AR inhibition may induce GR expression in some patients. The fact that poor PSA decline also correlates with GR expression raises the question of whether transcriptional regulation of a canonical AR target gene may be regulated by GR.

GR Expressing Drug-Resistant Tumors Show Uneven Restoration of AR Target Genes

Having implicated GR as a potential mediator of antiandrogen resistance, we next asked whether restored AR pathway activity also plays a role by comparing the mRNA transcript levels of 74 direct AR target genes in control, 4 day, and resistant tumors from the validation cohort (Figure 2F), as well as eight LREX- tumors (Figure 4A) (see Experimental Procedures and Table S2 for details on gene selection). Consistent with the data generated in the pilot cohort (Figure 1A), some AR target genes in resistant tissues showed elevated levels relative to control (SGK1 and STK39), whereas other genes (NDRG1, TIPARP, and PMEPA1) showed no evidence of restored expression.

To examine restoration of AR signaling across the entire set of 74 target genes, we calculated a fractional restoration value using log 2 transformed expression values and the equation (resistant – 4 day)/(control – 4 day). With this approach, a gene whose expression in resistant tissue equals the expression in control tumors calculates as 1, whereas a gene whose expression in resistance equals its expression after 4 days of antiandrogen treatment equals 0. (Values greater than one indicate hyperrestoration in resistance relative to control, and values below zero suggest further inhibition as compared to acute treatment.) These data confirmed that the pattern of restoration varied gene by gene, but this pattern was consistent in LREX xenografts and in the validation cohort tumors (Pearson r = 0.754; Figure 4B). This finding is most consistent with a model in which AR remains inhibited in drug-resistant tumors, but expression of certain AR target genes is restored by an alternative transcription factor, possibly GR. The fact that restoration values were somewhat higher in the LREX analysis correlates with higher GR expression in these tumors (Figure 4C).

GR Drives Expression of AR Target Genes in Resistant Tissues

To determine whether GR can drive expression of this subset of AR target genes, we compared, in vitro, DHT-induced (AR) and dexamethasone (Dex)-induced (GR) expression of seven AR targets that represent the spectrum of restoration noted in the in vivo analysis, as well as PSA (Figure 4D). All eight genes were regulated by DHT as expected, and this regulation was blocked by enzalutamide. Thus, AR signaling remains intact and can be inhibited by antiandrogens in these drug-resistant cells, making an AR-dependent mechanism of drug resistance less likely.

In contrast to DHT, the effect of Dex on these same target genes was variable but closely matched the pattern observed in drug-resistant xenografts. For example, Dex strongly induced SGK1 and STK39 but did not induce TIPARP, NDRG1, and PMEPA1. Of note, KLK3 (PSA) was comparably induced by either DHT or Dex, providing evidence that persistent PSA expression in patients responding poorly to enzalutamide could be driven by GR. As expected, enzalutamide did not notably
affect Dex activity. To confirm that this pattern of GR-dependent gene expression is not unique to LREX cells, we introduced a GR-expressing retrovirus into parental LNCaP/AR cells and observed a similar pattern of DHT- versus Dex-induced gene expression (Figures S4A and S4B). To be sure that the effects of Dex in these models are mediated through GR, we cotreated cells with a previously described competitive GR antagonist that lacks AR binding called compound 15 (Wang et al., 2006).

Figure 3. GR Induction in Disseminated Tumor Cells Is Associated with Poor Clinical Response to Enzalutamide and Persistence of Prostate-Specific Antigen

(A) Schematic of sample acquisition timeline and response groups.
(B) Number of good or poor responders who achieved prostate-specific antigen (PSA) decline greater than 50%.
(C) Examples of GR IHC images from matched samples at baseline and 8 weeks.
(D) Percent GR-positive epithelial cells in all tissue available at 0 and 8 weeks.
(E) Matched samples obtained from the same patient at 0 and 8 weeks ± SEM. Comparisons are by Mann-Whitney test.

See also Figure S2.
Compound 15 significantly decreased expression of Dex-induced genes, confirming that Dex activity in the LREX model is GR dependent (Figure S4C). Lastly, small interfering RNA (siRNA) experiments targeting AR confirmed that AR is not necessary for Dex-mediated gene activation (Figure S4D). Collectively, these experiments demonstrate that GR is able to drive expression of certain AR target genes independent of AR.

**AR and GR Have Overlapping Transcriptomes and Cistromes**

To explore AR and GR transcriptomes in an unbiased fashion, we performed expression profiling after short-term treatment of LREX cells with DHT or Dex in the presence or absence of enzalutamide. AR and GR signatures were respectively defined as all genes with absolute expression change greater than 1.6-fold (FDR < 0.05) after 1 nM DHT or 100 nM Dex treatment (Table S3). Of the 105 AR signature genes and 121 GR signature genes, 52 were common to both lists (Figure 5A). An even larger proportion of AR or GR signature genes (>80%) showed evidence of regulation by the reciprocal receptor using different thresholds for expression differences (Table S3). Heatmap analysis of these genes confirmed significant overlap in DHT- versus Dex-induced gene expression and showed that Dex-induced gene expression is not impacted by enzalutamide treatment (Figure 5B). These findings support the hypothesis that GR activity can bypass enzalutamide-mediated AR inhibition by regulating a distinct but significantly overlapping transcriptome.

We next addressed the question of whether transcriptomes of enzalutamide-resistant tumors are more likely to be explained by AR- or GR-driven gene expression using gene set enrichment analysis (GSEA). To define gene sets that distinguish AR and GR activity, expression of AR and GR signature genes was first evaluated by GSEA in the DHT- and Dex-treated samples from which they were derived. As expected, GR signature genes were enriched in the Dex-treated samples, and AR signature genes were enriched with DHT treatment (Figure 5C). Because several of the genes did not distinguish AR and GR status due to their overlapping transcriptional activities, we refined the lists into AR selective genes (defined as the AR-induced signature genes that were also more highly expressed in DHT-treated samples relative to Dex treated samples, n = 39) and GR selective genes (defined as the converse, n = 67) (Table S3). GSEA analysis of these selective genes lists revealed that GR selective genes were strongly enriched in the enzalutamide-resistant LREX tumors, whereas AR selective genes were strongly enriched in the control tumors (Figure 5D). These data provide compelling, unbiased evidence that drug resistance is associated with a transition from AR- to GR-driven transcriptional activity.

