BET Bromodomain Inhibitors Enhance Efficacy and Disrupt Resistance to AR Antagonists in the Treatment of Prostate Cancer

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Abstract

Next-generation antiandrogen therapies, such as enzalutamide and abiraterone, have had a profound impact on the management of metastatic castration-resistant prostate cancer (mCRPC). However, mCRPC patients invariably develop resistance to these agents. Here, a series of clonal cell lines were developed from enzalutamide-resistant prostate tumor xenografts to study the molecular mechanism of resistance and test their oncogenic potential under various treatment conditions. Androgen receptor (AR) signaling was maintained in these cell lines, which acquired potential resistance mechanisms, including expression of AR-variant 7 (AR-v7) and glucocorticoid receptor. BET bromodomain inhibitors were shown previously to attenuate AR signaling in mCRPC; here, we demonstrate the efficacy of bromodomain and extraterminal (BET) inhibitors in enzalutamide-resistant prostate cancer models. AR antagonists, enzalutamide, and ARN509 exhibit enhanced prostate tumor growth inhibition when combined with BET inhibitors, JQ1 and OTX015, respectively. Taken together, these data provide a compelling preclinical rationale to combine BET inhibitors with AR antagonists to subvert resistance mechanisms.

Implications: Therapeutic combinations of BET inhibitors and AR antagonists may enhance the clinical efficacy in the treatment of mCRPC.

Visual Overview: http://mcr.aacrjournals.org/content/14/4/324/F1.large.jpg.

Introduction

Metastatic castration-resistant prostate cancer (mCRPC) leads to nearly 30,000 deaths annually in the United States (1). Androgen receptor (AR) is a major driver alteration in mCRPC (2–4), and most tumors continue to rely on AR signaling (3). Second-generation antiandrogen therapies, such as abiraterone and enzalutamide, have become standard-of-care treatments for mCRPC (5, 6). However, most patients eventually develop resistance to these therapies, and thus, alternate approaches to target AR signaling are needed.

Recently, our group and others have shown that bromodomain and extraterminal (BET) inhibitors block mCRPC growth in animal models (7–9). BET inhibitors are epigenetic therapies that target bromodomain-containing proteins BRD2/3/4 and BRDT (10–12). They appear to preferentially affect oncogenic transcription through a super-enhancer–based mechanism (13, 14). mCRPC is a particularly attractive indication for BET inhibitors due to its reliance on oncogenic transcription factor signaling mediated by AR, ETS fusions, and MYC (7). Several groups, including our own, have shown that AR signaling is affected by BET inhibitors (7, 8, 15).

In this study, we report the efficacy of BET inhibitors in enzalutamide-resistant mCRPC models. We developed enzalutamide-resistant prostate cancer cells from murine xenograft models in an attempt to understand the mechanisms of resistance to enzalutamide. Clonal cell lines derived from distinct enzalutamide-resistant LNCaP-AR and VCAP tumors displayed significantly higher AR expression and signaling relative to controls. In addition, enzalutamide-resistant CRPC cell lines maintained sensitivity to BET inhibitors, leading to attenuation of AR signaling. Interestingly, AR-variant 7 (AR-v7), which has been reported to be associated with resistance to antiandrogen treatments (16), was specifically elevated in enzalutamide-resistant VCaP cells and was markedly repressed by BET inhibitors. In addition to inhibiting
the growth of enzalutamide-resistant CRPC cell lines, BET inhibitors displayed enhanced efficacy in vivo when combined with antiandrogens, such as enzalutamide and ARN509.

Materials and Methods

Murine xenografts

Procedures involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan (Ann Arbor, MI) and conform to all regulatory standards. Please refer to Supplementary Materials and Methods for details.

Cell culture and viability assays

Prostate cancer cells were grown in ATCC-recommended media. Please refer to Supplementary Materials and Methods for details.

qRT-PCR

Primer sequences are provided in the Supplementary Table S1. RNA extraction, cDNA synthesis, and qRT-PCRs were performed as described previously (7).

Western blot analyses

Antibodies used in this study are listed in Supplementary Table S2. Western blot analysis was performed as described previously (7).

