

# Targeting the Hsp40/Hsp70 Chaperone Axis as a Novel Strategy to Treat Castration-Resistant Prostate Cancer



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## Abstract

Castration-resistant prostate cancer (CRPC) is characterized by reactivation of androgen receptor (AR) signaling, in part by elevated expression of AR splice variants (ARv) including ARv7, a constitutively active, ligand binding domain (LBD)-deficient variant whose expression has been correlated with therapeutic resistance and poor prognosis. In a screen to identify small-molecule dual inhibitors of both androgen-dependent and androgen-independent AR gene signatures, we identified the chalcone C86. Binding studies using purified proteins and CRPC cell lysates revealed C86 to interact with Hsp40. Pull-down studies using biotinylated-C86 found Hsp40 present in a multiprotein complex with full-length (FL-) AR, ARv7, and Hsp70 in CRPC cells. Treatment of CRPC cells with C86 or the allosteric Hsp70 inhibitor JG98 resulted in rapid protein destabilization of both FL-AR and ARv, including ARv7, concomitant with reduced FL-AR- and ARv7-mediated transcriptional activ-

ity. The glucocorticoid receptor, whose elevated expression in a subset of CRPC also leads to androgen-independent AR target gene transcription, was also destabilized by inhibition of Hsp40 or Hsp70. *In vivo*, Hsp40 or Hsp70 inhibition demonstrated single-agent and combinatorial activity in a 22Rv1 CRPC xenograft model. These data reveal that, in addition to recognized roles of Hsp40 and Hsp70 in FL-AR LBD remodeling, ARv lacking the LBD remain dependent on molecular chaperones for stability and function. Our findings highlight the feasibility and potential benefit of targeting the Hsp40/Hsp70 chaperone axis to treat prostate cancer that has become resistant to standard antiandrogen therapy.

**Significance:** These findings highlight the feasibility of targeting the Hsp40/Hsp70 chaperone axis to treat CRPC that has become resistant to standard antiandrogen therapy. *Cancer Res*; 78(14); 4022–35. ©2018 AACR.

## Introduction

Surgical or chemical androgen deprivation therapy (ADT, encompassing both androgen depletion and antiandrogen strategies) is the first-line treatment for locally advanced or metastatic prostate cancer (1). However, ADT efficacy is limited and despite systemic androgen depletion, the cancer often continues to progress to a terminal disease state known as castration-resistant prostate cancer (CRPC; ref. 1). In support of the continuing

importance of androgen receptor (AR) signaling in CRPC, two potent second-line agents, abiraterone and enzalutamide, have been approved by the FDA. Abiraterone is a CYP17A1 inhibitor (blocking both 17 $\alpha$ -hydroxylase and 17,20-lyase, two enzymes important for androgen synthesis; ref. 2), whereas enzalutamide is a potent AR ligand-binding domain (LBD) competitive antagonist that blocks nuclear translocation and AR-dependent gene transcription (3). Although these agents extend overall survival, 20% to 40% of patients with metastatic CRPC have no response to these drugs, whereas those who initially respond eventually develop secondary resistance (4).

CRPC therapeutic resistance is associated with reactivated and sustained AR signaling mediated by several mechanisms including AR overexpression and gene amplification, AR point mutations, enhanced AR coregulator activity or expression, and intratumoral androgen synthesis (5). Notably, CRPC is frequently associated with expression of a number of AR splice variants (ARv), including, but not limited to, ARv7 and AR<sup>v567es</sup>, which lack a complete carboxy-terminal LBD. Consequently, these ARv are insensitive to antiandrogens or androgen ablation, and are constitutively active (6). In particular, ARv7 has been shown to provide a growth advantage in androgen-depleted environments, leading to castration-resistant growth *in vivo* (7). Furthermore, ARv7 expression in CRPC correlates with poor prognosis, shorter progression-free survival and overall survival, and

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resistance to standard of care treatments (4, 8). In part, these characteristics are likely the outcome of a distinct transcriptional program mediated by ARv7 that is able to sustain cancer cell growth in androgen-depleted environments. For example, ligand-dependent full-length AR (FL-AR) regulates the expression of *KLK3* (prostate-specific antigen, PSA), *TMPRSS2*, and *NDRG1* (9–11), whereas ligand-independent ARv7 transcriptionally activates *UBE2C* and *EDN2* (9–12).

In addition to increased expression of ARv7 and other ARv, upregulation of the glucocorticoid receptor (GR, like AR, is a member of the nuclear receptor family; ref. 13) provides an alternative mechanism for generating resistance to AR LBD-targeted therapy (14–16). GR can occupy approximately one-half of the AR cistrome and can induce expression of approximately one-third of AR-regulated genes (17). Although most prostate cancers express AR, approximately 30% of prostate cancers not exposed to LBD-targeted therapy also express GR (15, 16). Intriguingly, in the presence of androgen, AR-regulated transcription is frequently suppressed by the GR ligand dexamethasone. However, in the context of androgen deprivation, ligand-occupied GR drives the transcription of a host of AR target genes, many of which are overexpressed in prostate cancer. Thus, GR expression in CRPC also has been shown to promote clinical resistance to enzalutamide (14, 17). Because of the morbidity associated with late-stage disease, identification of novel strategies able to simultaneously disrupt the FL-AR/ARv7 and GR signaling axes in CRPC are of significant clinical importance and remain a critical unmet need.

Molecular chaperones (Hsp), including Hsp40, Hsp70, and Hsp90, participate in protein folding, quality control of misfolded proteins, and the stability of metastable proteins (e.g., protein homeostasis; ref. 18). Nuclear hormone receptors, including AR and GR, require the collaborative activity of the Hsp40 (DnaJ)/Hsp70/Hsp90 chaperone complex to maintain their LBD in an activation-competent, high-affinity ligand-binding state (13, 19–23). Hsp90 has been shown to be important for androgen binding to the AR LBD in yeast (24) and the chaperone interacts directly with the human AR LBD (25). Furthermore, Hsp90 inhibitors cause destabilization and degradation of FL-AR and GR (26, 27). However, recent studies have shown that ARv proteins lacking the LBD, including ARv7, are independent of both Hsp90 and androgen, and are insensitive to Hsp90 inhibitors (28, 29). It should be noted that two recent reports identify a role for Hsp90 in regulation of alternative splicing, including alternative splicing of AR (30, 31). Nevertheless, in contrast to the rapid FL-AR-destabilizing effects of Hsp90 inhibitors, indirect effects on AR splicing require longer drug exposure and have general effects on alternative splicing beyond AR (31).

This study is an outgrowth of a small-molecule screen whose aim was to identify novel dual inhibitors of both androgen-dependent and androgen-independent transcriptional activity. As shown here, a potent compound (C86) identified in this screen was found to be an Hsp40 interactor, suggesting a continued, Hsp90-independent role for Hsp40, and perhaps also Hsp70 (with which Hsp40 frequently associates), in functional regulation of ARv lacking an LBD. The current work uses C86 and the allosteric Hsp70 inhibitor JG98 to explore this possibility in detail, and our findings identify both Hsp40 and Hsp70 as potential therapeutic targets in CRPC that is resistant to abiraterone and enzalutamide.

## Materials and Methods

### Compounds/SAR/synthesis

C86 (3-nitro-2',4',6'-trimethoxychalcone) was obtained from Indofine Chemical Company and the inactive analogue (1,3,5-trimethoxybenzene) was obtained from Sigma. To synthesize biotin-C86 (b-C86) or biotin-inactive analogue (b-IA), C86 or the inactive analogue was conjugated with an iodinated biotin reagent in the presence of  $\text{Cs}_2\text{CO}_3$  and dimethylformamide. JG98 (32) and JG231 were synthesized and provided by J. Gestwicki. Synthesis and screening strategy of chalcone compounds have been described previously (33).

### Cell culture

LNCAp, 22Rv1, VCaP, HEK293, COS7, SkBr3, and A549 cells were obtained from ATCC and cultured in RPMI1640 (Corning) or DMEM (Corning), each supplemented with 10% FBS. The LNCAp<sup>AR-V7/pHage</sup> line, which expresses AR-V7 in response to doxycycline, was generated by lentiviral infection of a pHage vector containing the AR-V7 sequence as described previously (12). All cell lines used in this study were tested for *Mycoplasma* prior to use and were authenticated by ATCC using short tandem repeat analysis. For drug treatment, LNCAp cells were transferred to medium containing 10% charcoal-stripped serum (CSS) and cultured overnight prior to the addition of 10 nmol/L R1881 for 6 additional hours. LNCAp-ARv7 cells were transferred to medium containing 10% CSS and treated with drug 2 to 4 hours prior to the addition of 20 ng/mL doxycycline to induce ARv7. All cells were maintained in a 37°C and 5%  $\text{CO}_2$  humidified incubator, used for <20 passages following thawing, and cultured for no longer than 3 months.

