Second-generation HSP90 inhibitor onalespib blocks mRNA splicing of androgen receptor variant 7 in prostate cancer cells.

Running title: HSP90 inhibition decreases AR-V7 expression

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Abstract

Resistance to available hormone therapies in prostate cancer has been associated with alternative splicing of androgen receptor (AR), and specifically, the expression of truncated and constitutively active AR variant 7 (AR-V7). The transcriptional activity of steroid receptors, including AR, is dependent on interactions with the HSP90 chaperone machinery, but it is unclear if HSP90 modulates the activity or expression of AR variants. Here, we investigated the effects of HSP90 inhibition on AR-V7 in prostate cancer cell lines endogenously expressing this variant. We demonstrate that AR-V7 and full-length AR (AR-FL) were depleted upon inhibition of HSP90. However, the mechanisms underlying AR-V7 depletion differed from those for AR-FL. Whereas HSP90 inhibition destabilized AR-FL and induced its proteasomal degradation, AR-V7 protein exhibited higher stability than AR-FL and did not require HSP90 chaperone activity. Instead, HSP90 inhibition resulted in the reduction of AR-V7 mRNA levels, but did not affect total AR transcript levels, indicating that HSP90 inhibition disrupted AR-V7 splicing. Bioinformatic analyses of transcriptome-wide RNA sequencing data confirmed that the second-generation HSP90 inhibitor onalespib altered the splicing of at least 557 genes in prostate cancer cells, including AR. These findings indicate that the effects of HSP90 inhibition on mRNA splicing may prove beneficial in prostate cancers expressing AR-V7, supporting further clinical investigation of HSP90 inhibitors in malignancies no longer responsive to androgen deprivation.

Precis: A novel mechanism of action for HSP90 inhibitors involving the suppression of androgen receptor splicing strengthens predictions that these therapeutics may elicit potent clinical responses in advanced prostate cancers.

Introduction

Prostate cancer is the most common cancer in men and a leading cause of male cancer mortality in Western societies (1). Androgen deprivation remains the mainstay of treatment for patients with advanced stage disease. However, the response to suppression of gonadal androgens is not durable and transition of metastatic disease to a lethal castration-resistant prostate cancer (CRPC) is inevitable. It is now widely recognized that CRPC progression results from many different alterations of the androgen/AR axis that promote continued androgen receptor (AR) transcriptional activity. Novel androgen biosynthesis inhibitors (e.g. abiraterone acetate) and AR antagonists (e.g. enzalutamide) overcome several of the mechanisms underlying persistent AR transcriptional activity, and have been shown to extend overall survival of patients with CRPC (2, 3).

AR belongs to the nuclear receptor super family of transcription factors whose members are ligand-responsive transcription factors. The AR gene is located on Xq11–12; it contains eight exons that encode a protein of 919 amino acids (AR-FL). It is composed of four functional domains: the amino-terminal transcription activation domain (NTD), which possesses a ligand-independent transcriptional function (AF-1); the DNA-binding domain (DBD), which binds to specific DNA sequences named androgen-responsive elements (ARE); the hinge region, which includes a part of the nuclear localization signal; and the carboxy-terminal ligand-binding domain (LBD), which has a ligand-dependent transcriptional activity (AF-2). Multiple alternatively spliced AR variants (AR-V) have been identified and translated from alternatively spliced AR mRNAs (4). These variants typically contain exons 1-3, but lack portions of the carboxy-terminal LBD, and may be constitutively active (5, 6). AR-Vs have been shown to drive androgen-independent cell proliferation in a manner that is resistant to antiandrogens, including enzalutamide (7). The expression of AR-Vs is higher in CRPC compared to primary hormone-naïve prostate cancer and is reported to be mostly lacking in normal prostate epithelium (5, 6, 8-11). Constitutively active AR-Vs have been implicated as a mechanism of resistance to current hormonal therapies targeting the LBD (7, 12-15).