One prediction of this model is that GR should occupy a substantial portion of AR binding sites in drug-resistant cells. To address this question, we conducted chromatin immunoprecipitation sequencing (ChIP-seq) experiments to define AR and GR DNA binding sites in LREX cells after DHT and Dex treatment, respectively. Of note, 52% of the AR binding sites identified after DHT treatment were bound by GR after Dex treatment (Figure 5E). We examined the remaining 48% of AR peaks more closely to be sure that these peaks were not scored as GR negative simply because they fell just below the threshold set by our peak calling parameters. When we plotted the average AR and GR signal as a measure of the relative strength of AR and GR peaks, we found little evidence of GR binding at the AR unique sites (Figure S5A), confirming that these peaks were indeed unique to AR. Next we conducted motif analysis to explore potential differences between AR/GR overlap versus AR unique sites. The core ARE/GRE consensus sequence was present in both groups (66% and 68% of peaks), but AR/GR overlap peaks were relatively enriched for the FoxA motif (64% versus 45% of peaks; p = 2.2 x 10^{-16}) (Figure 5E). Similar analysis of the GR cistrome defined GR unique and AR/GR overlap peaks and revealed that a higher proportion of GR binding sites were unique to GR. Interestingly, GR unique peaks were highly enriched for the FoxA motif (Figure 5F), whereas the classic ARE/GRE was not reported by the motif discovery algorithm (MEME) and was found only 25% of the time.

Although these cistrome studies provide evidence of substantial overlap between AR and GR binding sites in enzalutamide-resistant cells, several lines of evidence indicate that the transcriptional differences in DHT- versus Dex-induced gene expression cannot be explained solely by DNA binding. For example, ChIP RT-qPCR experiments showed significant AR and GR DNA binding at genes induced by both receptors (SGK1, FKBP5, PSA) but also at genes such as NDRG1 that are transcriptionally activated by DHT, but not Dex (Figure S5B). Integrative ChIP-seq and transcriptome analysis provided further evidence that DNA binding is not sufficient to determine transcriptional competence. Of the 56 AR signature genes found to have an AR binding peak, 49 showed at least some transcriptional regulation by GR (1.2-fold expression change, p < 0.05). 38 of these 49 GR regulated genes (78%) had an overlapping AR/GR binding peak, confirming substantial overlap at coregulated genes. But GR peaks were also found in three of the seven AR targets genes (43%) with no apparent GR transcriptional regulation (Figure S5C). Others have reported evidence of allosteric regulation of hormone receptor complexes by specific DNA sequences independent of binding affinity (Meijsing et al., 2009), a phenomenon that may also be relevant here.

**Figure 4. Variable Expression of AR Target Genes in LREX In Vivo and after Glucocorticoid Treatment In Vitro**

(A) Normalized expression array signal (Illumina HT-12) of a suite of 74 AR target genes in control (n = 10), 4 day (n = 8), and LREX (n = 8, right) xenograft tumors. Genes are ranked by degree of restoration of expression in resistant tissue ([Res-4 day]/[Control-4 day]). All resistant tissues were continued on antiandrogen treatment through time of harvest.

(B) Fractional restoration values of each of the 74 AR targets in LREX xenografts (n = 8) or resistant tissues from the validation cohort (n = 12, see also Figure S3). (C) GR mRNA in resistant tissues used in (B).

(D) Relative Expression ± SEM of AR target genes in the LREX cell line in steroid depleted media after 8 hr of treatment with the indicated agonists in vitro. Enzalutamide, 10 μM; V, Vehicle.

See also Figures S3 and S4.
Figure 5. Comparative AR and GR Transcriptome and Cistrome Analysis in LREX

(A) Venn diagram of AR and GR signature gene lists. AR or GR signatures were defined as all genes showing >1.6 (or <1.6)-fold change (FDR < 0.05) after 8 hr of addition of DHT (1 nM) or Dex (100 nM) to charcoal-stripped media, respectively.

(B) Heatmap depiction of expression changes of AR signature genes (left) or GR signature genes (right) associated with the indicated treatment. Enzalutamide, 10 μM.

(C) Expression of AR- or GR-induced signature genes (as defined in A) were compared in DHT (1nM) or Dex (100 nM)-treated samples. GR signature genes that also had higher expression in Dex samples (>1.1-fold, FDR < 0.05) were designated as GR selective (n = 67), and AR signature genes that showed higher expression in DHT samples (>1.1-fold, FDR < 0.05) were designated as AR selective (n = 39).

(legend continued on next page)
Activation of GR by Dexamethasone Is Sufficient to Confer Enzalutamide Resistance

Whereas LNCaP/AR cells acquire GR expression after prolonged exposure to enzalutamide, some prostate cancer cell lines derived from CRPC patients (DU145, PC3, and VCaP) express endogenous GR (Figure 6A). DU145 and PC3 cells are AR negative and, hence, are resistant to enzalutamide, but (D) Expression of AR- and GR-selective genes in LREX0 and control tumors in vivo compared by GSEA.

(E) AR cistrome defined by AR ChIP-seq after DHT (1 nM) treatment of LREX0 in vitro in charcoal-stripped media. Percent of AR defined peaks that overlap with GR peaks found by GR ChIP-seq after Dex (100 nm) treatment of LREX0 in vitro are shown in pie graph. Top binding motifs in AR-unique and AR/GR overlap peaks are indicated below.