### Immuno and chemical precipitation

FLAG- or V5-tagged proteins were immunoprecipitated from cell lysate by incubation with FLAG- or V5-conjugated agarose beads for 1 hour (Sigma). For chemical precipitation, b-C86, GA beads, or PU-H71 beads (synthesized as described previously; refs. 34, 35) were incubated with recombinant Hsp40 (DnaJB1; Abcam), Hsp70 (provided by Sue Wickner; NCI, Bethesda, MD), Hsp90 (R&D Systems), or cell lysate for 1 hour, followed by streptavidin beads (Sigma) for 1 hour. In each case, beads were then washed at least three times with TNES buffer [50 mmol/L Tris (pH 7.5), 2 mmol/L EDTA, 100 mmol/L NaCl, 208 1% NP-40] and bound protein eluted with 3×FLAG peptide (Sigma), V5 peptide (Sigma), or 2× gel loading buffer. Samples were analyzed by SDS-PAGE and Western blot analysis.

### qRT-PCR

Total RNA was isolated from cultured cells using the TRIzol (Invitrogen) or the RNeasy RNA Isolation Kit (Qiagen) and reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Transcript levels were detected by RT-qPCR using an ABI 7500 Real Time Detection System (Life Technologies) with experiments performed in triplicate. Primer sequences are listed in Supplementary Information.

### Cell viability

Twenty-four hours following seeding (5,000 cells/well) in a 96-well plate, 22Rv1 cells (60%–70% confluent) were treated with vehicle (DMSO), C86, MDV3100 (enzalutamide; Selleck Chemicals), bicalutamide (Selleck Chemicals), or JG98 at

indicated concentrations and duration. Cell viability was then assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) or CellTiter-Glo (Promega) assay.

#### SDS-PAGE and Western blot analysis

Cells were rinsed with ice-cold PBS, lysed with TNES buffer containing fresh protease and phosphatase inhibitor (Roche) and centrifuged at 4°C, 13,200 × g for 30 minutes. Where applicable, soluble sample was removed and the insoluble pellet was rinsed with PBS following centrifugation. After pelleting and removal of PBS, TNES buffer was added and samples sonicated. All samples were quantified by BCA assay (Thermo Fisher Scientific). Ten to 20 µg of protein was separated by SDS-PAGE using Mini-PROTEAN TGX 4% to 20% precast gradient gels (BioRad), transferred to nitrocellulose membranes, blocked for 1 hour with TBS-T (50 mmol/L Tris [pH 7.5], 300 mmol/L NaCl, 0.5% Tween 20) containing 5% nonfat milk (blocking buffer), and incubated with primary antibody overnight at 4°C. The following day, membranes were rinsed in TBS-T and incubated with species-specific secondary antibody in blocking buffer for 1 hour at room temperature. Proteins were detected using the SuperSignal chemiluminescence detection system (Thermo Fisher Scientific) and films were scanned. The same blot was probed multiple times, and if necessary, was sliced horizontally for better exposure when the same antibody species was used. If brightness/contrast was adjusted, it was equivalently adjusted for the entire image. Antibodies used are listed in Supplementary Information.

#### Transfection

HEK293, COS7, or 22Rv1 cells were seeded at  $1 \times 10^6$  cells per 10-cm<sup>2</sup> dish. The following day, X-tremeGENE 9 (Roche) was used to transfect cells with 2 µg of: FLAG-DnaJA1, -A4, -B2a, -B4, -B6b, -C5, -C7 (provided by Chad Dickey, University of South Florida, Tampa, FL); tetracycline-inducible pcDNA3-V5-DnaJB6b, -B6b H/Q, -B8, -B8 H/Q (provided by Harm Kampinga, University of Groningen, Groningen, the Netherlands); pCMV-3xFLAG-ARv7, -FL-AR; pEGFP-ARv7, -FL-AR (provided by Lisa Butler, University of Adelaide, Adelaide, Australia); -3xFLAG-Hsp70. Cells were treated or lysate was collected 24 hours after transfection for binding assays (immunoprecipitations) and Western blotting.

#### Animals

All animal experiments were approved by the NCI-Bethesda Animal Care and Use Committee (approved animal protocol number UOB-013-A). Four-week-old male athymic mice (Charles River) were allowed to mature and acclimate for 2 weeks. To establish CRPC xenografts,  $1 \times 10^6$  low passage 22Rv1 cells in a 1:4 mixture of media/matrigel (v/v; BD Biosciences) were injected into the right flank of each mouse. Once tumors reached a volume of 75 to 125 mm<sup>3</sup>, mice were randomized and administered either vehicle [10% DMSO, 18% Cremophor RH 40 (Sigma), 3.6% dextrose, 68.4% 1 M HEPES] or 15 mg/kg C86 by tail vein (intravenous) injection every Monday, Wednesday, and Friday for 2 weeks. For experiments using JG231, tumor bearing mice were randomized and treated with either vehicle (as described above) or 8 mg/kg JG231 by intraperitoneal injection every other day for 3 weeks. Finally, for combination studies, tumor bearing mice were randomized and treated with vehicle or 4 mg/kg JG231 Monday, Wednesday, and Friday ± 15 mg/kg C86 Tuesday,

Thursday, and Saturday, with Sundays off, for 2.5 weeks. Tumor volumes and mouse body weights were measured two to three times per week. For pharmacodynamic analyses, tumors were excised from vehicle or drug-treated mice after 10 days, homogenized, and processed for Western blotting.

#### GR activity assay

The W303 yeast strain was utilized to measure GR activity and drug sensitivity. Cells were grown in YPD (1% Bacto yeast extract, 2% peptone, 2% dextrose) or defined synthetic media supplemented with 2% dextrose at 30°C unless otherwise specified. Yeast cells were transformed with the p/G795GR plasmid encoding the mammalian steroid hormone receptor GR in combination with either the β-galactosidase reporter *pUCASS-26X GRE*, *pRS416GPD* control vector, or the pLacZi reporter control (Addgene). Resultant colonies were grown in 50 mL of defined minimal media at 30°C overnight. Overnight culture density was measured and diluted to an O.D.<sub>600</sub> of 0.08 followed by incubation at 30°C for 30 to 45 minutes, until reaching an O.D.<sub>600</sub> of 0.1. Once cell density reached an O.D.<sub>600</sub> of 0.1, 100 µL of each culture was aliquoted in triplicate into a white 96-well plate, followed by the immediate addition of DMSO or C86. After an incubation period of 30 minutes, dexamethasone (10 µmol/L; Sigma) was added to each well and incubated for 2 hours at 30°C. Subsequently, 100 µL of Tropix Gal-Screen reagent (Applied Biosystems) was added to each well and incubated for 1.5 hours at room temperature. Luminescence was then measured using a microplate luminometer. To determine the effect of C86 on yeast growth, two isolated yeast colonies were inoculated in 50 mL of YPD media followed by an overnight incubation at 30°C. Inoculum densities were adjusted to an O.D.<sub>600</sub> of 0.5, either DMSO or C86 were added to the flasks, and cell density was subsequently measured at O.D.<sub>600</sub> every 2 hours for 8 hours.

## Results

### The small-molecule C86 inhibits FL-AR/ARv7 signaling in CRPC cells

To identify compounds that perturb the FL-AR/ARv7 transcriptional program, a library of chalcones was screened for their ability to simultaneously inhibit transcription of the androgen-responsive genes *KLK3* (PSA) and *TMPRSS2*, and the androgen-independent but AR-regulated gene *UBE2C*, that are endogenously expressed in 22Rv1 CRPC cells (which endogenously express both FL-AR and ARv7). Following several rounds of structure-activity relationship analyses, C86 (Supplementary Fig. S1A) was identified as the most potent inhibitor of the expression of these genes (red arrow, Fig. 1A), whereas bicalutamide, a clinically relevant FL-AR antagonist, was ineffective (green arrow, Fig. 1A). To further verify these results, C86 was tested in parental LNCaP cells (expressing only FL-AR) and in LNCaP cells stably expressing exogenous, doxycycline-inducible ARv7 (12). In agreement with the data obtained in 22Rv1 cells, C86 reduced androgen-dependent transcription of *NDRG1* (Supplementary Fig. S1B, top left; ref. 10), as well as transcription of the androgen-independent, ARv7-specific target gene *EDN2* (Supplementary Fig. S1B, top right; ref. 12).

