Co-targeting AR and HSP90 suppresses prostate cancer cell growth and prevents resistance mechanisms

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Abstract

Persistent androgen receptor (AR) signaling in castration resistant prostate cancer (CRPC) underpins the urgent need for therapeutic strategies that better target this pathway. Combining classes of agents that target different components of AR signaling has the potential to delay resistance and improve patient outcomes. Many oncoproteins, including the AR, rely on the molecular chaperone heat shock protein 90 (Hsp90) for functional maturation and stability. In this study, enhanced anti-proliferative activity of the Hsp90 inhibitors 17-allylamino-demethoxygeldanamycin (17-AAG) and AUY922 in androgen-sensitive and CRPC cells was achieved when the agents were used in combination with AR antagonists bicalutamide or enzalutamide. Moreover, significant caspase-dependent cell death was achieved using sub-optimal agent doses that individually have no effect. Expression profiling demonstrated regulation of a broadened set of AR target genes with combined 17-AAG and bicalutamide compared with the respective single agent treatments. This enhanced inhibition of AR signaling was accompanied by impaired chromatin binding and nuclear localization of the AR. Importantly, expression of the AR variant AR-V7 that is implicated in resistance to AR antagonists was not induced by combination treatment. Likewise, the heat shock response that is typically elicited with therapeutic doses of Hsp90 inhibitors, and is a potential mediator of resistance to these agents, was significantly reduced by combination treatment. In summary, the co-targeting strategy in this study more effectively inhibits AR signaling than targeting AR or HSP90 alone and prevents induction of key resistance mechanisms in prostate cancer cells. These findings merit further evaluation of this therapeutic strategy to prevent CRPC growth.

Key Words

- ▶ prostate cancer
- ▶ heat shock protein
- ▶ androgen receptor
- ▶ combination

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Introduction

Androgen deprivation therapy (ADT) is used palliatively to control tumor growth and metastasis in men with locally advanced prostate cancer (Labrie 2011). Despite an

initial response to ADT, the majority of patients will inevitably progress to castrate-resistant prostate cancer (CRPC) that is not curable by current treatments. CRPC

develops as prostate cancer cells adapt to a low androgen environment through a range of resistance mechanisms that are predominantly mediated by alterations to androgen receptor (AR) signaling (Karantanos *et al.* 2013, Maughan & Antonarakis 2015). These include AR overexpression or amplification (Chen *et al.* 2008), acquisition of gain-of-function point mutations in the AR gene (Buchanan *et al.* 2001), intra-tumoral androgen biosynthesis (Cai & Balk 2011) or emergence of C-terminally truncated AR variants (ARVs) that are constitutively nuclear and active (Sun *et al.* 2010, Li *et al.* 2011). This adaptation to therapy-mediated selection pressure is well recognized with the use of molecularly targeted cancer therapies (Izar *et al.* 2013) and has led to the growing paradigm that ablating a target with a single agent is unlikely to result in sustained growth inhibition. Recently, an integrative clinical genomics analysis of 150 metastatic CRPC cases revealed that 71.3% harbored aberrations in AR signaling (Robinson *et al.* 2015). This high frequency clearly demonstrates the continued reliance of CRPC on AR signaling and provides a biological rationale for the development of AR-targeted combination therapy. We hypothesize that using agents with different mechanisms of action, to simultaneously inhibit both the expression and activity of AR, will minimize the likelihood of an adaptive response and thereby improve disease outcomes.

Functional maturation of the AR protein is a critical step in the androgen signaling axis that can be targeted through inhibition of the molecular chaperone heat shock protein 90 (Hsp90) (Centenera *et al.* 2013). Molecular chaperones are required for the stabilization and activity of a diverse group of proteins, known as clients, which includes steroid receptors, transcription factors and protein kinases (Trepel *et al.* 2010). Importantly, Hsp90 folds the AR into the correct conformation for stable, high affinity ligand binding (Pratt & Toft 1997). As the chaperone activity of Hsp90 is ATP-dependent, most Hsp90 inhibitors are targeted to the conserved ATP-binding site and cause ubiquitin-mediated proteasomal degradation of more than 200 client proteins (Workman *et al.* 2007). The first Hsp90 inhibitor to enter clinical trials was the ansamycin derivative 17-allylamino-demethoxygeldanamycin (17-AAG). In pre-clinical studies, 17-AAG degraded the AR and other proteins (Her2, Akt, Bcr-Abl and Raf-1) implicated in prostate carcinogenesis, leading to cell cycle arrest and inhibition of prostate cancer cell growth *in vitro* and *in vivo* (Solit *et al.* 2002, Vanaja *et al.* 2002, Williams *et al.* 2007). Unfortunately, 17-AAG did not show its predicted clinical efficacy as a single agent in