Results and Discussion

The LNCaP-AR cell line was the key preclinical model used in the development of enzalutamide, where in castrated male mice, established LNCaP-AR xenograft tumors, regressed upon enzalutamide treatment (17). To study the mechanism of resistance to enzalutamide, we performed an in vivo drug efficacy experiment in castrated mice bearing LNCaP-AR tumor xenografts. Consistent with previous studies (17, 18), we initially observed robust tumor regression in enzalutamide-treated animals compared with vehicle-treated controls (Fig. 1A and B). However, after about 47 days, the tumors became refractory to enzalutamide treatment and tumor growth resumed. Next, we analyzed AR and AR target gene expression levels in enzalutamide-resistant clones (Fig. 1C and D). AR expression was elevated at the transcript and protein levels, as well as AR target genes, compared with vehicle controls. Interestingly, a few of the tumors displayed high levels of glucocorticoid receptor (GR) transcript and protein expression as reported earlier (18). However, we did not observe high AR signaling, as measured by AR target gene expression, in the GR-overexpressing tumors (Fig. 1C; green boxes, tumor with GR outlier).

Furthermore, to expand the model systems used to study the mechanisms of resistance to enzalutamide, we conducted a murine xenograft experiment using VCaP prostate cancer cells that harbor the TMPRSS2:ERG gene fusion and AR amplification, both of which are frequent molecular aberrations in patients with advanced mCRPC. Treatment of VCaP tumor–bearing mice with enzalutamide (30 mg/kg) for 5 days/week over 5 weeks led to a small but significant reduction in tumor volume compared with vehicle controls (Supplementary Fig. S1A). Furthermore, AR and ARv7 expression levels were elevated in the enzalutamide-treated tumors relative to vehicle controls (Supplementary Fig. S1B), consistent with a previous report that found elevated ARv7 expression in circulating tumor cells from mCRPC patients with acquired resistance to enzalutamide and abiraterone treatment (16). In addition, AR downstream targets, such as SLCA45A3, FKBP5, KLK3, TMPRSS2, and MYC, were also upregulated in enzalutamide-treated VCaP tumors.

To evaluate the efficacy of BET inhibitors in enzalutamide-refractory disease, we established multiple cell lines from enzalutamide-resistant LNCaP-AR and VCaP tumors that were harvested and cultured in vitro in the presence of 5 μmol/L enzalutamide (Fig. 2A and B). In LNCaP-AR enzalutamide-resistant cells, all clones expressed varying levels of AR and PSA, whereas only 1 of 5 clones displayed high GR expression (Fig. 2A). In VCaP enzalutamide-resistant cell lines, we observed a significant increase in full-length AR and ARv7 levels, with active AR signaling in all clones (Fig. 2B and Supplementary Fig. S1). As these tumor-derived cell line clones are enzalutamide-resistant, we asked whether these cells would respond to a BET inhibitor. We first directly compared the efficacies of three different BET inhibitors, JQ1, OTX-015, and I-BET762 in LNCaP versus LNCaP-AR cells and found the first two compounds to be equally efficacious in LNCaP-AR cells, with an IC50 value of approximately 65 nmol/L, whereas parental LNCaP cells displayed lower sensitivity to the compounds with an IC50 value of approximately 110 nmol/L (Supplementary Fig. S2A and S2B). We chose JQ1 as the representative BET inhibitor in subsequent studies. We treated 3 independent enzalutamide-resistant LNCaP-AR sublines with varying concentrations of JQ1 and analyzed for cell viability 4 days posttreatment and found that all of them were sensitive to JQ1, with IC50 values of approximately 100 nmol/L (Fig. 2C). To evaluate the long-term effects of JQ1 treatment in the presence of enzalutamide, we performed colony formation assays. As expected, parental LNCaP-AR cells were sensitive, whereas the enzalutamide-resistant clones were insensitive to enzalutamide treatment (Fig. 2D). However, even at low concentrations (100 nmol/L) of JQ1, proliferation of enzalutamide-resistant sublines was severely inhibited in the long term (Fig. 2D), demonstrating that these AR signaling–positive cells are inherently susceptible to BET inhibition.

In parallel experiments, we tested enzalutamide-resistant VCaP derivatives for sensitivity to BET inhibition: 3 independent enzalutamide-resistant VCaP sublines were treated with varying concentrations of JQ1 and analyzed for cell viability 4 days posttreatment. All 3 resistant sublines along with parental VCaP cells displayed sensitivity to JQ1 with IC50 values of less than 100 nmol/L (Fig. 2E). As expected, VCaP parental cells were sensitive to enzalutamide in long-term colony formation assays. Although enzalutamide-resistant VCaP sublines were insensitive to enzalutamide, these cells were sensitive upon JQ1 treatment in long-term colony formation assay (Fig. 2F), demonstrating that these AR-amplified, ARv7–overexpressing cell lines are also susceptible to BET inhibition.