To obtain further mechanistic insight, we examined effects of C86 on FL-AR and ARv7 protein levels. Treatment of 22Rv1



To explore whether C86 effects on FL-AR/ARv7 protein levels may be due to modulation of *AR/ARv7* gene expression, we evaluated effects on protein half-life. As shown in Fig. 1D, loss of FL-AR, ARv, and ARv7 proteins occurred more rapidly in 22Rv1 cells treated with cycloheximide and 10  $\mu\text{mol/L}$  C86 compared with cells treated with cycloheximide alone. Furthermore, treatment with the proteasome inhibitor MG132 enhanced the loss of FL-AR, ARv, and ARv7 (Fig. 1D) from the soluble lysate fraction and caused their accumulation in a detergent-insoluble lysate fraction (Supplementary Fig. S1D), suggesting C86 potentiates FL-AR/ARv7 aggregation and targets these nuclear receptors for degradation by the proteasome. Finally, although the clinically approved FL-AR antagonists bicalutamide and MDV3100 (enzalutamide) failed to display robust growth-inhibitory activity in these CRPC cells, C86 treatment led to a significant, dose-dependent reduction in 22Rv1 (Fig. 1E) and VCaP (Supplementary Fig. S1E) viability by 72 hours at concentrations that cause loss of FL-AR/ARv7 protein expression and reduced target gene expression. Taken together, these data identify C86 as a novel small molecule that affects FL-AR/ARv7 signaling *in vitro* in CRPC-derived cell lines with activity that is superior to that of FDA-approved standard-of-care treatments targeting FL-AR.

#### Importance of the highly conserved J-domain for C86 binding to Hsp40 (DnaJ) family members

In some respects, the activity of C86 resembles that of Hsp90 inhibitors, because both classes of inhibitors cause client protein instability and degradation (18). Furthermore, like many Hsp90 inhibitors (18), C86 appears to activate a heat shock response, as evident by induction of Hsp40 and Hsp70 (Fig. 1B and C), and to promote accumulation of clients in a detergent-insoluble lysate fraction when combined with proteasome inhibitor (Fig. 1D; Supplementary Fig. S1D; ref. 36). Thus, we first tested whether the mechanism of C86's anti-CRPC activity was due to its ability to interact with Hsp90. As shown in Supplementary Fig. S2A, biotinylated-C86 (b-C86; Supplementary Fig. S1A) was unable to bind recombinant Hsp90 protein (Supplementary Fig. S2A, top left) or endogenous Hsp90 from SkBr3 (Supplementary Fig. S2A, top right) and LNCaP (Supplementary Fig. S2A, bottom left) cell lysate, whereas a significant fraction of this Hsp90 was captured with Hsp90 inhibitor-conjugated agarose beads (Supplementary Fig. S2A, bottom left). The lack of direct Hsp90 interaction is also consistent with the recent observation that ARv lacking the LBD are resistant to Hsp90 inhibitors (28, 29), as Hsp90 is involved specifically in remodeling the LBD of nuclear receptors (23). Furthermore, Hsp90 inhibitor promoted degradation of FL-AR protein in 22Rv1 cells, but had no impact on ARv7 (Supplementary Fig. S2B).

Because of the collaborative nature of the AR chaperone cycle, we next tested whether C86 could interact with other chaperone proteins known to function together with Hsp90 or Hsp70. We found that b-C86 was also unable to bind recombinant Hsp70 protein (Supplementary Fig. S2A, bottom right). However, b-C86 bound a significant fraction of recombinant Hsp40 (DnaJB1), relative to a biotinylated-inactive control analogue (Supplementary Figs. S1A and S2C). In addition, b-C86 captured endogenous Hsp40 (DnaJB1) from 22Rv1 and A549 lung cancer cell lysates (Supplementary Fig. S2C), confirming that this interaction is not cell-type specific.

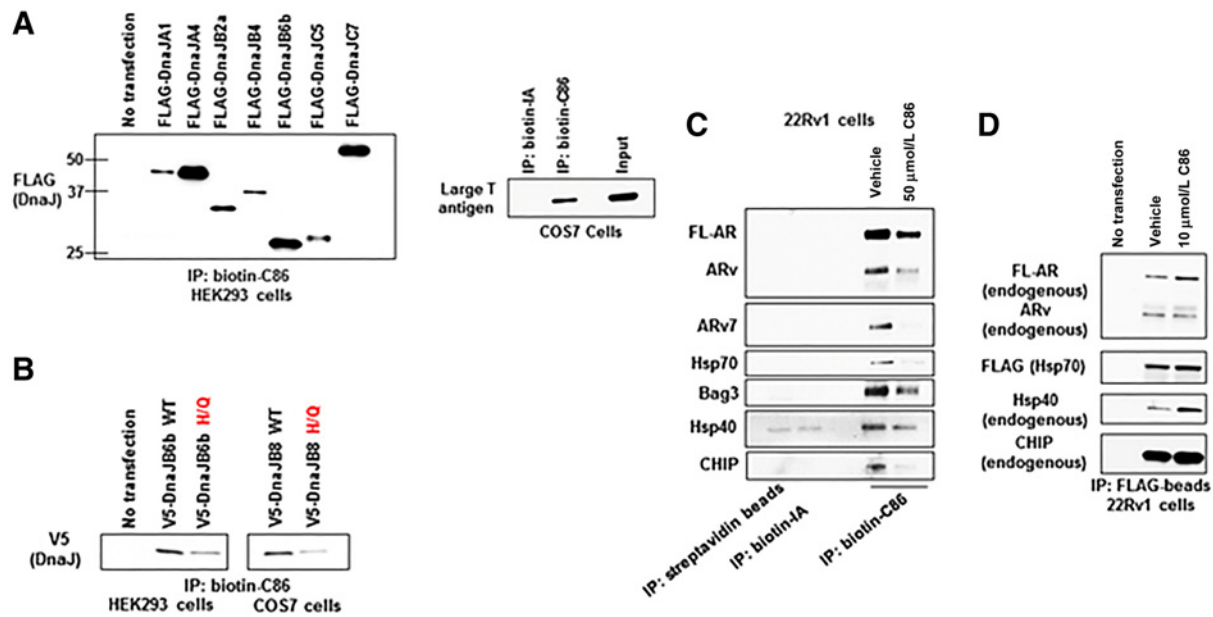
The mammalian Hsp40 (DnaJ) chaperone family consists of more than 40 members each with different functions and client binding profiles (37). Thus, it is possible that C86 may interact with some Hsp40 proteins and not with others. To determine whether C86 acts as a pan-DnaJ/Hsp40 inhibitor, b-C86 was incubated with cell lysates from HEK293 cells transfected with various FLAG-tagged DnaJ proteins. Our data revealed that C86 interacts with DnaJ/Hsp40 proteins representative of the three DnaJ families A, B, and C (Fig. 2A, left; Supplementary Fig. S2D, left; ref. 37). Because the presence of a J-domain (the site that interacts with Hsp70 for client protein transfer and to promote Hsp70 ATPase activity; ref. 37) is the only common structural motif shared by all DnaJs, our findings implicate the J-domain in C86 binding. This hypothesis is consistent with the observation that b-C86 recognizes the Simian virus 40 (SV40) large T antigen in COS7 cells (Fig. 2A, right), which is not a member of the Hsp40 family but also contains a J-domain (38).

To explore the possibility that J-domain structure is important for C86 binding, we transfected HEK293 or COS7 cells with DnaJs containing a known mutation in the highly conserved J-domain HPD motif (a three-amino acid motif comprised of histidine, proline and aspartic acid). This mutation (H/Q) perturbs the interaction of Hsp40 with Hsp70 by disrupting J-domain architecture (37). As shown in Fig. 2B, b-C86 was able to bind both wild-type (WT) V5-tagged DnaJB6b (confirming the interaction in Fig. 2A, left) and V5-DnaJB8 (Fig. 2B; Supplementary Fig. S2E). However, binding of V5-DnaJB6b-H/Q and V5-DnaJB8-H/Q to b-C86 was markedly decreased (Fig. 2B; Supplementary Fig. S2E), lending support to the importance of J-domain structural integrity for optimal recognition of Hsp40 by C86.

