Prostate cancer has the second highest incidence among cancers in men worldwide and is the second leading cause of cancer deaths of men in the United States. Although androgen deprivation can initially lead to remission, the disease often progresses to castration-resistant prostate cancer (CRPC), which is still reliant on androgen receptor (AR) signaling and is associated with a poor prognosis. Some success against CRPC has been achieved by drugs that target AR signaling, but secondary resistance invariably emerges, and new-generation drugs are urgently needed. Recently, inhibitors of bromodomain and extra-terminal (BET) family proteins have shown growth-inhibitory activity in preclinical models of CRPC. Here, we demonstrate that ARV-771, a small-molecule pan-BET degrader based on proteolysis-targeting chimera (PROTAC) technology, demonstrates dramatically improved efficacy in cellular models of CRPC as compared with BET inhibition. Unlike BET inhibitors, ARV-771 results in suppression of both AR signaling and AR levels and leads to tumor regression in a CRPC mouse xenograft model. This study is, to our knowledge, the first to demonstrate efficacy with a small-molecule BET degrader in a solid-tumor malignancy and potentially represents an important therapeutic advance in the treatment of CRPC.

**Significance**

We describe the development of a small molecule that mediates the degradation of bromodomain and extra-terminal (BET) proteins and its application in the treatment of castration-resistant prostate cancer (CRPC). Few therapeutic options exist to treat CRPC, especially CRPC tumors expressing constitutively active androgen receptor (AR) splice variants that lack the ligand-binding domain and can effect androgen-independent transactivation of target genes. Importantly, we demonstrate that targeted degradation of BET proteins using proteolysis-targeting chimera (PROTAC) technology causes cell death in cultured prostate cancer cells and results in tumor growth inhibition or regression in mouse models of CRPC, including models that express high levels of AR splice variant 7. Our work thus contains a significant potential therapeutic advance in the treatment of this cancer.

**Disclosure of Potential Conflicts of Interest**

K.R., J.L., Y.Q., J.D.W., A.P.C., C.M.C., and K.G.C. have a financial interest in Arvinas, LLC, which has licensed the BET PROTAC platform from the University of Connecticut. A.P.C. and C.M.C. are shareholders in Arvinas, LLC. J.L. and Y.Q. have contributed reagents/analytic tools. K.R., J.L., M.A., D.G., A.M.K.R., J.D.W., A.P.C., and K.G.C. designed research; K.R., J.L., Y.Q., J.D.W., A.P.C., C.M.C., and K.G.C. wrote the paper. All authors read and approved the final version of the manuscript.

Conflict of interest statement: C.M.C. is the founder and Chief Scientific Advisor of, and possesses shares in, Arvinas, LLC. K.R., J.L., and Y.Q. contributed equally to this work.


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ARV-771 Treatment of CRPC Cells Results in Apoptosis. We next examined the effect of ARV-771 on cell proliferation. In the three

Fig. 1. ARV-771 is a potent pan-BET degrader. (A) Chemical structures of ARV-771 and the inactive diastereomer ARV-766, which is unable to bind VHL. (B) Incubation of the indicated CRPC cell lines with ARV-771 for 16 h results in depletion of BRD2/3/4 in 22Rv1, VCaP, and LnCaP95 cells. The Western blot is representative of three independent experiments (n = 3). (C) ARV-771 treatment for 16 h results in suppression of cellular c-MYC levels measured by ELISA. The assay was performed in triplicate (n = 3). (D) ARV-771-mediated c-MYC suppression occurs at the mRNA level, as determined by qPCR analysis following 16-h treatment at the indicated concentrations. c-MYC levels were also monitored in the same cell lines by qPCR following a 16-h treatment with either 1 μM ARV-766 or 1 μM OTX015. The results shown represent an average of two biological replicates, each measured in triplicate (n = 3). (E) ARV-771–mediated BRD4 degradation at 8 h is blocked by 30-min pretreatment with either an excess of VHL ligand ARV-056 (10 μM) or the proteasome inhibitor carfilzomib (1 μM). The Western blot is representative of two independent experiments (n = 2). All data represent mean values ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). P values were determined using GraphPad Prism using an unpaired parametric t test with Welch’s correction.
cell lines tested (22Rv1, VCaP, and LnCaP95), ARV-771 was 10- to 500-fold more potent than JQ-1 or OTX015. Under our test conditions, both the diastereomer ARV-766 and enzalutamide had minimal effect on the proliferation of any of these cell lines, and the VHL ligand AR-V056 was completely inactive (Fig. 2 A and C). Notably, ARV-771 treatment had a pronounced effect on cell morphology consistent with apoptosis (Fig. S2E), which we corroborated by demonstrating that ARV-771 treatment was associated with significant caspase activation (Fig. 2 B and D).