As enzalutamide-resistant clones showed an increase in AR levels but nonetheless remained sensitive to BET inhibitors, we examined whether BET inhibitors would have an effect on AR-mediated gene expression in these cells. Three independent enzalutamide-resistant LNCaP-AR sublines were treated with vehicle or two different concentrations of JQ1, and the expression of AR target genes was analyzed by qRT-PCR and Western blot analysis. As shown in Fig. 3A, FKBP5, KLK3, TMPRSS2, and MYC were transcriptionally downregulated upon JQ1 treatment in the

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in all three enzalutamide-resistant sublines. Similarly, PSA and MYC proteins that were expressed in the presence of enzalutamide in the resistant sublines were significantly repressed upon JQ1 treatment (Fig. 3B). Interestingly, AR protein levels were elevated with increasing doses of JQ1 in the parental LNCaP-AR and resistant sublines (Fig. 3B), suggesting perturbation of a negative feedback loop controlling AR levels.

Similarly, we sought to determine the effect of JQ1 on AR signaling and, in particular, AR-v7 expression in enzalutamide-resistant VCaP sublines. Parental VCaP and 3 independent enzalutamide-resistant sublines described above were treated with JQ1, followed by qRT-PCR and Western blot analysis of AR target genes. FKBP5, KLK3, ERG, and MYC were transcriptionally downregulated upon JQ1 treatment (Fig. 3C); ERG, MYC, and PSA

Figure 1.
Active AR signaling in enzalutamide-resistant xenograft tumors. A, LNCaP-AR cells were implanted subcutaneously in castrated mice and grown until tumors reached a size of approximately 100 mm³. Xenografted mice were randomized and received vehicle or 10 mg/kg enzalutamide 5 days a week. Mean tumor volume ± SD is shown. B, individual tumors from A are shown. C, qRT-PCR analysis of AR and AR target genes in LNCaP-AR xenograft tumors. Blue box with green boundary indicates tumor sample with outlier GR expression. D, Western blot analysis in tumor. Parental LNCaP-AR and 22RV1 were positive controls. Note tumor sample 973L with outlier GR protein and corresponding PSA levels. The sample number denotes the animal ID.
Figure 2.
Enzalutamide-resistant tumor-derived cells maintain BET inhibitor sensitivity. A, Western blot analysis with lysates from enzalutamide-resistant tumor-derived LNCaP-AR cell lines (ERTC). B, Western blot analysis with lysates from enzalutamide (Enz.)-treated tumor-derived VCaP cell lines (n = 8). VCaP cells treated with DMSO or 5 μmol/L enzalutamide served as control. Note the overexpression of full-length AR and AR-variant in enzalutamide-resistant VCaP derivatives. C, cell viability curves for three independent enzalutamide-resistant LNCaP-AR cells treated with JQ1. Crystal violet imaging of the 96-well plate is shown. D, colony formation assay; cells were cultured in the presence or absence of drugs as indicated for 14 days, followed by staining. Quantification is shown at bottom right. E, cell viability curves for three independent enzalutamide-resistant VCaP cancer cells treated with JQ1. Parental VCaP cells served as control. F, colony formation assay; cells were cultured in the presence or absence of drugs as indicated for 14 days, followed by imaging. Quantification (relative number of cells) is shown at bottom right.
protein levels were also reduced (Fig. 3D). Surprisingly, AR-variant protein levels were downregulated, but not full-length AR, in all 3 resistant sublines upon JQ1 treatment (Fig. 3D, top); AR-variant was confirmed to be AR-v7 by AR-v7–specific antibody (Fig. 3E). Splicing factors, SRSF1 and U2AF65, reported to be involved in the generation of AR splice variants (19), were subject to downregulation by JQ1 and would explain the downregulation of AR-v7 observed here (Fig. 3F). Interestingly, the SRSF1 and U2AF65 promoter regions, enriched for BRD2/3/4 protein, displayed reduced levels upon JQ1 treatment (Supplementary Fig. S3). These data suggest that the antiproliferative effect of JQ1 in the enzalutamide–resistant cells is partly due to its inhibitory effect on AR-v7 generation, which has been proposed as one of the major drivers of resistance to androgen deprivation therapy (16).