CRPC, due to toxicity and poor pharmacodynamic properties that prevented therapeutic doses being achieved (Banerji *et al.* 2005, Heath *et al.* 2008, Ramanathan *et al.* 2010). While more potent inhibitors such as AUY922 are currently being developed and show promise as single agents (Samuel *et al.* 2010, Centenera *et al.* 2012), recent evidence of additive or synergistic activity between 17-AAG and cytotoxic agents or specific molecular therapeutics provides a promising new avenue of clinical development for this drug (Lu *et al.* 2012). In preclinical prostate cancer studies, 17-AAG or AUY922 combined with ionizing radiation has demonstrated supra-additive reductions in tumor growth and clonogenicity (Enmon *et al.* 2003, Ochel & Gademann 2006, Gandhi *et al.* 2013). Likewise, AUY922 sensitized CRPC prostate cancer cells lines to treatment with docetaxel chemotherapy (Ku *et al.* 2014). In a Phase I trial of 17-AAG plus docetaxel, 25% of the patients with prostate cancer exhibited a prostate-specific antigen (PSA) decline of $\geq 20\%$ (Iyer *et al.* 2012).

AR antagonists compete with androgens for binding to the AR. The AR antagonist bicalutamide is typically used in combination with ADT to inhibit signaling from residual circulating androgens in a strategy known as combined androgen blockade (CAB). CAB prolongs survival in both locally advanced and metastatic disease, particularly when administered as first-line systemic therapy (Labrie 2011); however, AR antagonists and ADT both essentially act to prevent ligand activation of the AR and patients inevitably develop resistance. The new generation antagonist enzalutamide also targets the AR ligand-binding domain but was specifically selected to have anti-tumor activity in prostate cancer cells that overexpress the AR (Tran *et al.* 2009) as this is a key aberration in CRPC (Robinson *et al.* 2015) and an important mechanism of resistance to androgen targeting agents (Chen *et al.* 2004). AR antagonists are a rational choice for use in combination with Hsp90 inhibitors due to the unique chaperone dependence of antagonist-bound AR. Several studies have demonstrated that bicalutamide stabilizes the interaction between AR and Hsp90, which prevents AR from acquiring its active state (Veldscholte *et al.* 1992, Kuil *et al.* 1995, Georget *et al.* 2002). As Hsp90 dissociates less readily from AR when bound to bicalutamide, compared to androgens, we propose that the use of bicalutamide maintains the AR as a vulnerable target for degradation by Hsp90 inhibitors. The aim of this study was to evaluate whether co-targeting Hsp90 and AR can achieve a better blockade of androgen signaling to enhance prostate cancer cell death.

Materials and methods

Cells and reagents

LNCaP, 22Rv1, C4-2B and PC-3 human prostate carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). All cell lines underwent verification by short-tandem repeat profiling in 2010 by CellBank Australia. LNCaP, 22Rv1 and C4-2B cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and PC-3 cells were supplemented with 5% FBS. The National Cancer Institute provided 17-AAG, AUY922 was provided by Novartis, bicalutamide was purchased from Sigma and enzalutamide was purchased from Selleckchem (Houston, TX, USA). All drugs were dissolved in dimethylsulfoxide (DMSO). Hsp27 (HPA000497) antibody was purchased from Sigma-Aldrich Co., Hsp70 (ADI-SPA-812) antibody from Enzo Life Sciences (Farmingdale, NY, USA), Hsp90 (SC-7947) and AR N-20 (SC-816) antibodies from Santa Cruz Biotechnology (Dallas, TX, USA), α -tubulin (05-829) antibody from Millipore (Billerica, MA, USA) and Histone H3 acetyl K27 (ab4729) antibody from Abcam (Cambridge, MA, USA). HRP-conjugated secondary antibodies were obtained from DAKO (Carpinteria, CA, USA). The tetrapeptide caspase inhibitor z-VAD-fmk was purchased from Calbiochem (Alexandria, NSW, Australia).

Measurement of cell viability

Cells were seeded in triplicate in 24-well plates at a density of 2.5×10^4 cells per well in RPMI medium containing 5% (PC-3) or 10% (LNCaP, C4-2B, 22Rv1) FBS. Cells were treated as indicated for 4 days and counted as previously described (Centenera et al. 2012). The caspase inhibitor z-VAD-fmk (50 μ M) was included for the duration of the relevant experiments.

Quantitation of drug synergy

The combination index (CI) theorem of Chou-Talalay (Chou & Talalay 1984) was used to quantify drug interactions with CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). This method uses the equation $Ac/Ae + Bc/Be = CI$ to determine the interaction between two drugs. Ac and Bc represent the concentration of drugs A and B used in the combination, whereas Ae and Be represent the concentration of drugs A and B that produced the same magnitude of effect when administered alone. The resulting CI quantitatively defines drug synergy ($CI < 1$), antagonism ($CI > 1$) and additive effects ($CI = 1$).

Cell cycle analysis

C4-2B cells were plated in six-well plates at a density of 1.5×10^5 per well in RPMI supplemented with 10% FBS. Two wells were used per treatment and all treatments were performed in triplicate. Cells were treated as indicated for 72 h then fixed as described previously (Centenera et al. 2012). Cell cycle analysis was performed on the LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA) running BDFACSDiva version 8.0 software (BD Biosciences) to generate frequency histograms.