One of the best characterized variants, and the most frequently detected in

CRPC to date (11), is the AR-V7 (also termed AR3), which contains exons 1–3 and 16 unique amino acids that arise from cryptic exon 3 (6). AR-V7 localizes constitutively to the nucleus, and facilitates AR-FL nuclear localization in the absence of androgen (14). AR-V7 upregulation mitigates the ability of the AR antagonist enzalutamide to inhibit AR-FL nuclear trafficking (14). AR-V7 may also direct a distinct transcriptional program to AR-FL, including the transcription of several cell-cycle-related genes in addition to canonical androgen-responsive genes (11, 16). Recent clinical studies have linked AR-V7 expression in circulating tumor cells (CTC) and CRPC tissue with resistance to enzalutamide and abiraterone (17, 18). This highlights an urgent need for alternative treatment strategies targeting AR-Vs.

HSP90 is a molecular chaperone essential for the late stage maturation, stability and function of a large number of client proteins involved in key signal transduction pathways in prostate cancer, including AR, AKT and the glucocorticoid receptor (GR) (19-21). A key requirement for the transcriptional activity of steroid receptors is the interaction of unbound receptor with the HSP90 chaperone machinery. This interaction occurs exclusively through the LBD via direct binding to HSP40 and HSP70 (22, 23). In prostate cancer cells inhibition of HSP90 chaperone activity impairs the nuclear translocation of AR-FL and enhances the degradation of AR-FL protein resulting in decreased AR-FL transcriptional output (20, 24, 25) and prostate cancer cell proliferation (26, 27). Conversely, recent studies have suggested that AR-V7 nuclear translocation and transcriptional activity does not require HSP90 activity (28, 29) although HSP90 has been proposed as a therapeutic target in CRPC.

Several HSP90 inhibitors are undergoing clinical trial (29, 30). In this study, we investigated the effect of HSP90 inhibition in prostate cancer cells expressing AR-V7. We demonstrate that HSP90 inhibition reduces expression of AR-V7. Interestingly, we show that AR-V7 down-regulation is not due to a traditional client-chaperone mechanism but results from disruption of AR splicing following HSP90 inhibition.

Methods

Cell lines and drug treatment

22Rv1 (#CRL-2505), VCaP (#CRL-2876) and PC3 (#CRL-1345) cells were obtained from the American Type Culture Collection (ATCC) and grown in their recommended culture medium, containing 10% FBS at 37°C in an atmosphere of 5% CO_2. LNCaP95, an androgen-independent cell line derived from long-term continuous culture of LNCaP cells, were cultured in red-phenol free RPMI-1640 supplemented with 10% charcoal-stripped FBS (CSS; Invitrogen). Tanespimycin (17-AAG; LC Laboratories), alvespimycin (17-DMAG; LC laboratories), R1881 (Steraloids), geldanamycin (Stratech), MG132 (Sigma-Aldrich), actinomycin D (Sigma), and cyclohexamide (Sigma) were obtained from commercial sources. Lactate salt of onalespib (AT13387) was synthesized by Astex Pharmaceuticals according to Woodhead et al (31).

Cell growth inhibition

Cell growth inhibition was assessed using the sulforhodamine B assay (SRB) (32). The GI_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth using GraphPad Prism software.

Cell-cycle analysis

Cell cycle distribution and sub-G1 populations were analyzed by flow cytometry using propidium iodide staining (33). Data were analysed with the Flowjo Software.

Cell lysis and Western blot analysis

Whole cells were lysed in RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (cOmplete, Roche). The nuclear and cytosolic subcellular fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce) according to manufacturer's instructions. To obtain detergent-soluble and detergent-insoluble fractions cells were lysed in TNESV buffer (1% NP40 detergent, 0.5% deoxycholate, 50 mM Tris–Cl (pH 7.4), 150 mmol/L NaCl, 0.5 mmol/L EDTA) for 20 min on ice. The NP40 detergent-insoluble pellet fraction was resuspended in RIPA lysis buffer with protease inhibitors by brief sonication. Protein extracts (20 µg) were separated on 4-12% NuPAGE® Bis-Tris gel (Invitrogen) by electrophoresis and subsequently electro-transferred onto Immobilon-P™ PVDF membranes of 0.45 µm pore

size (Millipore). Details of primary antibodies used are provided in **Supplementary Table 1**. Chemiluminescence was detected on Amersham Hyperfilm ECL (GE Healthcare Life Science). In some experiments the FluorChem™ Q Imager and Software was used for annotation and analysis of images including densitometry.

Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy® Plus Mini kit (Qiagen) and from formalin-fixed and paraffin-embedded (FFPE) tissue using the RNeasy FFPE kit (Qiagen) as per manufacturer's instructions. 5 µl of RNA was reverse-transcribed into cDNA using the First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR (qRT-PCR) was carried out on on ViiA™ 7 System Real-Time PCR System (Life Technologies) using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The assays used in this study are listed in **Supplementary Table 2**. Fold change in mRNA expression levels was calculated by the comparative Ct method, using the formula $2^{-(\Delta\Delta Ct)}$ and Human RPLPO (Large Ribosomal Protein) as calibrator. The vehicle-treated sample was set to 1.

Co-immunoprecipitation

Whole cell lysates were obtained using Pierce IP Lysis buffer (Thermo Scientific). Protein extracts (approximately 1 mg of total protein) were pre-cleaned by protein A/G Plus-agarose (Millipore) and extracts were incubated with the respective antibodies (1 µg) overnight at 4 °C. Immune complexes were collected by protein A/G Plus-agarose beads, analyzed by immunoblotting, probed with the protein A/G HRP-conjugated secondary antibody (Thermo Scientific), and visualized using an ECL chemiluminescence (Thermo Scientific).

Transfection

PC3 cells (3×10^5) were plated on 6-well plates and reverse transfected using the X-tremeGENE HP DNA Transfection Reagent (3 µl per well; Roche) (34). The plasmid constructs (1 µg DNA per well) used were pcDNA3.1-AR-V7 (encoding the V7 truncated AR isoform, a gift from Dr Jun Luo, Johns Hopkins University, Baltimore, USA), and F527-AR (encoding the full-length AR) (34).

Tumor xenograft studies

Subcutaneous 22Rv1 solid tumor xenografts were prepared by serial passaging in male NMRI nu/nu mice (Harlan). Tumors were measured twice weekly with micro-calipers and tumor volumes determined using the formula: (length x breadth^2 x 0.5). Drug treatment was started when tumors were well established (mean tumor volume 120 mm^3). Onalespib was dissolved in 17.5% (w/v) hydroxypropyl-β-cyclodextrin and administered intraperitoneally at 70 mg/kg twice a week for 2 weeks or 80 mg/kg once weekly (35). For pharmacodynamic analyses, tumor-bearing animals were sacrificed at indicated time points after a single dose of onalespib at 80 mg/kg (typically 4 per time point).

Immunohistochemistry

Immunohistochemistry (IHC) for AR N-terminus domain (AR-NTD), HSP72, Ki67 and cleaved caspase 3 was performed on 4 μm FFPE tumor sections. Details of the IHC assays are provided in **Supplementary Table 3.** Slides were counterstained with hematoxylin (TCS Biosciences Ltd), dehydrated, and mounted with DPX mountant (Sigma). All sections were scored by a pathologist blinded to treatment information.

RNA-Sequencing (RNA-Seq)

Total cellular RNA was extracted and purified using the RNeasy Mini Kit (Qiagen). RNA integrity was determined by Bioanalyzer 2100 (Agilent Technologies) and only samples with a RNA Integrity Numbers ≥9.5 were used. 500 ng of RNA was prepared into a library using the TruSeq RNA sample prep kit v2 (Illumina), and then clustered and sequenced on a Hi-Seq 2000 (Illumina) using TruSeq v3. An average of around 40 million paired end reads were sequenced per sample. The experiment was repeated three times. The paired end raw reads in FASTQ format were aligned to the reference human genome (hg19) using TopHat v2.0.7. The library and mapping quality were estimated using Picard tools (http://broadinstitute.github.io/picard). The alternative splicing events (SE, RI,

MXE, A5SS and A3SS), based on Ensembl v61 annotation, were accessed using MATS v3.0.8 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3333886/).

Statistical analyses

Effects of different treatments were compared using the Student's t test, Mann–Whitney test or ANOVA. Survival analysis was performed by Kaplan–Meier methods and log-rank analysis using quadruplication of the tumor as surrogate endpoint. In the RNA-seq analysis, the supportive reads of each alternative splicing event were counted, with the inclusion level being estimated using a Bayesian model as described by Shen et al., 2012 (36). Pairwise comparisons of each splicing event were performed among treatments using the Markov chain Monte Carlo method coupled with a simulation-based adaptive sampling procedure to calculate the p-value (36). Sample replicates were used to control variability within-treatment groups. The false discovery rate (FDR) was obtained by the Benjamini-Hochberg method with a FDR <0.05 being employed for the differential alternative splicing significance assessment.