Next, we expanded our b-C86 pull-down experiments in 22Rv1 cells to determine whether C86 can pull down Hsp40 proteins bound to FL-AR/ARv7. Indeed, probing cell lysate with b-C86 identified endogenous Hsp40 (DnaJB1) in a complex with FL-AR, ARv, and ARv7 (Fig. 2C). Compared with vehicle treatment, excess free C86 competed away Hsp40, AR, and ARv7, indicative of a specific interaction (Fig. 2C). Hsp70, and its cochaperones the nucleotide-exchange factor Bag3 and the E3 ubiquitin ligase CHIP, were also present in this complex and were similarly affected by addition of unlabeled C86, suggesting that b-C86, in addition to interacting with free Hsp40, also recognizes an Hsp40/Hsp70/Bag3/CHIP complex that associates with FL-AR and/or ARv7 (Fig. 2C). To further validate the interaction of C86 with an Hsp40/Hsp70 chaperone complex, we transfected 22Rv1 cells with FLAG-tagged Hsp70 and treated with 10  $\mu\text{mol/L}$  C86 (a concentration that causes AR/ARv7 destabilization at later time points, Fig. 1B) for 1 hour. Under these conditions, C86 exposure resulted in increased FL-AR, ARv, Hsp40, and CHIP association with Hsp70 (assessed following anti-FLAG immunoprecipitation, see Fig. 2D), whereas total FL-AR/ARv expression was diminished relative to vehicle-treated cells (Supplementary Fig. S2F). Taken together, these data are consistent with interaction of C86 with an Hsp40/Hsp70 multiprotein complex.

#### Multiple DnaJ families interact with AR and ARv7

To explore whether FL-AR and ARv7 preferentially interact with distinct Hsp40 proteins, first we transiently transfected HEK293 cells with either FLAG-tagged FL-AR or ARv7 and



**Figure 2.**

C86 binding to Hsp40/DnaJ family members likely involves the highly conserved J-domain. **A**, Biotin-C86 or -inactive analog (b-IA) binding to FLAG-DnaJ family members from transfected HEK293 cell lysates (left) or to endogenous Large T antigen from COS7 cell lysate (right) as assessed by streptavidin-bead immunoprecipitation and Western blotting. **B**, Biotin-C86 binding to WT or H/Q mutant V5-DnaJB6b (left) or V5-DnaJB8 (right) as assessed by streptavidin-bead immunoprecipitation and Western blotting. **C**, Biotin-C86 binding profile from 22Rv1 CRPC cells incubated with excess unlabeled vehicle (DMSO) or 50  $\mu\text{mol/L}$  C86 for 1 hour as assessed by streptavidin-bead immunoprecipitation and Western blotting. **D**, 22Rv1 CRPC cells were transfected with FLAG-Hsp70 and treated with vehicle or 10  $\mu\text{mol/L}$  C86 for 1 hour. Lysate was then probed with FLAG-beads and bound proteins were detected by Western blot. Experiments were repeated at least three times.

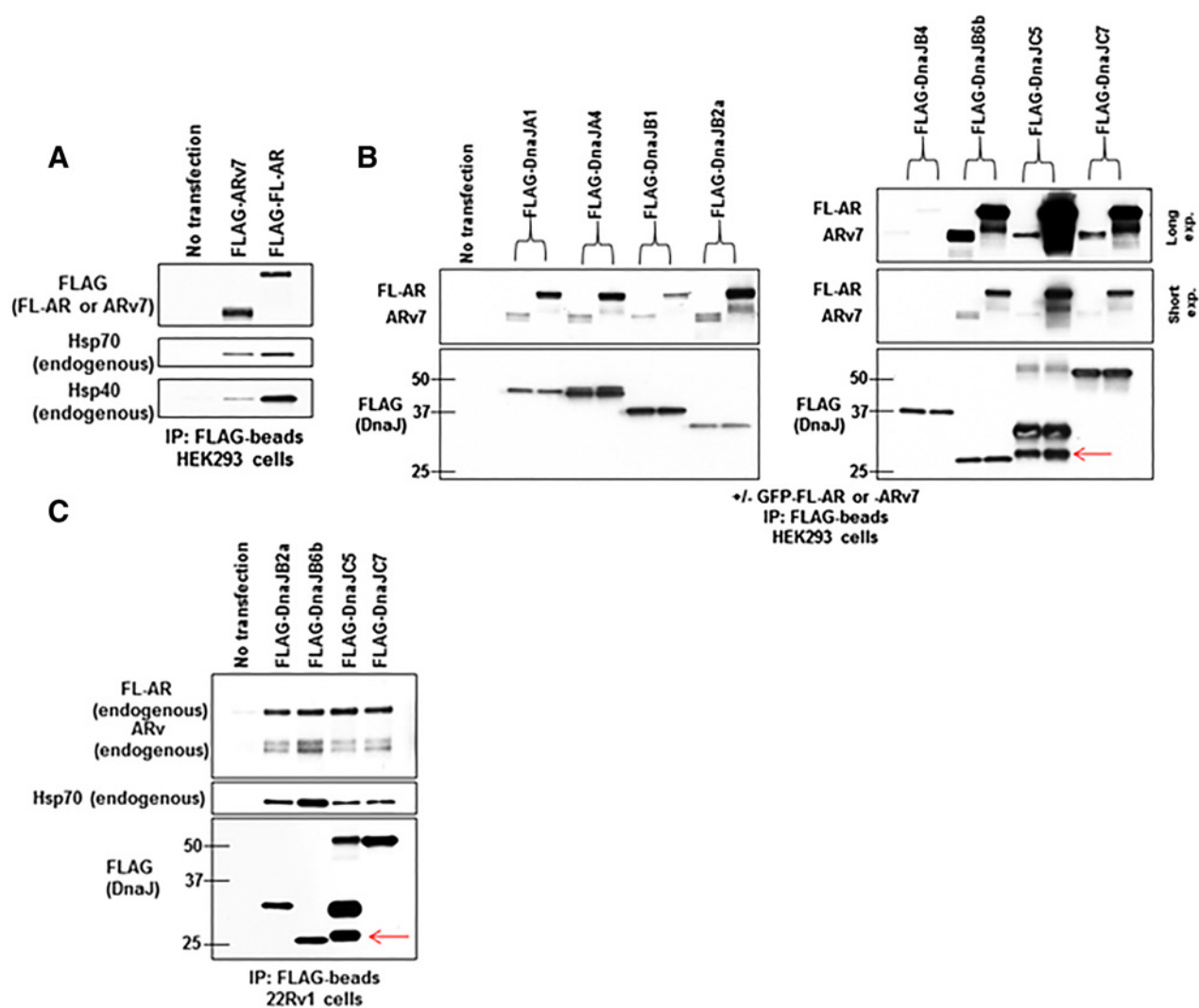
monitored interaction with endogenous Hsp40 (DnaJB1) by Western blot after immunoprecipitation with FLAG-beads. As shown in Fig. 3A (see also Supplementary Fig. S3A), endogenous Hsp40 coimmunoprecipitated with both FLAG-tagged FLAR and ARv7. Endogenous Hsp70 was also present in these pull downs (Fig. 3A; Supplementary Fig. S3A). We next examined whether various FLAG-DnaJ proteins shown to bind b-C86 may preferentially interact with either FLAR or ARv7. As demonstrated in Fig. 3B (see also Supplementary Fig. S3B), and similar to our previous b-C86 binding data (Fig. 2C), all FLAG-DnaJs tested interacted to some extent with FLAR and ARv7. For FLAG-DnaJA1, -A4, -B2a, -B6b, -C5, and -C7, interaction with FLAR was stronger relative to interaction with ARv7, while binding of FLAG-DnaJB1 and -B4 to FLAR and ARv7 was relatively equal (Fig. 3B; Supplementary Fig. S3B). From 22Rv1 cells, FLAG-DnaJB2a, -B6b, -C5, and -C7 each coimmunoprecipitated with endogenous FLAR and ARv (in this experiment, ARv was detected with FLAR antibody; Fig. 3C; Supplementary Fig. S3C). Endogenous Hsp70 was also present in these complexes (Fig. 3C; Supplementary Fig. S3C). Taken together, these data confirm that loss of the AR LBD does not confer loss of Hsp40/Hsp70 interaction.