(F) GR cistrome defined by GR ChIP-seq after Dex treatment of LREX0 in vitro in charcoal-stripped media. Percent of GR peaks that overlap with AR peaks found by AR ChIP-seq after DHT (1 nM) treatment of LREX0 in vitro are shown in the pie graph. Top binding motifs in GR-unique and AR/GR overlap peaks are indicated below.

See also Figure S5.

VCaP cells are enzalutamide sensitive in vitro (Tran et al., 2009). IHC analysis showed diffuse, primarily cytoplasmic GR expression under standard culture conditions that lack glucocorticoid supplementation (Figure S6A). To test whether GR activation by addition of glucocorticoids impacts antiandrogen sensitivity, we treated VCaP cells with enzalutamide in the presence or absence of Dex. Enzalutamide inhibited growth as expected, but cotreatment with Dex reversed this growth inhibition (Figure 6B). Additional studies with the GR antagonist, compound 15, or with GR shRNA restored enzalutamide sensitivity and provided pharmacologic and genetic evidence that GR confers resistance (Figures 6C–6E). Of note, GR knockdown (which inhibits GR more completely than compound 15, which has mixed agonist/antagonist properties [Wang et al., 2006]) augmented the activity of enzalutamide even in the absence of Dex (Figures 6D and 6F), suggesting that even the weak basal GR activity seen under our standard culture conditions can confer relative resistance to enzalutamide. This result also suggests that a pure GR

Figure 6. GR Activity Is Sufficient to Confer Enzalutamide Resistance in VCaP

For all panels: VCaP cells do not tolerate charcoal-stripped media and were cultured in standard culture conditions (fetal bovine serum with endogenous hormones). Enz, 10 μM; Dex, 100 nm; and CMP 15, 1 μM.

(A) Western blot analysis of prostate cancer cell lines.

(B–D) Cell viability assessed by CellTiter-Glo (Promega) assay and normalized to day 1 value ± SEM.

(E) Confirmation of GR knockdown by western blot after infection with GR targeting shRNA.

(F) Apoptosis as assessed by cPARP western blot after 3 days of indicated treatment.

(G) A suite of AR targets relevant to VCaP was defined (see Experimental Procedures) and normalized expression of each gene after 24 hr of indicated drug treatments is depicted by heatmap and ranked by degree of induction with Dex.

(H) Expression of the top two genes from (B) (KLK2 and FKBP5) after 24 hr of indicated treatments ± SEM.

See also Figure S6.
antagonist could enhance the activity of enzalutamide in prostate cancers coexpressing GR and AR.

To determine whether Dex activates a subset of AR target genes in VCaP (as we observed in the LREX model), we derived a list of AR target genes in VCaP cells exposed to DHT and asked whether Dex could modulate these same AR target genes in the presence of enzalutamide. Dex restored expression of some targets (KLK2, FKBP5, HOMER2, and SLC45A3), but not others (DHCR24, SLC2A3, TRPM8, and TMEM79), analogous to the uneven restoration we observed in the LNCaP/AR model (Figure 6G). Dex also induced expression of the clinical biomarker PSA in these cells, further supporting the hypothesis that GR can drive PSA progression in enzalutamide-resistant patients (Figures S6B and S6C). To confirm that Dex activated genes via the glucocorticoid receptor, we evaluated the effect of compound 15 on Dex-induced transcriptional activity. As expected, compound 15 reduced Dex induction of the GR targets KLK2 and FKBP5 (Figure 6H). Similarly, GR knockdown prevented Dex-mediated induction of target genes (Figure S6C). As in the LREX system (Table S3), the vast majority of genes robustly regulated by GR activation in VCaP cells were also regulated by AR activation with DHT (Table S4). Others have recently shown substantial overlap in the AR and GR cistromes in VCAP as well (Sahu et al., 2013). These findings extend our hypothesis that GR promotes enzalutamide resistance largely by replacing AR activity at a subset of genes to a second model system.

A Subset of Prostate Cancer Is Primed for GR Induction in the Setting of AR Inhibition

In considering potential mechanisms for increased GR expression in drug-resistant tumors, we noted several observations that suggested two distinct models. First, flow cytometry analysis of LNCaP/AR and CS1 cells revealed GR expression in a rare subset of cells (Figure 1E), raising the possibility that these cells clonally expand under the selective pressure of antiandrogen therapy. Consistent with this model, we observed rare GR-positive cells in a tissue microarray analysis of 59 untreated primary prostate cancers (Table S5). However, we also observed a modest (~2-fold) but significant increase in GR mRNA levels in LNCaP/AR xenografts after only 4 days of antiandrogen treatment, which is reminiscent of an older report of increased GR expression in normal ventral rat prostate after castration (Davies and Rushmere, 1990). These findings suggest a second model of adaptive resistance whereby AR inhibition causes an increase in GR levels due to loss of AR-mediated negative feedback.

To investigate the relationship between AR activity and GR expression, we first asked whether the high level of GR expression in LREX tumors is maintained after discontinuation of enzalutamide. Remarkably, GR mRNA levels dropped by ~5-fold 8 days after treatment discontinuation (Figure 7A). Because enzalutamide has a prolonged half-life in mice (Tran et al., 2009), it is difficult to make definitive conclusions about negative feedback loops using in vivo models. Therefore, we conducted similar enzalutamide withdrawal experiments in LREX cells cultured in vitro. GR mRNA levels dropped as early as 1 day after discontinuation and continued to decline throughout the 23 days of the experiment (Figure 7B). Additional experiments with LREX cells using earlier time points in charcoal-stripped media showed reduced GR mRNA levels after only 8 hr of DHT exposure, and this reduction was reversed by cotreatment with enzalutamide (Figure 7C). This reduction correlated precisely with the recruitment of an AR binding peak in an intronic enhancer of GR identified by ChIP, suggesting that AR directly represses GR expression in these cells (Figure 7D).

