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Glucocorticoid Receptor Confers Resistance to Anti-Androgens 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 novel 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., 2012b) (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,

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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 novel anti-androgens that restore AR inhibition in the setting of increased AR levels. These efforts yielded three second-generation anti-androgens: 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 FDA approval of enzalutamide in 2012 based on increased survival (Scher et al., 2012b).

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 novel and 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, Supplemental Table 1A). Tissue from fifteen resistant tumors obtained from long term antiandrogen treated mice (n=6 ARN-509, n=9 RD162) and from three 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 up-regulated genes in the resistant tumors was the glucocorticoid receptor (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 DHT treated LnCaP/AR cells, *in vitro* (Supplemental Table 1B)), 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,

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was among the most up-regulated genes, but several other androgen-induced genes (PMEPA1, SNAI2, KCNN2, LONRF1, SPOCK1) were among the most repressed. Conversely, several androgen-repressed genes (UGT2B15, PMP22, CAMK2N1, UGT2B17) were among the most up-regulated (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.

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 mRNA levels in 10 control, 8 short term treated (4 day) and 16 resistant tumors were substantially higher in resistant tissues 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 (Supplemental Table 1C). In contrast to GR, AR RNA or proteins levels were not consistently different across the treatment groups (Figure 1C,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 (Figure 2A,2B). Strong expression of GR was confirmed in multiple LREX' xenograft tumors by western blot and by IHC (Figure S1B, 2C). 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 stromal or endothelial cells (blue arrows), some appeared epithelial (black arrow), consistent the with flow cytometry analysis (Figure 1E, black arrows).

To determine whether GR expression is required to maintain the drug-resistant phenotype, LREX' cells were infected with a shRNA targeting GR (shGR) and stable knockdown of GR protein was confirmed (Figure 2F). Tumor growth of shGR infected LREX' cells was significantly delayed relative to shNT (non targeted)-infected cells in castrated mice treated with enzalutamide (Figure 2D). In contrast, shGR had no impact on the growth of GR-negative CS1 xenografts, diminishing the possibility of an off-target effect (Figure 2E). Of note, shGR LREX' xenografts harvested on day 49 showed decreased GR protein knockdown compared to the pre-implantation levels, indicative of selective pressure against

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GR silencing in the setting of enzalutamide treatment (Figure 2F). These findings provide direct evidence that GR drives enzalutamide resistance *in vivo*.

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 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, while 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 3C). 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 (Figure 3C,D). 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% vs 8%, p=.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=.02) (Figure 3C,D), 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 was 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 PSA levels also correlate with GR expression raises the question of whether transcriptional regulation of a canonical AR target gene may be regulated by GR.

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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 if 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 (Figures S3) as well as eight LREX' tumors (Figure 4A) (see experimental procedures and Supplementary Table 2 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, STK39*) while other genes (*NDRG1, TIPARP, PMEPA1*) showed no evidence of restored expression.

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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, while a gene whose expression in resistance equals its expression after 4 days of antiandrogen treatment equals 0. (Values greater than one indicate hyper-restoration 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 .64, $p = 7.54 \times 10^{-10}$, Figure 4B). This finding is most consistent with a model in which AR remains

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inhibited in drug-resistant tumors but expression of certain AR target genes is restored by an alternative transcription factor, possibly GR. The fact that AR 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 if 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 7 AR targets that represent the spectrum of restoration noted in the *in vivo* analysis, as well as PSA (Figure 4D). All 8 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 (Figure S4A, S4B). To be sure that the effects of Dex in these models are mediated through GR, we co-treated cells with a previously described competitive GR antagonist that lacks AR binding called compound 15 (Wang et al., 2006). Compound 15 significantly decreased expression of Dex-induced genes, confirming that Dex activity in the LREX' model is GR-dependent (Figure S4C). Lastly, 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<.05) after 1 nM DHT or 100 nM Dex treatment (Supplementary Table 3). 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 (Supplementary Table 3). 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

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