#### Pharmacologic targeting of Hsp70 destabilizes AR and ARv7 in CRPC cells

The fact that Hsp70 appears to be present in complexes with FLAR and ARv7, and that Hsp70 is found in endogenous complexes of Hsp40 and AR/ARv7 pulled down with either b-C86, FLAG-AR, or FLAG-DnaJ (Figs. 2C and 3A and C), suggests that Hsp70 contributes to the stability and/or function

of AR and ARv7 and provides a rationale for targeting Hsp70 in addition to Hsp40 in CRPC. JG98 is a potent allosteric inhibitor of Hsp70 derived from the rhodacyanine MKT-077 (32) that inhibits Hsp70 by preventing nucleotide exchange and thus stabilizing a high-affinity ADP-bound Hsp70/client interaction (32, 39). Preventing release of clients from Hsp70 eventually leads to degradation in proteasomes (20) and studies of JG98 in MCF7 breast cancer cells have demonstrated robust anticancer activity (39). To begin to explore the impact of JG98 on FLAR and ARv in CRPC, we first assessed whether Hsp70 directly interacts with endogenous FLAR and/or ARv in 22Rv1 cells, knowing that it coimmunoprecipitates with transiently expressed FLAG-FLAR and FLAG-ARv in HEK293 cells (Fig. 3A). As shown in Fig. 4A (see also Supplementary Fig. S4A), endogenous FLAR and ARv coimmunoprecipitated with FLAG-Hsp70 in 22Rv1 cells. Concurrently, and in agreement with our previous data (Fig. 3A), endogenous Hsp40 was also present in these FLAG pull-downs (Fig. 4A).

With evidence that endogenous FLAR and ARv engage with Hsp70 and with Hsp40, we next characterized the consequences of Hsp70 inhibition with JG98 in CRPC cells. A significant, dose-dependent decrease in cell viability was observed, with JG98 displaying an  $\text{IC}_{50}$  between 400 and 500  $\text{nmol/L}$  in 22Rv1 (Fig. 4B) and VCaP (Supplementary Fig. S1E) cells at 72 hours. Importantly, like 22Rv1, VCaP cells were highly resistant to enzalutamide (Supplementary Fig. S1E). Not surprisingly, we observed destabilization of FLAR, ARv, and ARv7 proteins with increasing JG98 dose and incubation time in both 22Rv1 and VCaP cells (Fig. 4C and D). Notably, coincident with destabilization of FLAR/ARv7 proteins, JG98 also led to a concomitant



**Figure 3.**

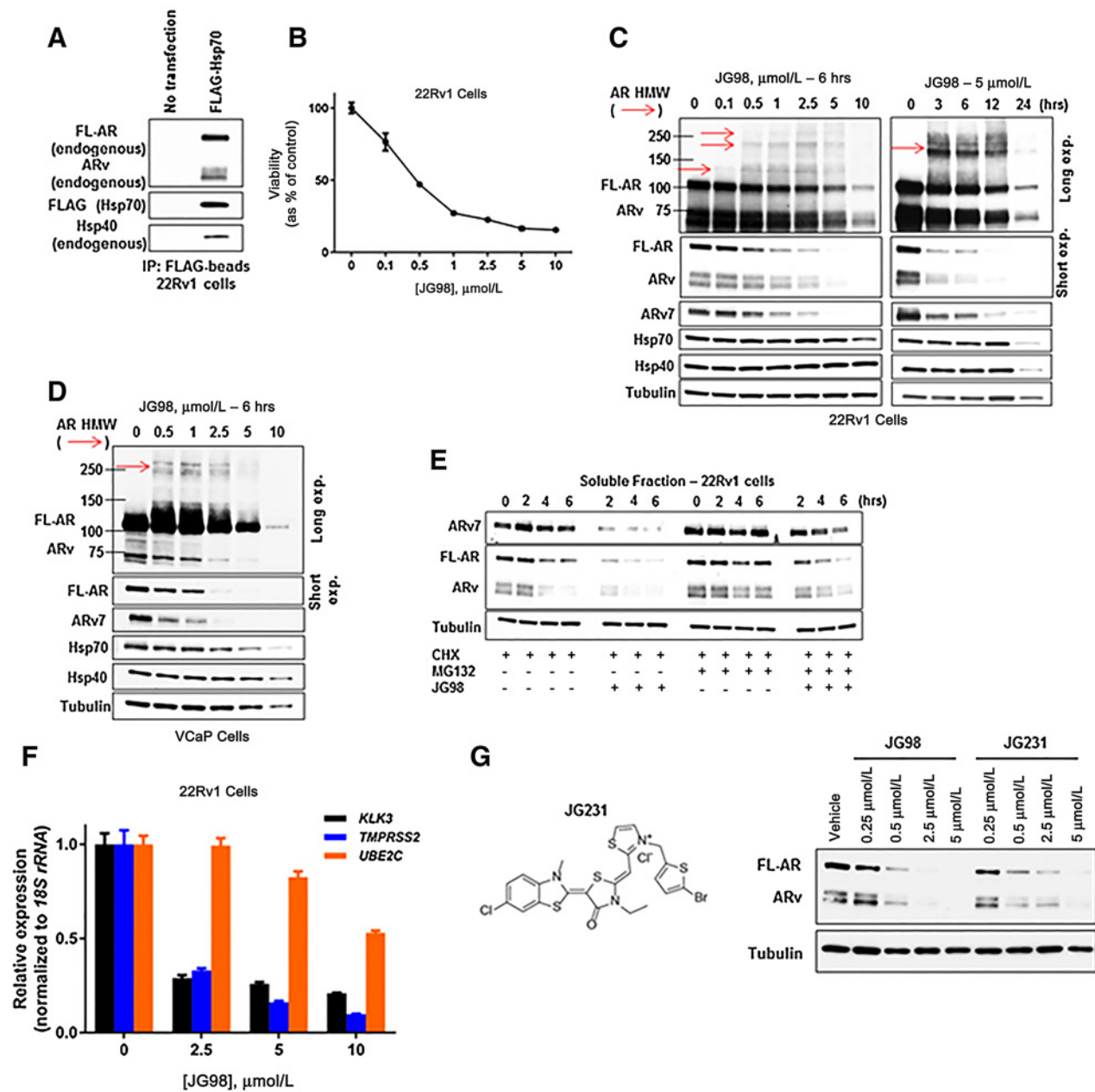
Multiple DnaJ isoforms interact with AR and ARv7. **A**, Lysate from HEK293 cells transfected with FLAG-ARv7 or FLAG-AR was probed with FLAG-beads. Bound proteins were detected by Western blotting. **B**, Lysate from HEK293 cells cotransfected with indicated FLAG-DnaJ family members and GFP-AR or GFP-ARv7 was probed with FLAG-beads. Bound proteins were detected by Western blotting. Red arrow notes detection of native FLAG-DnaJc5, which tends to oligomerize. AR antibody N-20 was used to detect FL-AR and ARv7. **C**, Lysate from 22Rv1 CRPC cells transfected with indicated FLAG-DnaJ family members was incubated with FLAG-beads. Bound proteins were detected by Western blotting. Red arrow notes detection of native FLAG-DnaJc5, which tends to oligomerize. Experiments were repeated at least three times.

increase in the formation of soluble high molecular weight (HMW) AR species (Fig. 4C and D, red arrows, detected with AR antibody N-20) in both cell types, which were not detected in vehicle control samples or C86-treated cells (Supplementary Fig. S1F). Unlike C86, JG98 did not induce a compensatory upregulation in either Hsp40 (DnaJB1) or Hsp70 *in vitro*. In fact, levels of these chaperones decreased in 22Rv1 cells after treatment with 10  $\mu\text{mol/L}$  JG98 for 6 hours (Fig. 4C, left) or after longer treatment (12–24 hours) with 5  $\mu\text{mol/L}$  JG98 (Fig. 4C, right). JG98 also caused a reduction in Hsp70 expression in VCaP cells after 6 hours (Fig. 4D).

To determine whether Hsp70 is present in these HMW AR species, we transiently coexpressed FLAG-Hsp70 with GFP-AR or GFP-ARv7 in HEK293 cells. JG98 caused a dose-dependent

loss of FL-AR (Supplementary Fig. S4B, left) and ARv7 (Supplementary Fig. S4B, right) in these cells, accompanied by formation of HMW soluble FL-AR and ARv7 species. JG98 treatment also led to formation of HMW FLAG-Hsp70 species (detected with FLAG-tag antibody), which appear to colocalize with HMW AR species (red arrows, Supplementary Fig. S4B).