Microarray and pathway analysis

LNCaP cells in six-well plates were treated as indicated for 6 h. Six replicates were prepared per treatment. Total RNA was extracted using TRIzol reagent (Invitrogen), and integrity was analyzed with an Agilent Systems Bioanalyser (Santa Clara, CA, USA). Microarray analysis was performed at the Adelaide Microarray Centre. Briefly, 300 ng of total RNA was labelled using the Affymetrix WT Sense Target labeling assay as per the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA, USA, p/n 701880). Samples were hybridized to Affymetrix Human Gene 1.0 ST Arrays for 17 h at 45 °C prior to washing, staining and scanning. Data was analyzed with Partek Genomics Suite (Partek Inc., St. Louis, MO, USA). Differential gene expression was assessed by ANOVA with the P value adjusted using step-up multiple test correction (Benjamini & Hochberg 1995) to control the false discovery rate (FDR). Adjusted P values < 0.05 were considered significant. Cluster and Treeview algorithms were used to generate self-organizing maps of the gene expression data sets (Eisen et al. 1998). Gene pathway analysis was conducted using core analysis in the Ingenuity Systems program (Ingenuity Systems, Redwood City, CA, USA) to identify molecular and cellular functions and canonical pathways that were enriched by the combination treatment. Differentially expressed genes were also analyzed for enriched gene ontology (GO) groups using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov/>; Huang da et al. 2009). Genes with a minimal twofold change and $P < 0.05$ were included for analysis and the background gene set comprised of all genes on the Affymetrix Human GeneChip ST 1.0 array.

Quantitative real-time PCR

Microarray results were validated using an independently generated sample set by real-time PCR (RT-qPCR).

RNA (1 µg) was DNase treated with Turbo DNA Free (Ambion, Carlsbad, CA, USA) and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed with a 1:10 dilution of cDNA using SYBR green (Bio-Rad) on a CFX384 Real-Time System (Bio-Rad) with three-step amplification for 40 cycles. Gene expression is presented relative to L19 and GUSB as suggested by GeNorm (Vandesompele *et al.* 2002). Primer sequences are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Western blotting

LNCaP cells were seeded in six-well plates at a density of 4×10^5 cells per well and allowed to attach for 24 h before being treated as indicated. Western blotting on whole cell extracts was performed as previously described (Centenera *et al.* 2012).

Visualization of green fluorescent protein-tagged AR

PC-3 cells were simultaneously seeded and transfected in six-well plates containing 20 mm round glass coverslips. Cells were seeded at a density of 5×10^5 cells per well in phenol red free (PRF)-RPMI 1640 and transfected with 10 µg pEGFP-ARwt using Lipofectamine 2000 (Life Technologies). After 24 h, cells were treated for 4 h with RPMI 1640 supplemented with 10% FBS containing DMSO or combination treatment, 20 nM 17-AAG + 2.5 µM bicalutamide, or PRF-RPMI supplemented with 10% dextran coated charcoal-treated FBS (dccFBS) containing vehicle (ethanol) or 10 nM 5- α -dihydrotestosterone (DHT). Cells were fixed in ice-cold methanol and immunostained with α -tubulin primary antibody (1:100) for 1 h, then Alexa Fluor 488 goat anti-mouse secondary antibody (1:400) for 1 h in the dark. Cells were mounted with ProLong Gold antifade reagent containing DAPI (Molecular Probes, Carlsbad, CA, USA). Slides were analyzed by the Confocal Microscopy Core Facility at the South Australian Health and Medical Research Institute on a Leica TCS SP8X/MP multifoton confocal microscope on an inverted DMI6000 microscope body with a resonance scanner (12 kHz), coupled with a Ludin Cube/Box/Brick environmental control system. Analysis of fluorescent images was performed using ImageJ version 3.91 software.

Subcellular fractionation

LNCaP cells were seeded in PFR-RPMI 1640 supplemented with 10% dccFBS at a density of 8×10^5 cells per 10 cm plate. After 3 days the cells were treated for 4 h with RPMI containing 10% FBS containing DMSO or combination

treatment, 20 nM 17-AAG + 2.5 µM bicalutamide, or PRF-RPMI containing 10% dccFBS containing vehicle (ethanol) or 10 nM DHT. Protein interactions were crosslinked with a 1/10 volume of fresh formaldehyde solution (50 mM HEPES-KOH, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% formaldehyde). Cells were washed in PBS and resuspended in lysis buffer (LB) 1 (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40 or Igepal CA-630; 0.25% Triton X-100). The cytoplasmic fraction was taken after centrifuging at 10,000 *g* for 10 s. The pellet was washed in 1 ml LB2 (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA) and nuclear fraction was obtained by resuspending and sonicating the pellet in LB3 (10 mM Tris-HCl, pH 8; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na-Deoxycholate; 0.5% N-lauroylsarcosine). Fractions were centrifuged and equivalent volumes of supernatant were separated by SDS-PAGE to assess AR location by probing with AR N-20 (1:1000). Appropriate subcellular fractionation was assessed using the cytoplasmic marker α -tubulin (1:20 000) and the nuclear marker H3 acetyl K27 (1:1000).