To determine whether the loss of GR expression upon enzalutamide withdrawal occurs across the entire cell population or is restricted to a subset of cells, we conducted flow cytometry experiments in which a shift in median signal intensity can be used to identify expression changes in the bulk cell population. (Expression changes limited to a minority subpopulation would not affect the median and would instead be identified as a tail population by histogram plot.) We observed an exponential decay in median GR protein signal (half-life 7.6 days) (Figure 7E, top row, and Figure 7F), confirming that the loss in GR expression occurs across the entire LREX cell population. Extension of this experiment to later time points (17 weeks) revealed a plateau in loss of GR expression by 7 weeks (Figure S7A).

Next, we conducted the reciprocal experiment of re-exposure of LREX cells to enzalutamide following GR downregulation after prolonged enzalutamide withdrawal (LREX<sup>−/−</sup>). GR expression was regained with induction kinetics essentially reciprocating the rate of decay previously seen with removal of drug (doubling time 6.8 days), establishing that the resistant line remained poised for GR induction in the setting of AR inhibition (Figures 7E and 7F). Consistent with the timescale, continued drug exposure for 7 weeks was associated with a clear shift in GR expression in essentially all cells (Figure S7A).

We next determined whether AR inhibition is sufficient to induce GR expression in LNCaP/AR or CS1 cells that had not previously been exposed to enzalutamide. In contrast to LREX, there was no change in median expression intensity in CS1 or LnCaP/AR over the 4 week experiment, indicating that most cells do not turn on GR expression simply as a consequence of AR inhibition (Figures 7E, 7F, and S7C). However, the area under

Figure 7. Resistant Cells Are Primed for GR Induction upon AR Inhibition

(A) GR mRNA in LREX xenografts. Tumors were injected into castrated mice and immediately treated with 10 mg/kg enzalutamide (n = 20) for 7 weeks. Half of the mice were then continued on 10 mg/kg enzalutamide (n = 10 tumors) or discontinued for 8 days (n = 10 tumors).

(B) LREX<sup>−/−</sup> are maintained in vitro in the presence of enzalutamide 1 μM. GR mRNA was assessed in LREX<sup>−/−</sup> cell line after passage for indicated number of days in standard fetal bovine serum containing media without enzalutamide.

(C) GR mRNA in LREX<sup>−/−</sup> cultured in charcoal-stripped media for 48 hr and then treated for 8 hr with vehicle or DHT with or without 10 μM enzalutamide.

(D) AR ChIP-qPCR with LREX<sup>−/−</sup> cultured in charcoal-stripped media and then treated for 1 hr with DHT (1 nM) or Dex (100 nM) at an intronic enhancer site 2 SD.

(E) Intracellular GR flow cytometric analysis of indicated cells at indicated time points. AUC, area under curve. Enzalutamide, 1 μM.

(F) Plotted median fluorescence (minus background) values from (E) and Figure S7C. For both LREX plots, R<sup>2</sup> values for nonlinear regression analysis is >0.96.

(G) Model of GR induction in resistant tissues. See also Figure S7.
the GR staining population did increase. Given the weak anti-proliferative effect of enzalutamide in vitro (Figure S7B), we conclude that this increase in GR expression is most likely explained by loss of AR-mediated negative feedback rather than by clonal expansion. Together, these findings support a model in which a subset of prostate cancer cells are “primed” for GR induction in the context of AR inhibition through an adaptive resistance mechanism (via AR-mediated negative feedback). We postulate that these cells then clonally expand under the selective pressure of AR blockade, eventually emerging as drug-resistant tumors whose expression profiles may resemble those of AR-driven tumors but are driven by GR (Figure 7G).

**DISCUSSION**

Following the recent approvals of the next-generation AR pathway inhibitors abiraterone and enzalutamide, the treatment of metastatic prostate cancer has evolved to a two-stage process. Initially, patients receive conventional androgen deprivation therapy, typically with a gonadotropin-releasing hormone agonist that lowers testosterone (castration), often in conjunction with an androgen such as bicalutamide. Preclinical and clinical studies have conclusively demonstrated that acquired resistance to conventional androgen deprivation therapy is caused by restoration of AR pathway activation, primarily due to increased AR expression. These discoveries provided the rationale for the development of next-generation AR therapies.

Here, we demonstrate that acquired resistance to at least one of these new next-generation therapies, enzalutamide, can occur via a different mechanism—increased expression of GR. The evidence for GR-driven resistance emerged from two independent preclinical models (LNCaP/AR and VCaP) and was supported by correlative data showing increased GR expression in patients with enzalutamide resistance. Consistent with our mechanistic studies showing that GR can function independently of AR, increased GR expression was also associated with ARN-509 resistance, potentially forecasting a general mechanism of resistance to androgens. Whether increased GR expression plays a role in abiraterone resistance remains to be determined. Unlike enzalutamide and ARN-509, abiraterone impairs AR signaling by lowering residual systemic and intra-tumoral androgen levels, and preclinical evidence suggests that abiraterone resistance may be associated with increased AR expression (Mostaghel et al., 2011). We speculate that tumors can efficiently overcome the ligand deficiency conferred by traditional androgen-deprivation therapy or abiraterone by simply elevating AR levels, whereas the increased selection pressure conferred by second-generation androgens requires an alternative strategy such as GR bypass or AR mutation (Balbas et al., 2013; Joseph et al., 2013; Korpal et al., 2013).