When FL-AR/ARv7 protein half-life was examined, we found that contemporaneous treatment with both cycloheximide and JG98 caused a greater loss (compared with cycloheximide alone) of "native" (e.g., with predicted mobility in SDS gels) AR, ARv, and ARv7, and increased accumulation of HMW AR species (Fig. 4E; Supplementary Fig. S4C). Blocking proteasome activity with MG132 somewhat reversed Hsp70-inhibitor mediated destabilization of native FL-AR/ARv7 and the



**Figure 4.**

Pharmacologic targeting of Hsp70 destabilizes the AR/ARv7 signaling axis in CRPC cells. **A**, Lysate from 22Rv1 CRPC cells transfected with FLAG-Hsp70 was probed with FLAG-beads. Bound protein was detected by Western blot. **B**, Viability of 22Rv1 CRPC cells treated with increasing concentrations of JG98 for 72 hours as determined by MTT assay. Data represent a mean of four to six replicates  $\pm$  SD. **C**, Western blot of cell lysate from 22Rv1 CRPC cells treated with increasing concentrations of JG98 for 6 hours (left) or 5  $\mu$ mol/L JG98 for up to 24 hours (right). Red arrows note detection of HMW AR species detected with AR antibody N-20. **D**, Western blot of cell lysate from VCaP CRPC cells treated with increasing concentrations of JG98 for 6 hours. Red arrow notes detection of HMW AR species detected with AR antibody N-20. **E**, Western blot of the stability of soluble AR, ARv, and ARv7 from 22Rv1 CRPC cells following preincubation with 10  $\mu$ mol/L MG132 and/or 10  $\mu$ g/mL cycloheximide (CHX) for 1 hour, followed by  $\pm$ 1  $\mu$ mol/L JG98 for six additional hours. Equal amounts of protein (10  $\mu$ g) were loaded for Western blot of soluble and insoluble fractions (see also Supplementary Fig. S4C). **F**, RT-qPCR of AR/ARv7 target genes, normalized to *18S rRNA*, from 22Rv1 CRPC cells treated with increasing concentrations of JG98 for 16 hours. Data represent a mean of three replicates  $\pm$  SD. **G**, Structure of JG231 (left). Western blot of cell lysate from 22Rv1 CRPC cells treated with increasing concentrations of JG98 or JG231 for 6 hours (right). Experiments were repeated at least three times.

appearance of HMW AR species (Fig. 4E; Supplementary Fig. S4C). However, in contrast to C86 (Supplementary Fig. S1D), JG98 did not promote accumulation of these nuclear

receptors in a detergent-insoluble protein lysate fraction (Supplementary Fig. S4C). Consistent with these data, and similar to inhibition of Hsp40 with C86, Hsp70 inhibition affected



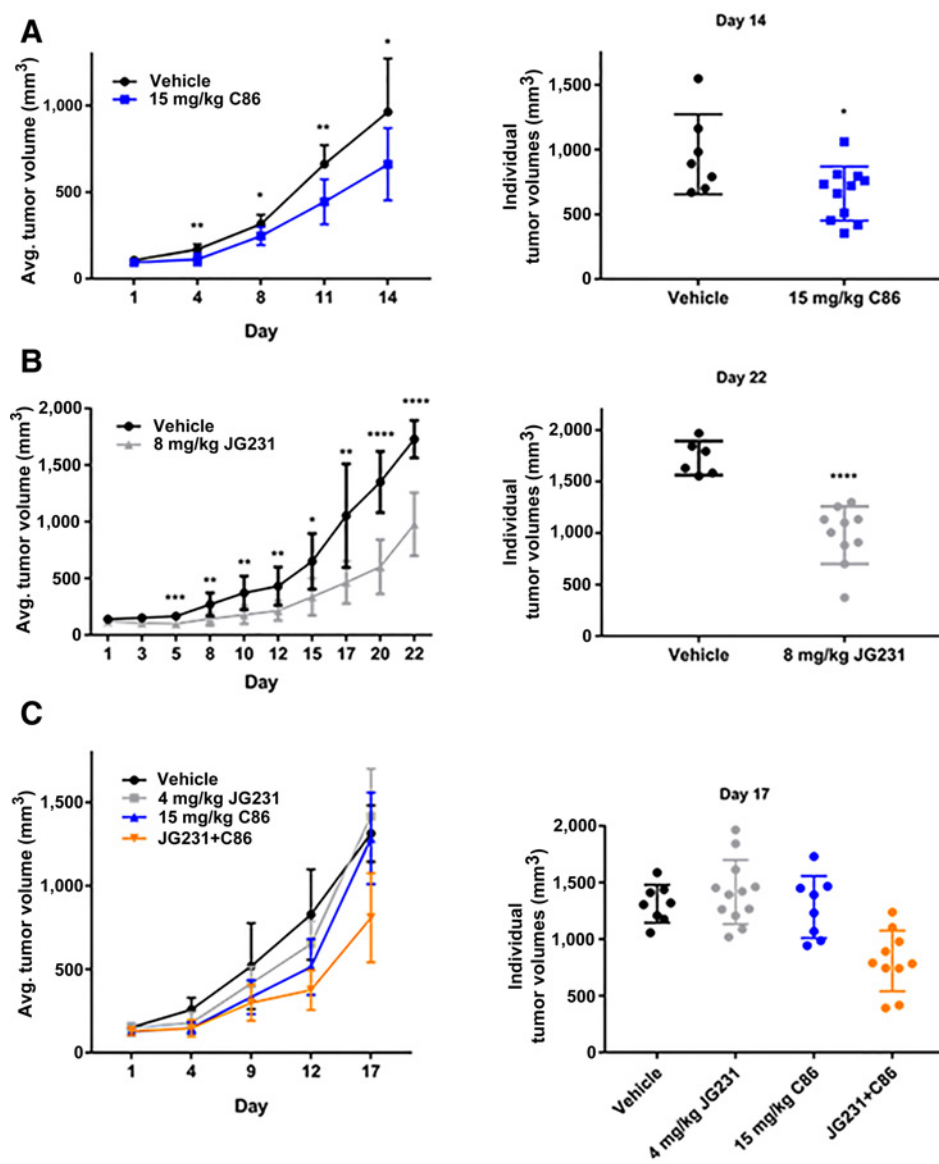
expression of FL-AR and Arv7 target genes, as treatment of 22Rv1 cells with JG98 led to a dose-dependent decrease in *KLK3*, *TMPRSS2*, and *UBE2C* gene expression (Fig. 4F). In addition, JG98 inhibited androgen-dependent *NDRG1* expression (Supplementary Fig. S4D, top left) and Arv7-dependent *EDN2* expression (Supplementary Fig. S4D, top right), while also destabilizing AR and Arv7 proteins (Supplementary Fig. S4D, bottom) in LNCaP and LNCaP-Arv7 cells.

**Inhibition of Hsp40 and/or Hsp70 demonstrates *in vivo* efficacy in CRPC-derived 22Rv1 xenografts**

With sufficient evidence in hand that AR/Arv7 rely on both Hsp40 and Hsp70 for protein stability, and that C86 and JG98 individually destabilize these nuclear receptors and inhibit their transcriptional activity *in vitro*, we next explored the efficacy of Hsp40 and/or Hsp70 inhibitor-mediated anti-CRPC activity *in vivo* (either single agent or in combination). After

confirming the *in vitro* equivalence of JG98 and JG231 (a JG98 analogue with improved *in vivo* characteristics) in promoting AR/Arv destabilization (Fig. 4G), we used JG231 for Hsp70 inhibition in these *in vivo* studies. For single-agent treatments, once palpable tumors were evident (tumor volume of 75–125 mm<sup>3</sup>), mice bearing 22Rv1 xenografts were treated by tail vein injection every Monday, Wednesday, and Friday for 2 weeks with vehicle or C86 at 15 mg/kg, or by intraperitoneal injection every other day for 3 weeks with vehicle or 8 mg/kg JG231. Relative to vehicle control animals, tumor growth was significantly inhibited in mice treated with either C86 (Fig. 5A) or JG231 (Fig. 5B) and both agents were well-tolerated, as no overt signs of toxicity were observed with either treatment (Supplementary Fig. S5A and S5B).

Because Hsp40 frequently interacts with Hsp70, it is possible that combined treatment with both JG231/JG98 and C86 would additively promote AR/Arv7 protein instability *in vitro*



**Figure 5.** C86 and the pharmacokinetically improved JG231 display anti-CRPC activity *in vivo*. **A**, Average tumor volume (±SD) of 22Rv1 CRPC xenografts in mice treated with vehicle (*n* = 7) or C86 (*n* = 11) every Monday, Wednesday, and Friday for 2 weeks (left). Individual tumor volume measurements for vehicle and C86 treated mice after 14 days (right). **B**, Average tumor volume (±SD) of 22Rv1 CRPC xenografts in mice treated with vehicle (*n* = 6) or JG231 (*n* = 10) every other day for 3 weeks (left). Individual tumor volume measurements for vehicle and JG231-treated mice after 22 days (right). **C**, Average tumor volume (±SD) of 22Rv1 CRPC xenografts in mice treated with vehicle (*n* = 8), JG231 (*n* = 12), C86 (*n* = 8), or JG231+C86 (*n* = 10) for 2.5 weeks (see Materials and Methods for schedule; left). Individual tumor volume measurements for vehicle, JG231, C86, and JG231+C86-treated mice after 17 days (right). Statistical analysis at day 17 – vehicle versus JG231 – NS; vehicle versus C86 – NS; vehicle versus JG231+C86\*\*\*. JG231+C86 versus vehicle\*\*\*; JG231+C86 versus JG231\*\*\*\*; JG231+C86 versus C86\*\*. Experiments were repeated at least two times. NS, not significant; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001 by unpaired *t* test or one-way ANOVA with multiple comparisons.