Chromatin immunoprecipitation

LNCaP cells were seeded in 15 cm plates at a density of 3×10^6 cells in RPMI 1640 containing 10% dccFBS. Cells were cultured for 3 days, then treated for 4 h with vehicle or combined 17-AAG and bicalutamide in RPMI 1640 containing 10% FBS. Chromatin immunoprecipitation (ChIP) was performed as described previously (Schmidt *et al.* 2009) with the following modifications. Samples were immunoprecipitated overnight with ARN-20x (Santa Cruz-816x) or Normal Rabbit IgG (Santa Cruz-2027) antibodies. DNA was cleaned up using the Qiagen PCR Purification Kit and eluted into 100 µl RNase free water. Enrichment analysis was performed by RT-qPCR as described above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 and statistical significance accepted at $P \leq 0.05$.

Results

Combining 17-AAG and bicalutamide enhances growth suppression and cell death in AR-sensitive prostate cancer cells

This study selected doses of 17-AAG and bicalutamide that individually elicit no significant growth inhibitory or cell

death effects (Fig. 1), as therapeutic doses of Hsp90 inhibitors and AR antagonists are known to induce mechanisms of resistance (Centenera *et al.* 2012, Maughan & Antonarakis 2015). Combination of these sub-optimal doses of 17-AAG and bicalutamide significantly inhibited the proliferation and induced death of LNCaP ($P < 0.05$; Fig. 1A) and C4-2B ($P < 0.05$; Fig. 1B) prostate cancer cells compared with either vehicle alone or individual agents. LNCaP cell proliferation was reduced by 75% and cell death induced by 3.2-fold with the most potent combination compared with vehicle treatment. C4-2B cells showed a similar response as LNCaP cells, with proliferation reduced by 81% and cell death induced by 3.5-fold compared with vehicle alone. To achieve comparable effects on cell proliferation and death with the agents individually, much higher concentrations of 80 nM 17-AAG or 25 μ M bicalutamide were required (Supplementary Figure 1, see section on supplementary data given

at the end of this article). Combination experiments were replicated in 22Rv1 and PC-3 prostate cancer cell lines that are considered androgen insensitive as they grow in the absence of androgens. No significant changes in 22Rv1 cell proliferation or death were observed compared with vehicle or equivalent doses of the individual agents (Fig. 1C). In PC-3 cells, the higher dose of 40 nM 17-AAG showed some anti-proliferative effect compared with vehicle (Fig. 1D); however, no further reduction in proliferation was observed when cells were co-treated with bicalutamide (Fig. 1D).

17-AAG and bicalutamide act synergistically to induce caspase-dependent cell death

CI_s were calculated using CalcuSyn software, in which synergy is achieved when a combination of drugs is more effective than each agent separately, resulting in a CI < 1 .

Figure 1

Combination treatment targeting Hsp90 and AR induces synergistic growth suppression and cell death in AR-sensitive prostate cancer cells. (A) LNCaP, (B) C4-2B, (C) 22Rv1 and (D) PC-3 cells were cultured with 17-AAG or bicalutamide, alone or in combination. Cells were counted after 4 days using a haemocytometer and cell viability assessed by trypan blue dye exclusion. Cell viability (left panels) and cell death (right panels) are presented as a percent of vehicle treatment $\pm$ s.e. of triplicate wells. Results are representative of at least three independent experiments. *ANOVA: $P < 0.05$ treatments vs control. (E) Combination indices (CI) from the above

growth curves were calculated according to the method of Chou-Talalay (Chou & Talalay 1984). The resulting CI defines whether the drug combination effect is synergistic (CI < 1), antagonistic (CI > 1) or additive (CI = 1). (F) LNCaP cells were cultured with 17-AAG and bicalutamide, alone or in combination, in the presence or absence of the z-VAD-fmk pan-caspase inhibitor. Cells were counted after 4 days as described above. Results are representative of three independent experiments and represent the mean $\pm$ s.e. of triplicate wells. *ANOVA: $P < 0.001$ treatments vs control. f.c., fold change.

The CI for 17-AAG and bicalutamide in LNCaP and C4-2B were both <1 , confirming a synergistic interaction between the two agents (Fig. 1E). In 22Rv1 and PC-3 cells, the CI was >1 indicating an antagonistic drug interaction (Fig. 1E). Combination-induced cell death was prevented in the presence of the pan-caspase inhibitor z-VAD-fmk, indicative of apoptotic cell death (Fig. 1F).