Comparative AR and GR transcriptome studies supported a model whereby GR bypasses enzalutamide-mediated AR blockade without the need for any restored AR function. This model is further supported by ChIP-seq analyses showing that GR can bind to just over half of all AR binding sites in enzalutamide-resistant cells. Importantly, GR occupied a large number of sites that are not bound by AR, raising the possibility of a distinct GR transcriptional program that could contribute to resistance. However, our transcriptome analysis found that a large majority of genes robustly regulated by GR were also regulated by AR. Notably, several canonical AR targets genes, including KLK3 and TMPRSS2, show regulation by GR (Table S3). For these reasons, we believe that the antiandrogen resistance conferred by GR is most likely mediated by one or more of the unevenly restored AR target genes rather than a distinct set of “GR only” target genes. It will be of interest to explore whether just one or a small number of downstream targets are responsible for resistance and also why GR fails to activate transcription at the vast majority of the “GR unique” binding sites. We postulate that variables such as chromatin context, cofactors, and other signaling events may be important.

The GR bypass model of AR pathway blockade reported here is reminiscent of recent reports that kinase inhibitor blockade in various cancers can be overcome by upregulation of other kinases and/or their ligands (Engelman et al., 2007; Johannessen et al., 2010; Strausssman et al., 2012; Wilson et al., 2012). To our knowledge, our observation is the first example of nuclear receptor bypass as a mechanism of acquired resistance to nuclear receptor blockade. In the case of kinase inhibitors, bypass is just one of many potential resistance mechanisms that also includes direct mutation of the kinase target and lineage switching to histologically distinct phenotypes that no longer require the drug target for survival (Sequist et al., 2011). We believe the same may be true here based on the fact that a subset of drug-resistant LNCaP/AR tumors had minimal GR expression, raising the possibility of other resistance drivers. For example, one of these low GR tumors contained the F876L AR mutation that converts both ARN-509 and enzalutamide to agonists and is associated with clinical resistance (Balbas et al., 2013; Joseph et al., 2013; Korpal et al., 2013). A second low GR tumor expressed high levels of N-cadherin (Table S1C), which can confer AR independence by morphological conversion to a tumor with mesenchymal features (Tanaka et al., 2010).

Expression of GR in antiandrogen-resistant prostate tumors appears to occur by a mechanism that includes features of adaptive resistance (via AR-mediated negative feedback of GR expression), as well as clonal selection. Our data showed that AR inhibition induced strong GR expression in drug-resistant prostate cancer cells, as well as in a subset of drug-naive cells that are somehow “primed” to respond. The molecular basis for this “primed” state remains to be defined but, based on the reversibility of GR expression in the presence or absence of AR inhibition, is likely to involve an epigenetic mechanism. Knowledge of baseline tumor GR expression in patients, as well as the “primed” state of these tumor cells, could have clinical relevance as a treatment response biomarker. We already have evidence that baseline GR expression may predict for a poor clinical outcome despite a limited clinical data set and, based on the increase in GR expression in some patients after 8 weeks of treatment, that the “priming” phenomenon observed in our models may also be relevant in patients.

Whatever the precise mechanism regulating GR expression, one immediate implication is that corticosteroid therapy could be detrimental to prostate cancer patients in certain clinical contexts. Corticosteroids are currently administered routinely with both docetaxel and abiraterone to prevent side effects.
from each of these therapies. Our data suggest that corticosteroids might promote tumor progression in men whose tumors express GR. Indeed, reanalysis of the phase 3 clinical trial AFFIRM that demonstrated a survival benefit with enzalutamide treatment found that men receiving corticosteroids had a significantly worse survival than those who did not (Scher et al., 2012, ESMO, abstract; Scher et al., 2012). It is worth noting that corticosteroids can also confer clinical benefit in CRPC, an effect attributed to feedback suppression of pituitary ACTH production and resultant decrease in adrenal androgen production (Tannock et al., 1989). This duality of potential glucocorticoid effects should prompt a reexamination of the appropriate clinical context for corticosteroid therapy.

Our findings also suggest that combined inhibition of both GR and AR could prolong the duration of response with next-generation AR antagonists. Clinical studies of the GR antagonist mifepristone in patients with excess glucocorticoid production (Cushing syndrome) demonstrate that GR can be inhibited in humans with an acceptable risk-benefit profile (Fleseriu et al., 2012). Unfortunately, both mifepristone and a related GR antagonist ORG34517 activate AR target gene expression, likely by direct AR agonism because mifepristone binds and activates AR (Klokk et al., 2007). The ability of compound 15 to overcome GR-driven resistance should stimulate further efforts to optimize GR-specific antagonists that lack “off target” AR effects for use in preventing or overcoming enzalutamide resistance.

**EXPERIMENTAL PROCEDURES**

**Bone Marrow Evaluation**

Patients were treated with enzalutamide 160 mg daily. Bone marrow biopsy and aspirate (~5 ml) were performed before treatment and at week 8. The bone marrow specimens were obtained by transiliac biopsy, and samples were processed according to standard MD Anderson Cancer Center decalcification and fixation procedures. After pathologic evaluation, samples were stored in the MD Anderson Cancer Center Prostate Cancer Tissue Bank. Imaging studies were performed at the time of suspected prostate cancer progression or at the treating physician’s discretion but generally were not performed prior to 12 weeks posttreatment initiation. Therapy was discontinued at the treating physician’s discretion in patients exhibiting progression. Retrospective analysis for GR was performed by IHC on 3.5 mm formalin-fixed, paraffin-embedded bone marrow biopsy sections with anti-GR at a dilution of 1:200 (BD Transduction Laboratories 611227). A Dako autostainer and standard 3,3-diaminobenzidine were used. GR expression was assessed in a blinded fashion by two pathologists scoring at least 100 tumor cells per specimen. Plotted are either data from all specimens or only from patients with usable material at baseline and 8 weeks.

**AR Target Gene List Derivation**

The 74 AR target gene list utilized for evaluation of AR pathway status in the LNCaP/PRAR model includes all genes that showed at least a 1.6-fold change (FDR < 0.05) when comparing control and 4 day treated xenografts and that were also found to have an AR binding peak by ChIP-seq analysis of LNCaP/PRAR in vitro (L.C., D.Z., and C.L.S., unpublished data). The VCaP AR target gene list includes all genes that showed reciprocal expression change with 24 hr DHT (1 nM) or enzalutamide (10 μM) of at least 1.4-fold (p < 0.05) (Illumina HT-12) and were also found to have an AR binding peak by ChIP-seq analysis of VCaP (L.C., D.Z., and C.L.S., unpublished data).