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and would display enhanced antitumor activity *in vivo*. Because of the observable increase in Hsp40/Hsp70 expression upon C86 exposure *in vitro* (Fig. 1B), we pretreated 22Rv1 cells with JG98 to attempt to counter any Hsp70-mediated compensatory effects caused by C86 treatment, followed by the addition of C86. A low dose of this combination (0.5  $\mu\text{mol/L}$  JG98 + 2.5  $\mu\text{mol/L}$  C86) more potently destabilized FL-AR, ARv, and ARv7 *in vitro*, relative to vehicle and single-agent treatments, but was unable to block the increase in Hsp40/Hsp70 expression caused by C86 (Supplementary Fig. S5C). Additive destabilization of AR/ARv7 was also observed at higher doses of JG98+C86 (1  $\mu\text{mol/L}$  JG98+5  $\mu\text{mol/L}$  C86) relative to vehicle and JG98 or C86 alone. Furthermore, this concentration of JG98 was also able to partially blunt the C86-induced increase in Hsp40/Hsp70 expression, suggesting it is possible to inhibit any potential chaperone-dependent compensatory effects caused by C86 treatment (Supplementary Fig. S5C). The combinatorial activity of JG98+C86 is also evident in the observed effect on viability *in vitro*, where a significant decrease was found in 22Rv1 cells treated with JG98+C86 compared with JG98, C86, or vehicle single-agent treatments (Supplementary Fig. S5D).

When tested against 22Rv1 xenografts *in vivo*, a significant inhibition of tumor growth was detected in JG231+C86-treated mice relative to vehicle and both monotherapies (Fig. 5C) with no overt toxicity observed (Supplementary Fig. S5E). Tumor homogenates from JG231 or C86-treated mice showed decreased FL-AR, ARv, and ARv7 protein levels compared with vehicle-treated mice (Supplementary Fig. S5F), thereby recapitulating our *in vitro* data (Figs. 1B and 4C). In addition, levels of c-IAP1, an Hsp70 client and biomarker of Hsp70 inhibition (40), were decreased in tumors from JG231-treated mice, confirming selective, on-target engagement with Hsp70. Combination treatment of JG231 with C86 also decreased these client proteins (Supplementary Fig. S5F), but the effects were not additive as in our *in vitro* data (Supplementary Fig. S5C). It is possible this combination impacts a number of other Hsp70 and Hsp90 client proteins, as several additional chaperone clients are affected by Hsp40 and Hsp70 inhibition *in vitro* (Supplementary Fig. S5G). Finally, it should be noted that no induction of the heat shock response by C86 was found *in vivo* because Hsp27/Hsp40/Hsp70 protein levels were not induced by JG231, C86, or JG231+C86 (Supplementary Fig. S5F). In fact, expression of these chaperone proteins was decreased relative to vehicle-treated mice, which may also play a role in the *in vivo* efficacy observed (Fig. 5). Collectively, these data suggest multiple dynamic client interactions with the Hsp40/Hsp70/Hsp90 chaperone machinery in CRPC cells, consistent with the pleiotypic nature of molecular chaperones. Importantly, these data also support the feasibility and need for further exploration of targeting the Hsp40/Hsp70 chaperone axis as an alternative, multifactorial therapeutic strategy to treat CRPC.

#### Hsp40 and Hsp70 inhibition destabilizes GR in CRPC cells

Increased GR expression in a significant percentage of CRPC and in a drug-resistant CRPC xenograft model, coupled with the ability of GR to occupy and regulate a portion of the AR cistrome, supports GR upregulation as an alternative mechanism of resistance to enzalutamide and other AR-targeted therapies in a subset of patients with CRPC (14–17). Like AR, GR is a

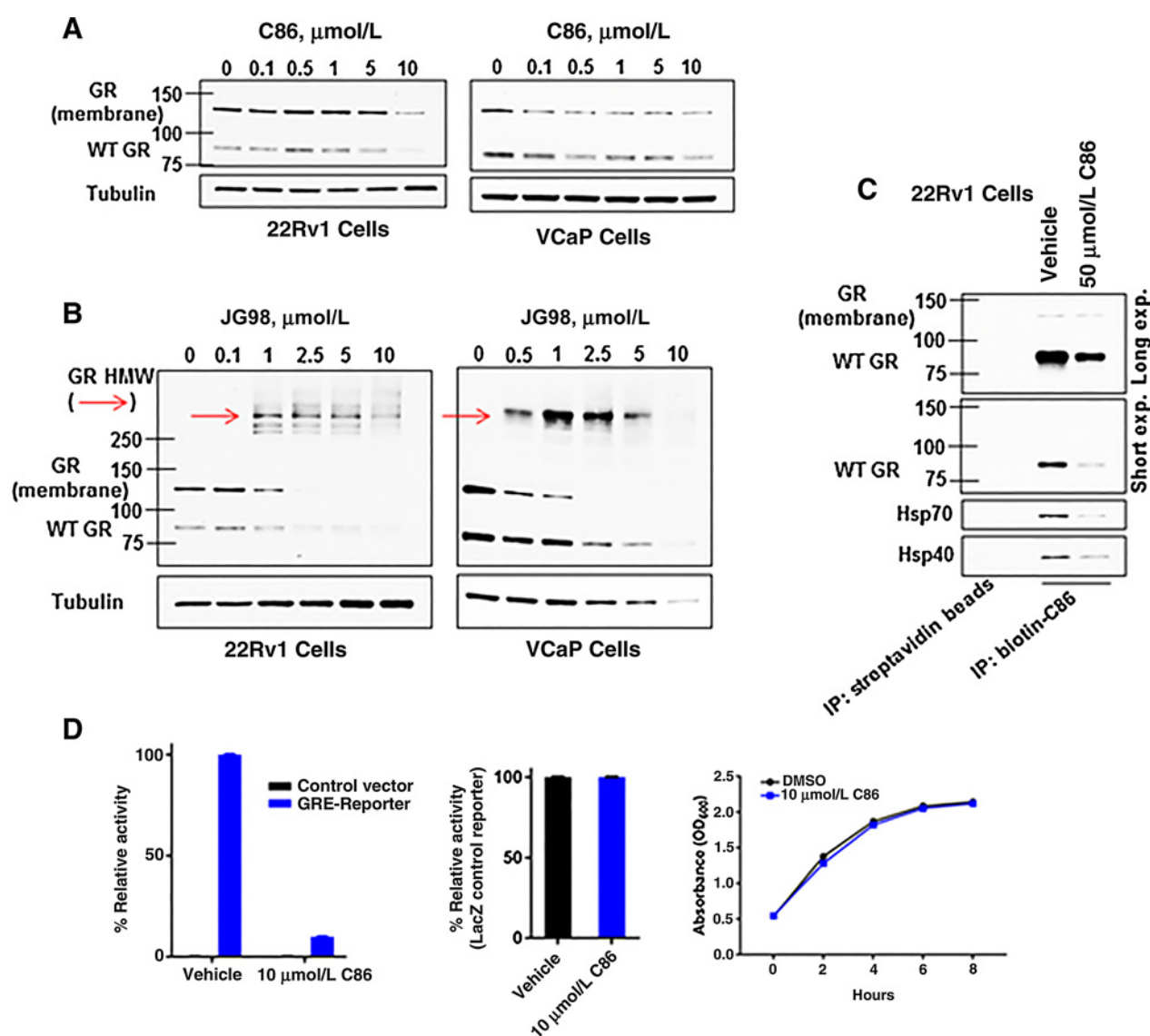
nuclear receptor that requires interaction with the Hsp40/Hsp70/Hsp90 chaperone axis for stability and function (41). Thus, we tested the ability of C86 and JG98 to affect GR protein levels. Using the D8H2 GR antibody (Cell Signaling Technology), we detected two distinct GR bands in an SDS gel: one pertaining to "native" (e.g., having the predicted mobility) GR and the other consistent with a lesser studied membrane-associated form of GR, which has also been reported to interact with Hsp70 and Hsp90 (Hsp40 was not tested; ref. 42). In response to either C86 or JG98, levels of both forms of GR protein in 22Rv1 and VCaP cells were decreased (Fig. 6A and B). These effects were confirmed *in vivo*, where a reduction in membrane-associated GR and native GR was found in 22Rv1 tumor lysate from JG231, C86, or JG231+C86-treated mice (Supplementary Fig. S5F). Similar to its impact on AR, JG98 induced the formation of soluble HMW GR species (Fig. 6B, red arrows). We also found both forms of GR interacted with b-C86, were pulled down in a complex containing Hsp40/Hsp70, and that b-C86-mediated pull-down of these proteins was competed by excess unlabeled C86 (Fig. 6C).