Global gene expression changes following combination treatment

To explore the potential mechanisms underlying synergy between 17-AAG and bicalutamide in androgen-dependent cells, gene expression profiling was performed using Affymetrix microarray. Single-agent treatment with 17-AAG or bicalutamide significantly ($P < 0.05$) altered a similar number of transcripts (790 and 609 genes respectively), which increased markedly to 2158 transcripts when the two agents were used in combination (Fig. 2A). Although some overlap with single-agent

17-AAG (14%) or bicalutamide (16%) treatment was evident, the majority of genes (59%) significantly affected by the drug combination were unique. Ingenuity Pathway Analysis (IPA) revealed cell cycle to be the most highly enriched molecular and cellular function by the combination, followed by drug and lipid metabolism (Fig. 2B). Enrichment of these molecular and cellular functions was confirmed using DAVID, in which cell cycle was again the top biological process in combination-treated cells (Supplementary Table 2, see section on supplementary data given at the end of this article). The most highly enriched canonical pathway was protein ubiquitination (Fig. 2C). Several pathways associated with steroid receptor signaling featured in the top 10 canonical pathways, including aldosterone signaling, glucocorticoid receptor signaling, Huntington's disease signaling and, importantly, prostate cancer signaling (Fig. 2C). According to DAVID, prostate cancer signaling was the most significant KEGG pathway in combination-treated cells (Supplementary Table 3).

Figure 2

17-AAG works in synergy with bicalutamide to induce caspase-dependent cell death and amplify global gene expression changes in LNCaP cells. (A) Venn diagram represents the overlap between significantly expressed genes as identified by Affymetrix gene expression profiling in treated LNCaP cells compared with vehicle-treated cells (Benjamini-Hochberg adjusted P values; $P < 0.05$). (B) Molecular and cellular functions and

(C) canonical pathways enriched by combination treatments as determined by Ingenuity Pathway Analysis. (D) Representative flow cytometry histograms depicting cell cycle distribution in C4-2B cells treated with combined 17-AAG and bicalutamide for 72 h. Bar graph presents the mean $\pm$ s.e. of triplicate wells and is representative of three independent experiments. *ANOVA: $P < 0.05$ treatments vs control.

Combination treatment induces cell cycle inhibition

Based on the outcomes of the pathway analysis, we investigated how combined 17-AAG and bicalutamide treatment influences the cell cycle. When compared with vehicle treatment, combination-treated cells displayed a significant decrease in G0/G1 phase from 83% to 63% ($P < 0.05$), and accumulation of cells in the sub-G1 phase increased from 3% in controls to 20% with combined 17-AAG and bicalutamide ($P < 0.05$; Fig. 2D). This data not only verifies the pathway analysis but provides further support that the combination treatment induces cell death through apoptosis.

Inhibition of AR signaling is enhanced by co-treatment with 17-AAG and bicalutamide

The specificity of the combination treatment for AR-dependent cells suggests that AR signaling has a mechanistic role in treatment efficacy. This idea was directly interrogated by cross-referencing the list of genes regulated by the combination treatment with the 1755 androgen-regulated genes identified in LNCaP cells by Wang *et al.* (2009). Approximately 29% (505/1755) of androgen-responsive genes were significantly ($P < 0.05$) altered by combined 17-AAG and bicalutamide. Cluster

analysis revealed that the majority (87%; 439/505) of those androgen-regulated genes are antagonized by combination treatment (Fig. 3A). As single agents, 17-AAG and bicalutamide significantly altered the expression of 171 and 300 androgen-regulated genes, respectively, whereas combination treatment markedly increased this number to 505 androgen-regulated genes (Fig. 3A). The Venn diagram in Fig. 3B illustrates that the 505 genes not only were the sum of the individual treatments but also include a set of 207 androgen-regulated genes that were novel to combined 17-AAG and bicalutamide. Of the 2158 transcripts significantly altered by combination treatment, only 48 genes displayed $\geq$ twofold change in expression compared with vehicle treatment (Supplementary Table 4, see section on supplementary data given at the end of this article) and, notably, 58% (28/48) of those were androgen regulated (Fig. 3C). RT-qPCR analysis of two well-known androgen-regulated genes, *PMEPA1* and *NKX3.1*, using RNA from the microarray analysis and an independently generated RNA sample set validated these findings (Fig. 3D). Expression of AR was not altered by the low doses of 17-AAG or bicalutamide, either alone or in combination (Fig. 3E). Collectively, these findings suggest that using 17-AAG and bicalutamide in a combinatorial strategy broadens the

Figure 3

Combining 17-AAG with bicalutamide enhances inhibition of AR signaling. (A) Heat map represents androgen-regulated genes that were significantly ($P < 0.05$) up-regulated (blue) or down-regulated (red) by the indicated treatments. Hierarchical clustering was performed by applying the complete linkage rule in Cluster3.0 and heat maps constructed using Java TreeView (<http://rana.lbl.gov/eisensoftware.htm>). (B) Venn diagram represents the overlap between androgen-regulated genes significantly altered by 17-AAG or bicalutamide, alone or in combination (Benjamini-Hochberg adjusted P values; $P < 0.05$). (C) Heat map represents the subset of

androgen-regulated genes that were up-regulated (blue) or down-regulated (red) by at least twofold by the indicated treatments. (D) Enhanced expression of a subset of androgen-regulated genes in combination-treated LNCaP cells was validated by qPCR analysis of *PMEPA1* and *NKX3.1*. Gene expression was normalized to *GUSB* and *L19* and represents the mean $\pm$ s.e. of three biological replicates. *ANOVA: $P < 0.05$ treatment vs control. (E) LNCaP cells treated for 24 h with the indicated doses of 17-AAG and bicalutamide, alone or in combination, were analyzed by western blot for modulation of AR. HSP90 was used as a loading control.

blockade of AR signaling over a larger number of AR-regulated target genes.