Results

HSP90 targeting inhibits proliferation of prostate cancer cells expressing AR-V7

We initially examined the effects of the first-generation HSP90 inhibitors tanespimycin (17-AAG) and alvespimycin (17-DMAG) and the second-generation HSP90 inhibitor onalespib (AT13387) on cell proliferation in human prostate cancer cell lines expressing the AR-V7 including 22Rv1, VCaP and LNCaP95. Previous studies had established the expression status for both AR-FL and AR-Vs in these cell lines (5, 6, 28). HSP90 inhibition reduced cell proliferation in a dose-dependent manner (data not shown). Onalespib and alvespimycin potently inhibited the growth of the androgen-dependent cell line VCaP, and also the growth of the androgen-independent 22Rv1 and LNCaP95 cell lines, which are resistant to the AR antagonist enzalutamide (7, 11) (**Table 1**). Our results confirmed previous observations that AR-V7 expression does not confer resistance to HSP90 inhibitors in prostate cancer cells (29).

HSP90 targeting depletes AR-FL and AR-V7 in prostate cancer cell lines

We then investigated the effect of HSP90 inhibition on AR-FL and AR-V7 protein levels in prostate cancer cells expressing both the AR-FL and AR-V7. Onalespib reduced AR-FL protein irrespective of AR-FL status (i.e. wild-type or mutant), in a concentration- and time-dependent fashion in VCaP and 22Rv1 cell lines (**Figure 1**) Notably, onalespib caused similar depletion of AR-V7 protein in the 3 cell lines tested (**Figure 1**). AR-FL and AR-V7 depletion was accompanied by a concentration-dependent (data not shown) and time-dependent depletion of various HSP90 clients including AKT, ERBB2, CRAF and GR (**Supplementary Figure 1**). HSP72 and HSP27 were induced by HSP90 inhibition, associated with the heat shock factor-1 (HSF-1)-mediated stress response, confirming HSP90 target engagement (37). Similar results were observed with first generation HSP90 inhibitors (geldanamycin, tanespimycin, alvespymycin); however tanespimycin resulted only in modest depletion of AR-FL and AR-V7 at the concentrations used in VCaP and 22Rv1 cells (**Supplementary Figure 2 and 3**). Since DT-diaphorase/NQO1 plays a role in primary and acquired resistance to tanespimycin (32, 38), we

evaluated constitutive protein expression levels in prostate cancer cell lines to clarify if lower sensitivity to tanespimycin was due to different expression of the enzyme. Low levels of DT-diaphorase/NQO1 protein were detected in 22Rv1 and VCaP cells, respectively by immunoblotting, possibly explaining the differences in effect seen with tanespimycin compared to alvespimycin and onalespib (data not shown). We should note that all the HSP90 inhibitors we had tested inhibit both alpha and beta HSP90 isoforms. We confirmed depletion of AR-V7 protein when the expression of both the HSP90 isoforms was reduced simultaneously by siRNA knockdown (**Supplementary Figure 4**).

Importantly, in time-course experiments, the kinetics of AR-V7 protein loss in response to HSP90 inhibition differed from that for AR-FL (**Figure 1C and 1D**). Onalespib induced a reduction of AR-FL protein within 4 hours. In VCaP cells AR-FL was reduced in a time-dependent fashion with maximal depletion reached at 48-72 hours, but in LNCaP95 AR-FL protein level remained constant. Depletion of AR-V7 was first noted after 12-24 hours reaching maximal depletion at 48-72 hours, when AR-V7 was hardly detectable. The timing and degree of AR-V7 depletion was similar in VCaP and LNCaP95. These results confirmed AR-FL depletion following HSP90 inhibition and also indicated for the first time that AR-V7 protein level is also decreased when HSP90 is inhibited.