Finally, we confirmed the rapid induction of apoptosis with ARV-771 by demonstrating significant poly (ADP-ribose) polymerase (PARP) cleavage in 22Rv1 cells 16 h after PROTAC treatment (Fig. 2E). Under the same conditions, ARV-766 and the BET inhibitor OTX015 failed to induce any detectable PARP cleavage.

**ARV-771 Suppresses FL-AR and AR-V7 Expression.** Although BET inhibitor activity against CRPC cells arises from mechanisms such as c-MYC suppression as well as from the inhibition of AR-driven transcription (19, 20), we hypothesized that BET depletion with ARV-771 may result in additional cellular effects. Interestingly, ARV-771, but not JQ-1 or OTX015, significantly lowered AR protein levels in VCaP cells as measured by ELISA (Fig. 3A) and immunoblotting (Fig. 3B). Although only FL-AR was detectable at the protein level in VCaP cells, expression of the mRNA encoding both FL-AR and the AR-V7 could be measured readily. AR-V7 is the best studied of the transcriptionally active AR splice variants detected in the clinic and has been hypothesized to play a role in acquired resistance to enzalutamide and abiraterone (6, 11, 12, 32–34). We observed downregulation of both FL-AR and AR-V7 mRNA upon treatment with 10 nM ARV-771 in VCaP cells (Fig. 3C). Although the BET inhibitor JQ-1 lowered AR-V7 levels in this assay, it had no effect on FL-AR. Similar results were obtained in LnCaP95 cells (Fig. S34). In 22Rv1 cells, however, the levels of FL-AR showed more complex regulation in response to ARV-771, whereas AR-V7 levels were attenuated (Fig. S3B). As expected, ARV-766 showed no effect on either FL-AR or AR-V7 levels. The time course of AR downregulation and BRD4 degradation also was established in VCaP cells. Interestingly, although the loss of BRD4 was complete by 6 h following ARV-771 treatment, levels of FL-AR took longer to attenuate (Fig. S3C). Treatment with the synthetic androgen R1881 down-regulated both transcripts, and enzalutamide had the opposite effect, as has been reported previously (35–37). Finally, we showed that ARV-771 has an antitumorogenic effect on a number of AR-regulated genes in VCaP cells (Fig. 3D). In addition, we carried out an RNA-sequencing experiment in 22Rv1 cells in which we analyzed changes in gene expression upon treatment with either 30 nM ARV-771 or 500 nM OTX015 (Datasets S1 and S2). Interestingly, although AR signaling was not identified by an unbiased bioinformatics analysis as one of the top five networks targeted by ARV-771 (Dataset S3), the levels of a number of AR-regulated genes (ELL1, PMEP1A1, STEAP1, FAM105A, ATAD2, ENDO1, and ZNF189) were found to be attenuated by >50% in this experiment. Similarly, immunoblotting for ERG, which also is regulated by AR in VCaP cells, revealed that the induction of this gene by the synthetic androgen R1881 could be blocked by ARV-771 pretreatment, providing further evidence that BET degradation with the PROTAC blocks AR signaling (Fig. 3E).
We next established that ARV-771 possesses physicochemical attributes that are favorable for in vivo experiments (Table S1). Consistent with these data, the PK profile of ARV-771 revealed that a single subcutaneous administration of a 10-mg/kg dose resulted in plasma drug levels significantly above the predicted efficacious concentration [c-MYC IC₅₀ = 100 nM with 50% (vol/vol) mouse serum] (Table S1) for 8–12 h (Fig. S4A). Importantly, treatment of noncastrated male Nu/Nu mice bearing AR-V7+ 22Rv1 tumor xenografts with daily subcutaneous injections of ARV-771 at 10 mg/kg for 3 d resulted in 37% and 76% down-regulation of BRD4 and c-MYC levels, respectively, in tumor tissue (Fig. 4A). Separately, 2 wk of daily dosing resulted in a dose-dependent suppression of BRD4 and c-MYC in tumors with 10 mg/kg ~80% knockdown of both at 8 h following the last 10-mg/kg dose. (Fig. 4B and C and Fig. S4B). The corresponding 8-h ARV-771 plasma concentration of 1,200 ± 230 nM in these mice (Table S2) was significantly higher than its c-MYC IC₅₀ in mouse serum, consistent with the robust BRD4 and c-MYC knockdown that was observed. Interestingly, administration of c-MYC 50 mg/kg OTX015 by oral gavage resulted in c-MYC down-regulation but also in an accumulation of BRD4 protein (Fig. 4B and C). Finally, we also observed a marked down-regulation in levels of AR-V7 in the 22Rv1 tumors after ARV-771 treatment (Fig. 4D and Fig. S4C).