Finally, we coexpressed human GR and a GR reporter in yeast to examine the influence of C86 on GR transcriptional activity (JG98 was not used in this experiment as it directly affected the LacZ reporter). Consistent with the effects of C86 on GR protein levels (Fig. 6A), C86 markedly reduced GR transcriptional activity in this yeast model system (Fig. 6D, left), while negligibly affecting the LacZ control reporter (Fig. 6D, middle) or yeast viability (Fig. 6D, right). Together, these data provide evidence of the reliance of GR on the Hsp40/Hsp70 chaperone axis and they further emphasize the potential benefit of developing inhibitors of these two chaperones to combat therapeutic resistance in CRPC.

## Discussion

Current standard-of-care therapies for the treatment of CRPC, including enzalutamide and abiraterone, disrupt AR signaling by targeting the AR LBD (directly or indirectly, respectively). Although these agents prolong patient survival, they are not curative, and alternative splicing of the AR to a constitutively active, LBD-deficient form (e.g., ARv7) represents one mechanism of resistance (6). Furthermore, expression of the nuclear receptor GR is significantly increased in an enzalutamide-resistant CRPC model and in a portion of human CRPC patient tumors, and GR has been shown to regulate a subset of AR-dependent genes in CRPC (14, 17). Thus, identification of agents able to simultaneously target FL-AR and GR, as well as ARv lacking the LBD, may provide a novel strategy to treat several mechanisms of ADT resistance. In this study, we have identified Hsp40 and Hsp70 chaperones as critical regulators of these nuclear receptors, including ARv lacking the LBD.

These observations stem from our initial identification of C86 as a small molecule able to inhibit transcription of both androgen-dependent and androgen-independent AR target genes, and by our subsequent characterization of C86 as an inhibitor of Hsp40. Furthermore, our data showing that b-C86 recognizes a number of structurally diverse Hsp40 proteins (and even SV40 large T antigen), all of which have in common a J domain, which allows Hsp40 to interact with Hsp70, are key observations suggesting that C86 is a modulator of pan-Hsp40-dependent proteostasis. In 22Rv1 and VCaP CRPC cells, C86



**Figure 6.**

Hsp40 and Hsp70 inhibition destabilizes GR in CRPC cells. **A** and **B**, Western blot of cell lysate from 22Rv1 or VCaP CRPC cells treated with increasing concentrations of C86 (**A**, left, right, respectively) or JG98 (**B**, left, right, respectively) for 6 hours. Red arrows note detection of HMW GR species. **C**, Biotin-C86 binding profile of 22Rv1 CRPC cells incubated with excess unlabeled vehicle (DMSO) or 50  $\mu\text{mol/L}$  C86 for 1 hour as assessed by streptavidin-bead immunoprecipitation and Western blotting. **D**, GRE-(left) or LacZ Control-Reporter (middle) assay of yeast cells treated with vehicle or C86 as detected by chemiluminescence. Absorbance at O.D. 600 was utilized to measure yeast viability  $\pm$  C86 (right). Experiments were repeated at least three times.

was able to simultaneously affect FL-AR, ARv7, and GR protein stability and transcriptional activity.

Using b-C86 to pull down Hsp40-containing complexes, we not only identified FL-AR, ARv, including ARv7, and GR, but also Hsp70 and its cochaperones Bag3 and CHIP. Thus, we explored whether targeting Hsp70 may also impact FL-AR/ARv/GR protein stability and transcriptional activity. For that purpose, we used JG98, a potent allosteric inhibitor of Hsp70 (32, 39). We found that, similar to Hsp40 inhibition with C86, Hsp70 inhibition with JG98 also destabilized these nuclear receptor proteins and inhibited their transcriptional activity. However, unlike C86, which promoted accumulation of the nuclear receptors in a detergent-insoluble fraction upon pro-

teasome inhibition, JG98 promoted the formation of detergent-soluble HMW nuclear receptor species in which Hsp70 is present. This is a likely consequence of this inhibitor's mechanism of action, which involves stabilization of a high-affinity client-ADP-bound Hsp70 complex (32, 39).

Given the ability of C86 and JG98 to simultaneously target FL-AR, ARv, and GR, Hsp40/Hsp70 inhibition has the ability to affect multiple mechanisms of CRPC therapeutic resistance. For example, ADT can lead to reactivated AR signaling due to AR gene amplification, which sensitizes prostate cancer cells to low androgen levels, or to point mutations in the AR, which promote promiscuous/nonandrogenic ligand binding capable of upregulating AR transcriptional activity (5). Our data using

VCaP cells (which overexpress wild-type FL-AR due to gene amplification, while also expressing a number of ARv), show that both C86 and JG98 promote the degradation of overexpressed FL-AR as well as ARv. Furthermore, our data show that the endogenous FL-AR H874Y mutant expressed in 22Rv1 cells, which can be activated by the antiandrogen flutamide (43), remains sensitive to inhibition of Hsp40 and Hsp70. FL-AR mutations in response to selective pressure from enzalutamide have been described in an *in vitro* random mutagenesis screen (44) and have also been detected in human patients with CRPC (45). On the basis of our data, it is likely that these resistance-mediating AR mutants will also retain sensitivity to C86 and JG98.

There are a number of ARv whose contributions to CRPC and development of therapeutic resistance are not completely understood. For example, in addition to ARv7, both 22Rv1 and VCaP cells, and some human CRPC patient tumors, express AR<sup>v567es</sup>, another constitutively active AR splice variant (6). Expression of AR<sup>v567es</sup> promotes tumor growth in castrated mice (46), its transcript level has been shown to be increased in CRPC bone metastases, and its expression correlates with reduced survival (8). Like ARv7, AR<sup>v567es</sup> is also resistant to Hsp90 inhibitors (28). Although we did not specifically examine the effect of C86 or JG98 on AR<sup>v567es</sup> expression, it would be worthwhile to investigate if it and other ARvs also require the Hsp40/Hsp70 chaperone machinery for stability and transcriptional activity. This is likely to be the case, because the AR antibody N-20 (used to detect FL-AR) appears to recognize additional splice variants besides ARv7 that are similarly sensitive to both C86 and JG98.

Although there is strong evidence to implicate the Hsp40/Hsp70/Hsp90 chaperone complex in FL-AR/GR LBD refolding and stabilization (20, 23), other observations support an LBD-independent role for Hsp40/Hsp70 chaperones in regulating nuclear receptor stability. As general modulators of protein homeostasis, Hsp40 and Hsp70 are known to interact with and stabilize hydrophobic patches of nascent proteins (41), and mutations in Hsp40 result in AR aggregation (47). Furthermore, exogenous expression of the Hsp40 family members DnaJB6b and DnaJB8 reverses the aggregation propensity of poly-glutamine-expanded AR (48). Taken together, these observations suggest that ARv7 (and other ARv) likely require Hsp40 and Hsp70 for proper folding and prevention of aggregation. In addition, each of the currently identified ARv share a common N-terminal transactivation domain, which is required for interactions with cofactors to activate transcription (6). Because Hsp40/Hsp70 may reassociate with the FL-AR after ligand binding and assist with its nuclear translocation (41), and given that our data confirm LBD-deficient ARv7 interacts with Hsp40 and Hsp70, it is likely that one or both of these chaperones may play a role in stabilizing FL-AR and ARv7 for dimerization events (including heterodimerization; refs. 49, 50), assistance in nuclear translocation,

and interactions with cofactors and/or DNA to stimulate transcriptional activity.

No matter the ultimate mechanism or mechanisms by which C86 and JG98 affect the stability and function of these overexpressed, mutated, or aberrantly spliced nuclear receptors driving CRPC, this study provides an *in vitro* and *in vivo* proof-of-concept of the potential benefit of inhibiting the Hsp40/Hsp70 chaperone axis in ADT-resistant CRPC.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

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**Other (These contributions except for overall review pertain to the experiments carried out in my laboratory):** N.L. Weigel

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