**AR/GR Signature Analysis and GSEA**

AR and GR signature genes were defined as all genes showing >1.6-fold (FDR < 0.05) expression change with either 1 nM DHT or 100 nM Dex treatment, respectively, of LREX cells for 8 hr in charcoal-stripped media. For GSEA, signature genes induced by either DHT or Dex treatment were used. GR selective genes showed at least 1.1-fold higher expression in Dex-treated samples compared to DHT-treated samples (FDR < 0.05). AR selective genes showed at least a 1.1-fold higher expression in DHT-treated samples compared to Dex-treated samples (FDR < 0.05).

**Statistics**

Microarray data analysis and comparisons were performed with Partek Software. All RT-qPCR comparisons are by two-sided t test. Xenograft volumes and GR IHC of clinical specimens are compared by one-sided Mann-Whitney test. In vitro growth comparisons are by two-sided t test. GSEA statistical analysis was carried out with publicly available software from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp). In all figures, *p = < 0.05, **p = < 0.01, ***p = < 0.001, and ****p = < 0.0001.

**ACCESSION NUMBERS**

The GEO accession number for mRNA expression data reported in this paper is GSE52169, and the GEO accession number for DNA sequencing data reported in this paper is GSE51497.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.11.012.

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**Cell Lines**

LNCaP/AR and VCaP cells were maintained as previously described (Tran et al., 2009). LREX cells were derived from a single enzalutamide resistant tumor that was harvested, disaggregated with collagenase treatment, and then maintained in RPMI supplemented with 20% FBS and 1 μM enzalutamide. Cells were initially grown on collagen-coated flasks until confluent and then were maintained on standard tissue culture dishes. CS1 were similarly derived from vehicle treated tumors and maintained in standard LNCaP/AR media. LNCaP/AR and LREX cells were cultured in phenol-red free RPMI with 10% charcoal-stripped FBS prior to drug treatments.

**Xenografts**

For all experiments, tumors measurements were obtained weekly using the average of three consecutively obtained volume measurements calculated from three-dimensional calipers measurements. LNCaP/AR xenografts were established in castrate mice as described previously (Tran et al., 2009). Once tumor where established, mice were treated with either enzalutamide, ARN-509, or RD612 (10 mg/kg), or vehicle alone (1% carboxymethyl cellulose, 0.1% Tween-80, 5% DMSO) 5 days a week by oral gavage. 4 day treated mice received ARN-509. Vehicle treated mice were harvested after either 4 or 28 days of treatment. For the validation cohort, 25 tumors were initiated on treatment with intention to continue until resistance, from which 19 resistant tissues were harvested (16 of which had attained a volume greater than at start of treatment.) Xenografts with LNCaP/AR sub-lines were established by injecting two million cells per flank into castrate mice. Mice injected with resistant sub-lines were initiated on treatment with enzalutamide (10 mg/kg) immediately after injection. For xenograft knock-down experiments, cells were infected with virus expressing a control (NT) or GR targeting hairpin, selected with puromycin treatment, and then implanted.

**Global Transcriptome Analysis**

RNA extracted from xenograft tumors was analyzed by either Affymetrix HuEx1 (pilot cohort) or Illumina HT-12 (validation cohort, LREX) microarray. (A technical note: NR3C1 probe in Illumina HT-12 array appears to be non-functional and did not detect GR in any tissue, including LnCap/AR cells engineered to express high levels.) For LREX in vitro analysis, cells were plated into steroid depleted media for 48 hr prior to drug treatment. Drug treatments were performed in triplicate with a final concentration of 1nM DHT, 10nM or 100nM dexamethasone, and/or 10 μM enzalutamide for 8 hr. For VCaP in vitro analysis VCaP cells were maintained in standard media with complete fetal bovine serum and were treated in triplicate for 24 hr with vehicle, 0.1 nM DHT, 100nM Dex, and/or 10 μM enzalutamide. For analysis of Illumina HT-12 expression data, probes values were gene summarized using the mean of the probe values. Data were quantile normalized and analyzed with Partek software with correction for batch effects.

**Chromatin Immunoprecipitation**

LREX cells were maintained in steroid depleted media for 4 days. The day prior to drug treatment, cells were given fresh media. Material from two 15 cm plates of cells were divided for ChIP. For ChIP-seq, agonist stimulation was carried out for 30 min prior to harvest. Fixation and processing for was carried out as described by others (Goldberg et al., 2010). Immunoprecipitation was carried out with Anti-Androgen Receptor Antibody, PG-21 (Millipore) or Glucocorticoid Receptor Antibody #7437 (Cell Signaling). Immunoprecipitated DNA was quantified by picogreen and size was evaluated on a HighSense BioAnalyzer chip. Fragments between 100 and 600 bp were collected using an automated system (Pippin Prep, Sage Science) then end repaired, ligated and amplified for 15 cycles using reagents included in the Truseq DNA Sample Preparation kit from Illumina. Experimental conditions followed strictly the instructions of the manufacturer, with the exception of the adaptors being diluted 1/10 for the input DNA and 1/50 for all other samples. Barcoded libraries were run on a HiSeq 2000 in a 50bp/50bp paired end run, using the TruSeq SBS Kit v3 (Illumina). For ChIP-qPCR, ligand treatments were performed for 1 hr and fixation and processing was carried out using a chromatin immunoprecipitation assay kit (Millipore) in accordance with the manufacturer’s protocol. Immunoprecipitation was carried out with Anti-Androgen Receptor Antibody, PG-21 (Millipore), Glucocorticoid Receptor Antibody #3660 (Cell Signaling), or Normal Rabbit IgG (Millipore: 12-370).