ARV-771 Induces Degradation in Vivo. We next confirmed that ARV-771 efficacy in 22Rv1 tumor xenografts was not an artifact of cellular lineage. Specifically, we chose the VCaP tumor model, which represents the clinical setting of AR overexpression following androgen-deprivation therapy. Because the CB17 SCID mice bearing VCaP xenografts did not tolerate daily dosing of either ARV-771 or OTX015, we explored intermittent dosing in this experiment. Noncastrated male CB17 SCID mice bearing VCaP tumor xenografts were treated with two intermittent dosing schedules of ARV-771 (3 d Q4D or 3 d Q3D) for a total of 16 d, during which the vehicle arm underwent a quadrupling of tumor size (Fig. 5C and D). Both dosing schedules resulted in an identical 60% TGI over this time course without significant loss in body weight in either arm (Fig. S5B). In comparison, enzalutamide had a marginal impact on tumor growth. Although we observed no alopecia with ARV-771 dosing in the CB17 SCID mice, the lack of tolerance for daily dosing does suggest potentially significant toxic effects. With chronic intermittent dosing in our experiment, toxicity presented primarily as hunching of the spine, along with lethargy and decreased mobility in PROTAC-tubular animals (Fig. S5F). Surprisingly, although enzalutamide had little impact on tumor growth, it resulted in a 40% reduction in PSA serum levels. Finally, we confirmed our findings of BRD4 and c-MYC suppression with immunohistochemical analysis of tumor samples collected from the vehicle and ARV-771 Q3D cohorts (Fig. 5G).

Discussion

Despite recent advances in androgen therapy, 20–40% of patients with metastatic CRPC demonstrate de novo resistance to the newly FDA-approved drugs abiraterone and enzalutamide, and the remaining patients acquire resistance during treatment (8–11). Several BET inhibitors have recently shown promising efficacy in preclinical models of CRPC (19–21). Although the specific mechanisms behind this activity are a subject of intense scrutiny, BET inhibitors are thought to function partly by blocking BRD4 localization to AR target loci, thereby inhibiting...
Here we have described a VHL-based BET targeting PROTAC, which usually is not a clinically relevant route of administration. To date, the first-generation BET PROTAC shown to be efficacious in vivo is ARV-771, which shows \(<5\) nM potency of BRD2/3/4 degradation in several prostate cancer cell lines. ARV-771 also has an antiproliferative effect that is up to 500-fold more potent than the BET inhibitors JQ-1 and OTX015 in these cell lines. Although we believe that ARV-771 and BET inhibitors share some common mechanism(s) of action, we hypothesize that protein depletion with a PROTAC could result in pleiotropic outcomes that would not be accessible with traditional inhibitors. Interestingly, it recently has been discovered that one of the mechanisms of acquired resistance to BET inhibitors in breast cancer involves completely BRD-independent transcriptional regulation mediated by BRD4 (39). Similarly, in the same study, BRD4 knockdown by shRNA was shown to have much more significant antitumor effects than mere small-molecule inhibition. A separate study reported increased sensitivity of breast cancer lines to BRD4 siRNA compared with BET inhibition (40). Given these emerging data, and because BET family proteins are known to have scaffolding functions whereby they interact with a variety of transcriptional regulators through their extraterminal and C-terminal domains (41, 42), we hypothesized that a BET degrader would have a more profound effect than a BET inhibitor on the growth and/or survival of prostate tumor cells. Our observation that ARV-771 lowers levels of FL-AR in addition to AR-V7 in VCaP cells, whereas BET inhibitors impact only the latter, supports this hypothesis. These data are consistent with other reports that show no impact of BET inhibitors on FL-AR levels (19, 20), although one recent study claims that BET inhibitors do decrease FL-AR levels (22). Accumulating evidence in the literature suggests that AR splice variants may mediate castration resistance, in part by heterodimerization with FL-AR and activation of the latter in an androgen-independent manner (15, 16, 37). Although the attenuation of AR transcript variant levels is likely only one among many antiproliferative mechanisms downstream of the depletion of important epigenetic regulators such as BRD2/3/4, it nonetheless is of considerable importance in the context of PCa. The superiority of a BET PROTAC compared with a BET inhibitor is demonstrated by the observations that ARV-771 induces apoptosis in CRPC cells grown in vitro, whereas JQ-1 and OTX015 have only a cytostatic effect in the same time frame. Furthermore, ARV-771 induces regression of 22Rv1 xenografts compared with the 80\% TGI that occurs in mice treated with OTX015. This effect clearly establishes the value of BET degraders over inhibitors, which, although efficacious in vivo, still result in progressive disease. Taken together, our results strongly support pursuing PROTAC-mediated BET degradation as a therapeutic strategy in CRPC.