AR-V7 protein is more stable than AR-FL and is not directly affected by HSP90 inhibition

HSP90 inhibition results in protein client destabilization and degradation by the ubiquitin-proteasome pathway (39). Ubiquitinated proteins first accumulate in the cytosol and become aggregated and relocated in the detergent-insoluble fraction of cells when proteasomes are inhibited. To evaluate whether AR-V7 protein depletion was due to increased proteasomal degradation after HSP90 blockade, we examined the impact of the proteasome inhibitor MG132 on AR-V7 depletion by onalespib. VCaP cells were treated with onalespib in the presence or absence of proteasome

inhibitor MG132 for 24 hours. As expected, addition of MG132 to onalespib treatment induced accumulation of high-molecular weight ubiquitinated AR-FL forms that were redistributed to the detergent-insoluble fraction (**Figure 2A**). Such accumulation of high-molecular weight AR-V7 forms was not observed, suggesting the lack of involvement of the proteasomal pathway in AR-V7 depletion by onalespib (**Figure 2A**).

To determine whether the observed decrease in AR-V7 protein in onalespib-treated cells was the result of altered protein stability, we treated VCaP with the protein synthesis inhibitor cycloheximide (CHD) in the presence or absence of onalespib **(Figure 2B and 2C)**. When protein synthesis was inhibited by CHD, AR-V7 stability was greater than AR-FL (**Figure 2B**) suggesting that AR-V7 has a longer half-life than AR-FL. Onalespib treatment significantly reduced AR-FL protein levels, indicating increased degradation of AR-FL **(Figure 2B and 2C top panel).** Conversely**,** AR-V7 protein stability was not affected as demonstrated by similar levels of AR-V7 in the presence of onalespib (**Figure 2B and 2C bottom panel**). To further confirm that the effect of onalespib was not due to protein destabilization and increased protein degradation, we also investigated the effects of onalespib in the AR-negative PC3 cell line transiently transfected with plasmid constructs carrying AR-FL and AR-V7 cDNA. Onalespib treatment in this transient transfection system resulted in depletion of transiently-expressed AR-FL but not AR-V7 protein, confirming that the effect of HSP90 inhibition on AR-V7 was not due to destabilization or a direct effect on AR-V7 protein (**Supplementary Figure5**).

AR-V7 bypasses direct HSP90 binding

A key requirement for steroid receptor activity is the interaction of unliganded receptors with the HSP90 chaperone machinery (40). To further examine the relationship between AR-V7 and HSP90, we determined by co-immunoprecipitation assays whether AR-V7 and HSP90 protein complexes exist in prostate cancer cells that endogenously express the variant (**Figure 2D**). As expected, AR-FL formed a complex with HSP90 when the cells were cultured in androgen-depleted media (**Figure 2D top panel**). In contrast, AR-V7 did not co-immunoprecipitate detectable amounts of HSP90 (**Figure 2D**

bottom panel). This was in agreement with results of Gills et al. in PC3 cells exogenously expressing the AR-V7 (29). Also, in keeping with a previous report (7), using subcellular fractionation we confirmed that AR-V7 entered the nucleus independently of HSP90 activity (data not shown). Taken together, these results indicated that AR-V7 protein, unlike AR-FL, does not require HSP90 chaperone.

HSP90 inhibition induces AR-V7 mRNA downregulation

To evaluate whether AR-V7 depletion was due to down-regulation of AR mRNA expression we next examined levels of AR transcripts following onalespib treatment (**Figure 3A**). In VCaP, 22Rv1 and LNCaP95 cells treated for 24 hours with onalespib, AR-FL mRNA levels were not significantly altered (data not shown). AR-V7 mRNA levels, however, were significantly decreased by onalespib in a concentration-dependent fashion (**Figure 3A**, **Supplementary Figure 6)**. Onalespib treatment at 0.6 µmol/L for 48 hours decreased AR-V7 mRNA level by 72 % (**Figure 3A**). To distinguish between transcriptional and post-transcriptional effects on AR-V7 mRNA we examined the effect of onalespib treatment on mRNA stability. VCaP cells were treated with onalespib or vehicle in combination with the transcriptional inhibitor actinomycin D (ACTD). Onalespib treatment did not significantly alter the AR-V7 (**Figure 3B**) and AR-FL (data not shown) mRNA levels in the presence of ACTD, suggesting that the decrease in AR-V7 mRNA in onalespib-treated cells was not due to reduced stability of the mRNA. These results, coupled with the lack of depletion of AR-FL mRNA, suggested that onalespib reduced the generation of AR-V7 mRNA. To test this, we evaluated levels of unspliced AR pre-mRNA in 22Rv1 cells using three different sets of primers/probes (AR-5; AR-6; AR-7 **Supplementary Table 2**) directed against different regions of the unspliced AR cDNA (intron8-exon9; intron 3; exon2-intron2). Onalespib did not reduce levels of unspliced AR and AR-FL but only AR-V7 mRNA levels **(Figure 3C)** suggesting that HSP90 inhibition specifically altered pre-mRNA splicing and impaired the generation of AR-V7 mRNA.