Co-treatment with 17-AAG and bicalutamide prevents chromatin binding and nuclear localization of AR

Combining 17-AAG and bicalutamide markedly decreased the binding of AR to regulatory regions of the *KLK3*, *FKBP5* and *C1ORF116* genes (Fig. 4A), consistent with a loss of AR

chromatin occupancy underlying the observed silencing of AR-regulated genes. To evaluate whether AR nuclear translocation was also impaired, we assessed the effect of the combination on subcellular localization of an enhanced green fluorescent protein (EGFP)-tagged AR. AR was predominantly localized to the nucleus in vehicle-treated PC-3 cells expressing EGFP-AR cultured in full serum, similar to that observed in cells cultured in androgen-deplete (charcoal-stripped) serum containing

Figure 4

Combined 17-AAG with bicalutamide inhibits AR nuclear translocation and prevents DNA binding. (A) Recruitment of AR to the promoter of AR-regulated genes *KLK3*, *FKBP5*, *C1ORF116* was prevented by treatment with combined 17-AAG and bicalutamide. Values are presented as percentage input. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. NC2 is an irrelevant region of the *PSA* gene. (B) Confocal microscopy of representative PC-3 cells transfected with pEGFP-AR and treated for 4 h with vehicle or combined 17-AAG and

bicalutamide in medium containing full serum (upper two panels) or vehicle or 1 nM DHT in medium containing steroid-deplete serum (lower two panels). The nucleus and cytoplasm were stained with DAPI and α-tubulin respectively. (C) Evaluation of fluorescent localization images represented as the percent of transfected PC-3 cells with nuclear AR as determined by ImageJ analysis. *Unpaired t test: $P < 0.05$ treatment vs vehicle. (D) LNCaP cell fractionation demonstrating the location of AR following the indicated treatments. α-tubulin was used as a cytoplasmic marker; acetylated H3 was used as nuclear marker.

1 nM DHT (Fig. 4B and C). In contrast, combination treatment blocked the nuclear import of AR, which remained in a diffuse pattern, similar to cells that had been grown in androgen-deplete serum for 3 days (Fig. 4B and C). As combination treatment does not significantly alter AR steady-state levels (Fig. 3E), the decrease in nuclear AR cannot be attributed to altered stability or turnover. Subcellular fractionation of LNCaP cells verified a decrease in nuclear AR with combined 17-AAG and bicalutamide treatment (Fig. 4C).

Combinatorial synergy is retained by new generation inhibitors AUY922 and enzalutamide

We extended our studies to investigate whether newer generation agents AUY922 and enzalutamide would also exhibit synergy when used in combination. AUY922 is a synthetic Hsp90 inhibitor with much greater potency than 17-AAG but also improved solubility and reduced toxicity (Eccles et al. 2008, Centenera et al. 2012). When treated in combination with bicalutamide, highly similar patterns of cell growth inhibition (80% vs vehicle) and death (4.4-fold vs vehicle) were achieved using AUY922 in place of 17-AAG ($P < 0.05$; Fig. 5A). We then alternated the agents and investigated whether the second generation AR antagonist enzalutamide is capable of synergy as part of a combinatorial treatment. Enzalutamide is

superior to bicalutamide in terms of binding affinity and disruption of AR localization and DNA binding and is clinically approved for use in CRPC (Tran et al. 2009, Scher et al. 2010). Significant cell growth inhibition and death of LNCaP cells was observed when enzalutamide was used in combination with either 17-AAG ($P < 0.05$; Fig. 6B) or AUY922 ($P < 0.05$; Fig. 6C).

Co-treatment with AUY922 and enzalutamide inhibits growth of androgen-insensitive 22Rv1 cells and prevents induction of AR-V7

Previously we have demonstrated that AR-V7 expressing prostate cancer cells are sensitive to AUY922 (Gillis et al. 2013). Given the efficacy of co-treatment with AUY922 and enzalutamide we observed in LNCaP cells, we evaluated the combination of these two agents in AR-V7 expressing 22Rv1 cells and found that cell growth inhibition was reduced significantly by 40% compared to vehicle treatment ($P < 0.05$; Fig. 5D). In addition, we demonstrated that expression of AR-V7 is not induced with combined AUY922 and enzalutamide treatment (Fig. 5E), whereas therapeutic doses of both enzalutamide (Supplementary Figure 2, see section on supplementary data given at the end of this article) (Li et al. 2013) and AUY922 (Gillis et al. 2013) do acutely induce AR-V expression.

Figure 5

Combinatorial synergy is retained with new generation Hsp90 inhibitor AUY922 and AR antagonist enzalutamide. LNCaP cells were treated with (A) AUY922 and bicalutamide, (B) 17-AAG and enzalutamide or (C) AUY922 and enzalutamide, alone or in combination. (D) 22Rv1 cells were treated with AUY922 and enzalutamide, alone or in combination. Cells were counted after 4 days using a haemocytometer and cell viability assessed by trypan blue dye exclusion. Cell viability (left panel) and cell death

(right panel) are presented as fold change from vehicle treatment $\pm$ s.e. of triplicate wells. Results are representative of at least three independent experiments. *ANOVA: $P < 0.05$ treatments vs control. (E) 22Rv1 cells treated with the indicated doses of AUY922 and enzalutamide, alone or in combination, were analyzed by western blot for modulation of ARfl and ARV7. HSP90 was used as a loading control. f.c., fold change.