Materials and Methods

All experiments described in this paper were approved by the Arvinas Senior Management Team. All human-derived materials used in this study were obtained from commercial vendors and did not require informed consent. For full methods, see SI Materials and Methods.

Reagents. The 22Rv1 and VCaP cell lines were purchased from ATCC. LncCap95 cells were a generous gift from Alan Meeker at The Johns Hopkins University School of Medicine. BRD2 (5848), BRD4 (13440), PARP (9532), and c-MYC (5605) antibodies were purchased from Cell Signaling Technology. BRD3 (sc-81202) antibody was purchased from Santa Cruz Biotechnology. Antibodies used for immunohistochemistry were c-MYC (ab32072, Abcam) and BRD4 (a301-985a50, Abgent Laboratories). Actin and tubulin antibodies were purchased from Sigma.

Immunoblotting. Cells were lysed in RIPA buffer (catalog no. 89900, Thermo Fisher) supplemented with protease inhibitors (EDTA-free Protease Inhibitor Tablets, catalog no. 88266, Pierce). Lysates were centrifuged at 16,000 \( \times \) g, and the supernatants were used for SDS/PAGE. Western blotting was carried out following standard protocols.

RT-qPCR. Cells were treated as indicated and were pelleted by centrifugation at 2,000 \( \times \) g for 2 min, followed by washing with PBS. Total RNA was extracted using the Qiagen RNeasy Kit (catalog no. 74904), and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (catalog no. 4368814, Thermo Scientific).
Tumor tissue was dissected, fixed in 10% (vol/vol) formalin for 10 min; antigen retrieval was performed in citrate buffer (pH = 6) at 100 °C for 10 min and then was blocked with Avidin-Biotin Reagents (SP-2001, Vector Laboratories), followed by 10% (vol/vol) horse serum block. Slides then were incubated with either anti-c-MYC (rabbit monoclonal, ab30272, Abcam) or anti-BRD4 (a301-985a50, Bethyl Laboratories) at a 1:1,000 dilution overnight at 4 °C. On the next day, after washing with PBS, slides were blocked with 3% (vol/vol) H2O2 (in PBS), followed by incubation with biotinylated horse anti-rabbit secondary antibody for 30 min. Peroxidase ABC Substrate (PK-6100, Vector Laboratories) was applied for 30 min, and 3, 3′-diaminobenzidine-tetrahydrochloride (SK-4100, Vector Laboratories) was used for color development. Nuclei were counterstained with Gill III hematoxylin and lithium carbonate.

Cell Proliferation Assay Protocol. 22Rv1 cells (5,000 cells per well) were dosed with compounds serially diluted at 1:3 ratio for a 10-point dose curve. The medium was aspirated and cells were washed once with PBS. RIPA buffer (50 μL) supplemented with protease and phosphatase inhibitors was used to lyse cells. Lysates were centrifuged and transferred to a 96-well c-MYC ELISA plate (catalog no. KH02041, Novex, Life Technologies). AR ELISA. VCaP cells (40,000 cells per well) were dosed with compounds serially diluted at 1:3 ratio for an eight-point dose curve. Medium was aspirated, and cells were lysed in cell lysis buffer (9803, Cell Signaling Technology) supplemented with protease and phosphatase inhibitors. Lysates were centrifuged and transferred to a 96-well Androgen Receptor ELISA plate (PathScan Total Androgen Receptor Sandwich ELISA Kit 12850, Cell Signaling Technology).

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