HSP90 inhibition disrupts alternative splicing in prostate cancer cells

HSP90 inhibition elicits a dual effect in cells: The depletion of client proteins

and the activation of a Heat Shock Response (HSR) mediated by HSF1) activation (41). It has been reported that the splicing of mRNA precursors is broadly repressed on heat shock (42-44). To evaluate global changes in splicing pattern associated with HSP90 inhibition we treated VCaP cells with onalespib (0.3 and 0.6 μmol/L) for 24 hours and performed RNASeq analysis. The two different concentrations of onalespib (0.3 and 0.6 μmol/L) resulted in largely overlapping splicing changes (**Supplementary Table 4 and Figure 4A**). Onalespib treatment introduced 743 alternative splicing events involving 557 genes (FDR <0.05) (**Supplementary Table 4**). In keeping with data following heat shock we observed a significant increase in the retention of introns in transcripts and decreased exon skipping (42, 43) (**Figure 4 C**). Furthermore, RNAseq analyses confirmed a reduction of AR-V7 cryptic exon expression (**Figure 4B**). Pathway analyses showed that genes involved in splicing, including processing of Capped Intron-Containing Pre-mRNA(R) and Spliceosome(K), as well as heat shock related pathways, were significantly enriched (FDR <0.05) in the alternative splicing events induced by HSP90 inhibition (**Supplementary Table 5**).

Onalespib exhibits antitumor activity against AR-V7-expressing 22Rv1 tumor xenografts

We next evaluated the effect of onalespib on tumor growth and AR-V7 levels in 22Rv1 subcutaneous solid tumor xenografts. Onalespib was administered intraperitoneally at 70 mg/kg in a twice a week schedule (35). Onalespib reduced tumor growth significantly when administered twice a week ($P <$ 0.001 against the vehicle control; **Figure 5A**) and significantly prolonged survival (**Figure 5B**). Weekly treatment at 80 mg/kg did not show significant benefit, suggesting that more frequent dosing is required in this fast growing model (data not shown). A single administration of onalespib at 80 mg/kg caused increases in cleaved caspase 3 and wide areas of necrosis together with reduced Ki67 expression were observed 48 and 72 hours after drug administration (**Figure 5C**). Reduction in total AR as assessed by an antibody directed against the AR-NTD was observed (**Supplementary Figure 8**). After a single dose of onalespib AR-V7 mRNA were reduced by 20% after 6 and 24

hours (*P*<0.05) (**Figure 5D**) while AR-FL levels were not significantly altered (**Figure 5E**). Levels of mRNA at 48 and 72 hours after drug administration were not evaluated due to the low amount and poor quality of RNA extracted from these tumors in which significant tumor cell kill and necrosis was observed (**Figure 5C**). Onalespib treatment also significantly downregulated PSA expression in tumors 24 hours following treatment (**Supplementary Figure 7**). Upregulation of HSP72 mRNA and protein by IHC confirmed HSP90 target engagement (**Figure 5F and Supplementary Figure 8 and 9**).