Figure 6

Combination treatment minimizes mechanisms of resistance to Hsp90 inhibitors and AR antagonists. LNCaP cells were treated with low dose 17-AAG (20 nM) and bicalutamide (5 μM), alone or in combination, or with a therapeutic dose of 17-AAG (80 nM). (A) Total RNA was extracted after 6 h and expression of genes encoding heat shock proteins Hsp70, Hsp40, Hsp27 and CLU were evaluated by RT-qPCR. Expression was normalized to L19 and GUSB. Error bars represent mean $\pm$ s.e. of three biological

replicates. *ANOVA: $P < 0.05$ combination vs 80nM 17-AAG. (B) Whole cell lysates were analyzed by western blotting with AR N-20. Densitometry was used to quantitate AR steady state levels relative to HSP90 loading control. Values are presented as fold change over vehicle treatment. (C) LNCaP cells were treated with low doses of AUY922 (10 nM) and bicalutamide (2.5 μM), alone or in combination. Total RNA was extracted and RT-qPCR performed as in panel A. *ANOVA: $P < 0.05$ combination vs 40 nM AUY922.

Co-treatment with 17-AAG and bicalutamide minimizes the heat shock response

The most significantly up-regulated transcripts in combination-treated LNCaP cells were those that encode members of the heat shock protein family, including *HSPA1A*, *HSPA1B*, *DNAJB1*, and *CLU* (Supplementary Table 4). Of the 13 genes that were up-regulated by $\geq$ twofold compared with vehicle-treated cells, 77% (10/13) encode heat shock proteins (Supplementary Table 4). The transcriptional induction of heat shock proteins in response to cellular stress, known as the heat shock response, serves to repair damaged proteins or prepare them for proteasomal degradation (Zou et al. 1998). The observed up-regulation of heat shock proteins is therefore consistent with protein ubiquitination being the most significantly enriched canonical pathway in combination-treated cells (Fig. 2D). Increased expression of genes encoding heat shock proteins HSP70 (*HSPA1A/B*), HSP40 (*DNAJB1*), HSP27 (*HSPB1*) and CLU (*CLU*) with combined 17-AAG and bicalutamide treatment was compared with vehicle or the equivalent dose of bicalutamide alone, but there was no significant difference to the equivalent dose of 17-AAG alone ($P < 0.05$; Fig. 5A). Moreover, induction of heat shock proteins in LNCaP cells co-treated with 17-AAG and bicalutamide was significantly lower than in cells treated with therapeutic doses (80 nM) of 17-AAG alone ($P < 0.05$, Fig. 5A), despite the fact that both treatments induced equivalent cell growth suppression and death

(Fig. 1A and Supplementary Figure 1). Concomitant changes in the protein levels of HSP70 and HSP27 were observed (Fig. 5B). Combining AUY922 with bicalutamide also minimized induction of the heat shock response when compared with a lethal dose (40 nM) of AUY922 alone ($P < 0.05$; Fig. 5D).

Discussion

With recent compelling evidence that CRPC arises as an adaptive response to maintain AR signaling despite castrate levels of circulating testosterone, we propose that combinatorial targeting of the AR using drugs with different mechanisms of action may provide a more effective strategy to control CRPC and its associated mortality (Chen et al. 2008). This study demonstrates that Hsp90 inhibitors act in synergy with AR antagonists to enhance AR blockade and reduce survival of prostate cancer cells while also minimizing known mechanisms of resistance. Importantly, these effects were demonstrated using doses of each agent that individually were not effective and are both clinically achievable and tolerable (Kelly et al. 2003, Tyrrell et al. 2006, Solit et al. 2007).

Using four different prostate cancer cell lines, we found that combining 17-AAG and bicalutamide is effective in cells that are sensitive to androgens (LNCaP, C4-2B) but is not effective in cells that are weakly activated by androgens and express constitutively activated AR