**ChIP-Seq Data Analysis**

The sequencing reads (50 bp, paired-end) were aligned to the human genome (hg19, build 37) using the program Bowtie (Langmead et al., 2009). 8,201,777 and 18,876,986 reads from DHT-treated AR ChIP-seq and Dex-treated GR ChIP-seq LREX’ samples were aligned to a single genomic location with no more than two mismatches. These aligned reads were analyzed by the software MACS (Zhang et al., 2008) for peak identification with data from ChIP input DNAs as controls. The top 5,217 AR and 15,851 GR peaks were selected based on analysis of false discovery rate and peak intensities. Genes with peaks located from −50 kb of their transcription start sites to +5 kb of their transcription termination sites were defined as AR or GR targets, using the human RefSeq annotation as reference. The MEME software suite (Bailey et al., 2009) was applied to 100-bp sequences around the AR or GR peak summits for finding motifs, with the program MEME for motif discovery and MAST for motif scanning (p value < 0.001).
**ChIP-PCR Primers**

- **SGK1**
  F: CTTCCCACCACCTTGCTT, R: GAAAGTGCCAGAGGAGAC;
- **FKBP5**
  F: CCCCCTATTTTCAATCGGAGTAC, R: TTTTTGAAGCACAGAACACCCT;
- **KLK3**
  F: ATGTTCACATTAGTACACCTTGCC, R: TCTCAGATCCAGGCTTGCTTACTGTC;
- **NDRG1**
  F: ATGGCCCCAGATATGTTCCA, R: CCCAAGGTCTCAGAGCCAGT;
- **TIPARP**
  F: CGTCTGGGGAGTAGGCAAAAT, R: CCCGAGGGAGGATGTGAAC;
- **NR3C1**
  F: ACCAGACTGAATGTGCAAGC, R: AGGGTTTTTGATGGCACTGA.

**GR Expression and GR/AR Knockdown**

shRNA knock-down experiments were carried out by infection of LREX or VCAP cells with MISSION TRC2 pLKO.5-puro containing a nontargeting or GR specific hairpin (NT: GGGATAATGGTGATTGAGATGGCTCGAGCATTCAATCACCATTATCCTT, GR: CCGGCACAGGCTTGATCGAGGCGAGATCGGCTTGATCGAGGCGAG). siRNA knock-down experiments were performed Dharmacon SMARTpool: ON-TARGETplus AR siRNA, L-003400-00-0005 or ON-TARGETplus Non-targeting Pool, D-001810-10-20 according to manufactures protocol with a final concentration of 50 nM siRNA. For GR expression experiments, a stop codon was engineered into the NR3C1 alpha ORF (Origene RC204878) by PCR and then it was sub-cloned in pMIdT (a generous gift from Dr. Yu Chen, MSKCC.) pMIdT-EGFP was introduced into control cells. Infected cells were sorted by tdTomato expression using flow cytometry.

**In Vitro Growth Assays**

VCaP: Cells were plated in triplicate and then assayed in triplicate at the time points indicated using CellTiter-Glo (Promega). Viability is plotted normalized to day 1. For knockdown studies, cells were infected and then plated 3 days later for the experiment without prior drug selection. LnCaP/AR and sub-lines: Equivalent numbers of cells were plated and then harvested and counted in triplicate at indicated time points using the Beckman Coulter Vi-Cell XR. Cells were passaged at each time point and identical numbers of cells re-plated. Fold increase in cell numbers were determined for each time interval.

**Intracellular Staining and Flow Cytometric Analysis**

Cells were re-suspended in Fixation/Permeabilization working solution (eBioscience; San Diego, California, USA) at a concentration of 1-2 x 10^6 cells/ml for 30 min at room temperature. The cells were subsequently stained with primary antibodies, Rabbit (DA1E) mAb IgG XP Isotype Control, androgen receptor (D6F11) XP Rabbit mAb, or glucocorticoid receptor (D6H2L) XP Rabbit mAb (Cell Signaling Technology; Danvers, Massachusetts, USA) for 20 min at room temperature. The cells were washed twice with Flow Cytometry Staining Buffer (eBioscience; San Diego, California, USA), and then washed and then stained with secondary antibody, Allophycocyanin-AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; Westgrove, Pennsylvania, USA) for 20 min at room temperature. Following two more washes, the cells were re-suspended in Flow Cytometry Staining Buffer and analyzed by flow cytometry on a LSR II (BD Biosciences; San Jose, California, USA) using FlowJo software (Tree Star, Ashland, Oregon, USA). For GR staining, cells were maintained in their standard media and treated with dexamethasone for 20 min prior to harvest to fully expose antigen. For AR staining, cells were cultured in charcoal stripped media without added ligands for 3 days prior to harvest.

**RNA Extraction and RT-qPCR Analysis**

RNA was extracted from cell lines using the RNeasy kit (QIAGEN). Frozen tumors were lysed with lysing matrix A using the Fast-Prep24 tissue homogenizer system (MP BIOMEDICALS) in Trizol (Invitrogen) followed by clean up with RNeasy (QIAGEN). cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems.) Data were quantified relative to either beta Actin or GAPDH expression and relative expression was generally plotted. Primers for ACTB (PPH00073E), NDRG1 (PPH02202B), NR3C1 (PPH02652A), and SGK1 (PPH00387E), STK39 (PPH14239B), GRB10 (PPH05866B), TIPARP (PPH07883A), PMEPA1 (PPH01013B), GAPDH (F: TGGTTGCGTCAATGTATGAAAGC, R: AGCTCTTGGTTGTCTCTCAGT), FKB5 (F: CAGATCTCCATGTGCCAGAAG, R: CTCGCCCTGTTTATTGG), GAPDH (F: TGCCAGAACCACCTGCTT, R: GGCATGGACTGTGGTCATGAG) and KLK3 (F: GCTCT GGCTTGTTTCT, R: TGCCAGACCAGCAAGATC).