Discussion

In this study we examined the effects of HSP90 inhibition in prostate cancer cell lines expressing the AR-V7 variant. In vitro, the first-generation HSP90 inhibitors tanespimycin and alvespimycin and the second-generation HSP90 inhibitor onalespib induced cell growth inhibition and degradation of client proteins including AR-FL, AKT and GR. For the first time we demonstrated that the truncated splice variant AR-V7 protein is depleted when HSP90 is inhibited. Our results confirmed that AR-V7 protein, unlike AR-FL, does not require the HSP90 chaperone for its function and suggested that the mechanism underlying AR-V7 depletion upon HSP90 blockade was not directly linked to interaction of AR-V7 protein with the HSP90 chaperone. We demonstrated that AR-V7 mRNA levels were downregulated when HSP90 was inhibited in a concentration-and time-dependent fashion. Previous studies have linked heat shock to widespread but selective repression of splicing (42, 43). We therefore hypothesized that the heat shock response induced by HSP90, through the release of HSF1, can similarly broadly impact alternative splicing. We have shown that onalespib treatment introduced 743 alternative splicing events involving 557 genes. In keeping with published data evaluating the impact of heat stress we observed a significant increase in retention of introns in transcripts and decreased exon skipping (42, 43). Pathway analyses showed that genes critically important to splicing, including processing of capped intron-containing pre-mRNA and spliceosome genes, as well as heat shock related pathways were significantly enriched in the alternative splicing events induced by HSP90 inhibition.

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Further studies are required to elucidate the complex and multiple mechanisms that can underlie the alternative splicing disruption observed upon HSP90 inhibition. A previous study identified the serine/arginine-rich (SR) splicing factors U2AF65 and ASF/SF2 as critical to AR-V7 splicing (45). The function of SR splicing factors is regulated by their complex interaction with kinases, including the SR protein kinases (SRPKs) and Clk/Sty, as well as phosphatases that regulate their phosphorylation status (46). SRPK1 interacts with various chaperones and co-chaperones including HSP70, HSP40 and Aha1 (47). Alternative splicing is also extensively regulated by signal transduction pathways, whereby signalling cascades can link the splicing machinery to the exterior environment (48, 49). HSP90 is heavily involved in the function of key nodes in these pathways. For instance, AKT can directly phosphorylate SR proteins (50) or indirectly regulate SR protein by activating SRPK (51). In addition, repression of splicing following heat stress has been related to the SR protein SRp38 dephosphorylation by the phosphatase PP1 (52, 53).

Other groups have evaluated the effect of HSP90 inhibition on AR-V7 function and activity. Shafi et al. used LNCaP stably transfected with AR-V7 cDNA and demonstrated no effect on AR-V7 protein levels (54). Gillis et al. demonstrated that AR-V7 transcriptional activity is not altered by HSP90 inhibition in PC3 cells transfected with prespliced AR-V7 driven by an ARR2-probasin-luciferase (29). They also demonstrated AR-V7 does not bind HSP90 by co-immunoprecipitation using a FLAG-tagged AR-V7 transfected in PC3 cells. Our results in cell lines with endogenous AR-V7 support these observations. However, Gills et al. also reported that treatment of VCaP cells with the second-generation HSP90 inhibitors, luminespib (AUY922) and HSP990, increased AR-V7 protein (29) and Shafi et al reported no effect of geldanamycin on AR-V7 in 22Rv1 cells (54). Their results are incongruent with what we have observed with different HSP90 inhibitors in three prostate cancer cell lines expressing the AR-V7 (VCaP, LNCaP95 and 22Rv1).

In conclusion, our observations confirm that AR-V7 protein functions independently of HSP90, and that AR-V7 is not a direct HSP90 client (29). However, we have demonstrated for the first time that HSP90 inhibition leads

to the depletion of AR-V7 protein by downregulating AR-V7 mRNA splicing. AR-V7 splicing was decreased by HSP90 inhibition in prostate cell lines resistant to androgen depletion and enzalutamide, suggesting that HSP90 inhibition remains a viable approach to block the generation and possibly the upregulation of AR-V7 in CRPC. Our data demonstrate a novel mechanism of action for this class of drugs in prostate cancer cells. Onalespib and other HSP90 inhibitors are now being tested in Proof of Concept clinical trials (29, 30). These have included a Phase I study of onalespib in combination with abiraterone acetate in patients no longer responding to abiraterone (ClinicalTrials.gov Identifier: NCT01685268) (55). The impact of HSP90 inhibitors on not only AR-FL and AR-V7, but also AKT and GR, makes HSP90 inhibitors a class of drugs that merit further evaluation against metastatic prostate cancer.

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