spliced variants (22Rv1) or are androgen independent and AR negative (PC-3). These findings focused our investigation into androgen signaling as a key mechanism of action underpinning the efficacy of the combination treatment. Minimal changes in AR transcript or protein levels were observed in combination-sensitive cells. This was unexpected, in light of previous reports of enhanced interaction between bicalutamide-bound AR and Hsp90 and the fact that Hsp90 inhibitors are well documented to induce proteasomal degradation of the AR (Centenera et al. 2013), but likely reflects the low doses that were used in this study. ChIP and localization analysis revealed instead that the AR is held in the cytoplasm by combined 17-AAG and bicalutamide, thereby preventing nuclear translocation and loss of AR occupancy at AR-regulatory regions in chromatin. These results echo previous biochemical studies that observed cytoplasmic aggregates in cells treated with bicalutamide and geldanamycin, the predecessor of 17-AAG (Georget et al. 2002). Reduced AR nuclear transport and impaired chromatin binding explains the pronounced antagonistic effects of the combination treatment on androgen signaling. This mechanism of action is similar to that observed for the latest generation AR antagonist enzalutamide (Tran et al. 2009) and provides a major advantage for combination treatment over bicalutamide as a single agent. Bicalutamide does not prevent AR from translocating to the nucleus or binding DNA but rather stimulates the assembly of an inactive receptor complex at AR binding sites (Masiello et al. 2002). Increased expression of AR or coactivator proteins can reduce the efficacy of bicalutamide and even cause a shift in action from antagonist to agonist. Use of bicalutamide in a combinatorial strategy with Hsp90 inhibition therefore provides an opportunity to markedly enhance its activity, even in the context of aberrant AR signaling that is characteristic of CRPC (Robinson et al. 2015).

Inhibition of androgen signaling as the primary mechanism of action of 17-AAG and bicalutamide is consistent with the associated molecular and cellular functions of cell cycle, cell death and lipid metabolism that were identified through genome-wide profiling. Cross-talk between AR and the cell cycle is well known to influence the mitogenic response to androgens (Schiewer et al. 2012). Androgen-sensitive cells that are deprived of androgen exit the cell cycle and arrest in G0 (Knudsen et al. 1998, Agus et al. 1999), much like the cell cycle effects of combined 17-AAG and bicalutamide treatment observed here. Finally, lipid metabolism is highly regulated by androgens in prostate cancer cells

(Swinnen et al. 2004). Bicalutamide inhibits lipid synthesis in LNCaP cells (Swinnen et al. 1996), and increased levels of key lipogenic enzymes have been reported in CRPC patients (Rossi et al. 2003, Ettinger et al. 2004).

Although AR-V7 expressing 22Rv1 cells (Dehm et al. 2008, Guo et al. 2009) were refractory to combined 17-AAG and bicalutamide, they were highly sensitive to a combination of the new generation agents AUY922 and enzalutamide. This is a critical finding as ARV expression represents a potential mechanism of resistance to ADT in prostate cancer cells, driving the progression to CRPC (Nadiminty et al. 2013). In particular, detection of AR-V7 in circulating tumor cells has been associated with resistance to enzalutamide (Antonarakis et al. 2014), which is consistent with the resistance of 22Rv1 cells to this AR antagonist. Here we demonstrate that, unlike therapeutic doses of enzalutamide (Hu et al. 2012, Li et al. 2013) or AUY922 (Gillis et al. 2013, He et al. 2013, Shafi et al. 2013) that increase AR-V7 expression in cell lines, xenografts and *ex vivo* cultured prostate tumors, treating 22Rv1 cells with low dose combined AUY922 and enzalutamide prevents induction of AR-V7 expression.

The heat shock response is an ancient physiological reaction to cellular stress, at times when protein aggregation and denaturation is prevalent such as heat, hypoxia and toxicity, and is characterized by induction of stress response proteins Hsp70, Hsp27 and Clusterin (Whitesell et al. 2003). This cytoprotective response has been posited as a mechanism of resistance to Hsp90 inhibitors and an underlying reason for the failure of 17-AAG as a single agent therapy (Bagatell et al. 2000). Our results demonstrate that the heat shock response to combined 17-AAG and bicalutamide is significantly reduced compared with single agent doses of 17-AAG or AUY922 that have equivalent anti-proliferative effects. This reveals a key mechanistic advantage for the combination, compared to single-agent treatment with N-terminal Hsp90 inhibitors, that may markedly improve patient responses. Similar to combined 17-AAG and bicalutamide, C-terminal targeted Hsp90 inhibitors do not elicit a heat shock response (Conde et al. 2009, Eskew et al. 2011), and development of these agents is currently being pursued to take advantage of this characteristic.

The AR antagonists bicalutamide and enzalutamide are established prostate cancer therapies that target AR ligand binding, but resistance to these agents is inevitable. This study demonstrates that both current and emerging clinical Hsp90 inhibitors work in synergy with bicalutamide or enzalutamide to markedly enhance growth suppression and death of prostate cancer cells.

Two major advantages of this combination treatment are noteworthy for their clinical implications. First, anti-proliferative and pro-apoptotic effects were observed using up to fourfold less Hsp90 inhibitor and up to tenfold less AR antagonist than the respective single agent treatments. Secondly, the capacity to use reduced doses of Hsp90 inhibitor or AR antagonist minimizes the potential for resistance through induction of the heat shock response or through increased ARV expression. Collectively, these results provide a strong rationale for evaluation of Hsp90 inhibitors in combination with AR antagonists for treatment of advanced prostate cancer.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0541>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M M Centenera, D L Marrocco-Tallarigo and L M Butler were involved in the concept and design of the project. M M Centenera, S L Carter, D L Marrocco-Tallarigo, J L Gillis and R H Grose performed experiments and analyzed data. M M Centenera and L M Butler wrote the manuscript. All authors provided critical input throughout the project and revised the manuscript.

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