**Protein Extraction and Western Blot Analysis**

Protein was extracted from cell lines using M-PER Reagent (Thermo Scientific). Protein was extracted from frozen tumors with lysing matrix A using the Fast-Prep24 tissue homogenizer system (MP BIOMEDICALS) using 1% SDS, 10 mM EDTA and 50 mM Tris, pH 8.0. Protein was quantified by BCA Protein Assay (Thermo Scientific). The following antibodies were used for Western blots: anti-AR PG-21 at 1:5000 (Milipore 06-680), anti-GR at 1:1000 (BD Transduction Laboratories 611227), b-actin at 1:20,000 (AC-15, Sigma), anti-cPARP at 1:1000 (Cell Signaling #9541).

**Cell Line, Xenograft, and Tissue Microarray IHC**

Cell line pellets or tumor pieces were fixed in 4% PFA prior to paraffin embedding and then were stained for GR at 1:200 with anti-glucocorticoid receptor (D6H2L) XP Rabbit mAb (Cell Signaling Technology, #12041) using the Ventana BenchMark ULTRA. TMA
was stained for GR at 1:200 with anti-glucocorticoid receptor (BD Transduction Laboratories #611227) using the Ventana BenchMark ULTRA.

Drugs
DHT and Dexamethasone were purchased from Sigma. ARN-509, RD162, and enzalutamide were all synthesized by the organic synthesis core at MSKCC. Compound 15 was a gift from Tom Scanlan (OHSU). All drugs were dissolved in DMSO in 1000X stocks.

SUPPLEMENTAL REFERENCES


Figure S1. AR Expression in LREX, Related to Figures 1 and 2

(A) Indicated cells were cultured in vitro, in charcoal-stripped media without enzalutamide for 3 days and then analyzed for AR expression by intracellular AR flow cytometric analysis.

(B) LnCaP/AR control xenografts (n = 6, same samples as in Figure 1D) or enzalutamide (10 mg/kg) treated LREX xenografts (n = 8) were analyzed by GR and AR Western blot. AR Western blot signals were quantified using Image J software.
Figure S2. GR Induction Dichotomized Based on PSA Response, Related to Figure 3
GR IHC scores in matched baseline and 8 week samples (same as in Figure 3E) dichotomized based on maximal PSA response ± SEM. Comparisons are by Mann-Whitney test.
Figure S3. Expression of AR Target Genes in Resistant Tumors from Validation Cohort, Related to Figure 4
Normalized expression array signal (Illumina HT-12) of a suite of 74 AR target genes in control (n = 10), 4 day (n = 8), and resistant tissues from the validation cohort described in Figure 1 (n = 12 of 16). The bottom quartile of GR expressing tissues were excluded from the analysis of the validation cohort tissues to minimize contamination from other resistance drivers (see Table S1C). Genes are ranked by degree of restoration of expression in resistant tissue (Res-4 day) / (Control-4 day). All resistant tissues were continued on anti-androgen treatment through time of harvest.
Figure S4. Dexamethasone Activity Is GR, but Not AR, Dependent, Related to Figure 4
(A) LnCaP/AR cells engineered to express GFP or GR were treated with indicated drugs.
(B) Western blot confirmation of GR expression in cells used in A.
(C) Cotreatment of LREX cells with Dex and compound 15 and assessment of target gene expression.
(D) Control or AR siRNA knock-down in LREX followed by treatment with indicated drugs. For all panels: V = Vehicle, DHT = 1nM, Dex = 100nM (unless otherwise indicated), CMP 15 = 1 μM, Enz = 10 μM. Cells were treated in charcoal-stripped media. Expression determined by RT-qPCR ± SEM.
Figure S5. Comparative AR and GR Cistrome Analysis, Related to Figure 5

(A) ChIP-seq signal strength for AR or GR at unique and overlap peaks in the AR or GR defined cistromes.

(B) AR and GR ChIP-qPCR at indicated AR target genes after treatment of LREX in steroid depleted media with DHT (1nm), Dex (100nM), and/or enzalutamide (10 μM) for 1 hr as indicated ± SD.

(C) Integration of transcriptome and cistrome analysis. 56 AR signature genes transcriptionally regulated by DHT in LREX were also found to have AR binding peak. Of those, 49 also showed at least modest regulation by Dex (1.2 fold, p < 0.05). The percent of the 49 genes showing Dex regulation (yes) or the 7 showing no Dex regulation (no) that have an AR/GR overlap peak is shown.
Figure S6. GR Expression and Activity in VCaP, Related to Figure 6

(A) GR IHC of VCAP of cells in standard media treated with vehicle or Dex 100nM + Enz 10 μM for 30 min prior to fixation.
(B) KLK3(PSA) Western blots of VCaP lysates generated from cells in standard media treated with indicated drugs for 3 days. DHT = 0.1 nM, Dex concentrations are indicated (nM), Enz = 10 μM.
(C) Expression analysis using RT-qPCR of VCaP infected with a non-targeting or GR-targeting hairpin. Cells were treated in standard media as indicated for 24 hr prior to harvest. Dex = 100nM, Enz = 10 μm. ± SEM.
Figure S7. GR Expression in Resistant and Sensitive Cells, Related to Figure 7

(A) GR intracellular staining and flow cytometric analysis of LREX⁺ or LREX⁻ cells after either vehicle (left) or 1 μM enzalutamide (right) treatment for indicated time.

(B) Relative cell numbers determined by cell counting (Vi-cell) of indicated cells with vehicle or 1 micro-molar enzalutamide treatment.

(C) Intracellular GR flow cytometric analysis of indicated cells at indicated times points. AUC = area under curve. Enzalutamide = 1 μM.