To extend upon the in vitro studies above, we next sought to conduct BET inhibition studies in vivo using the castrated VCaP...
Figure 4.
BET inhibitor in combination with enzalutamide or ARN-509 demonstrates enhanced antitumor activity: A, castrated mice bearing VCaP CRPC xenograft received vehicle or 10 mg/kg enzalutamide or 50 mg/kg JQ1 or enzalutamide/JQ1 combination as indicated 5 days/week. Percentage tumor volume ± SEM is shown. Statistical significance by two-tailed Student t test. *, P = 0.012; **, P < 0.0001; ***, P = 0.005. B, cumulative incidence plot depicting the percentage of tumors in each treatment group that have doubled in volume as a function of time. C, qRT-PCR analysis of indicated target gene expression in xenograft tumors. Relative fold expression with mean ± SEM is shown. Statistical significance by two-tailed Student t test. *, P = 0.0045; **, P < 0.001; ***, P = 0.0001; P = 0.007. E, same as in B. F, same as in C.
xenograft mouse model (7). Although VCaP cells are originally derived from a patient with CRPC (20), these cells require androgen supplementation for growth in culture, and therefore, VCaP tumor xenografts are castration resistant in mouse models (21). Castrated VCaP tumor–bearing mice were maintained until the tumors reached their original precastration volume and then treated with JQ1 (50 mg/kg/day), enzalutamide (10 mg/kg/day), or a combination. As observed previously (7), JQ1 alone led to approximately 45% reduction in the castration-resistant VCaP tumors compared with vehicle controls at 30 days after treatment. However, when JQ1 was combined with enzalutamide, tumor growth was inhibited by 62% (Fig. 4A). Furthermore, we assessed the doubling of tumor volume (time interval between initiation of treatment and tumor progression) by generating Kaplan–Meier survival curves and compared the treatment groups using log rank test. Tumor progression was delayed for the JQ1-treated group (P = 0.0008), with a median tumor doubling of 15 days, whereas tumor doubling for vehicle-treated mice was 10.5 days, with no significant difference in the enzalutamide alone treatment group (12 days; P = 0.215). However, the combination treatment group displayed a marked delay in tumor progression (P < 0.0001), with a median doubling of 22 days (Fig. 4B). The qRT-PCR analysis of tumors from the various treatment groups showed robust silencing of driver oncogenes, AR-v7, ERG, and MYC in mice treated with JQ1 alone or in combination with enzalutamide, whereas treatment with enzalutamide alone showed no change or an increase in AR and AR-v7 expression, respectively, compared with vehicle controls (Fig. 4C). In addition, in an AR- and ERG-positive patient-derived xenograft model, we observed greater antitumor efficacy of JQ1 and enzalutamide in combination than JQ1 alone (Supplementary Fig. S4). Interestingly, JQ1 treatment did not show antitumor activity in AR- and ERG-negative patient-derived xenografts.

Next, we tested the in vivo efficacy of OTX-015, a BET inhibitor that is being evaluated in a phase I/II clinical trial that includes metastatic prostate cancer, alone or in combination with ARN-509, a second-generation antiandrogen related to enzalutamide (22), in the castrated VCaP xenograft mouse model. As shown in Fig. 4D, treatment with a combination of OTX-015 and ARN-509 led to robust antitumor activity, resulting in 82% growth inhibition in mice, in contrast to 53% and 60% inhibition in mice treated with ARN-509 or OTX-015 alone, respectively. Likewise, the progression-free survival as measured by tumor doubling was significantly increased for all the treatment groups (P < 0.0001), with median doubling of 21 and 21.5 days for ARN-509 and OTX-015, respectively, compared with 7 days for vehicle-treated group. The median doubling time for the combination treatment group could not be determined over the course of the experiment as the group did not achieve threshold progression (Fig. 4E). Interestingly, ARN-509 alone had improved antitumor activity in this model than enzalutamide as was observed in LNCap-AR model by Clegg and colleagues (23). Furthermore, gene expression analysis of tumors from the OTX-015 and ARN-509 combination treatment groups showed robust silencing of AR-v7, ERG, and MYC, whereas AR and AR-v7 levels were elevated in tumors from mice treated with ARN-509 alone compared with vehicle controls (Fig. 4F). These in vivo data demonstrate enhanced efficacy of combined BET inhibitors and antiandrogens.

Multiple paths leading to primary or acquired resistance to antiandrogen therapies have been reported including overexpression of androgen synthesis enzymes, amplification, and point mutations in AR, overexpression of AR splice variants, and induction of GR (16, 18, 24, 25). Our cell line and in vivo mouse data recapitulated several of these paths to resistance to enzalutamide, and we demonstrated that BET inhibitors could overcome this resistance. The BET inhibitor efficacy data presented here have implications on the design of subsequent clinical trials that will most likely follow the ongoing multiple phase I trial of BET inhibitors in CRPC (www.clinicaltrials.gov). We anticipate that combining BET inhibitors with second-generation antiandrogens, such as enzalutamide or ARN-509, will result in more durable therapeutic responses.

Disclosure of Potential Conflicts of Interest
A.M. Chinnaiyan and S. Wang are co-founders of Oncofusion Therapeutics, which is developing novel BET bromodomain inhibitors. I.A. Asangani has served as consultant to Oncofusion Therapeutics.

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References


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A diagrammatic summary of the major findings and biological implications:
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