Cotargeting HSP90 and Its Client Proteins for Treatment of Prostate Cancer

Long Chen1, Jie Li1, Elia Farah1, Sukumar Sarkar2, Nihal Ahmad3, Sanjay Gupta4, James Larner2, and Xiaoqi Liu1,5

Abstract

Castration-resistant prostate cancer (CRPC) is the later stage of prostate cancer when the disease has stopped responding to androgen deprivation therapy (ADT). It has been established that androgen receptor (AR) reactivation is responsible for the recurrence of prostate cancer after ADT. Thus, targeting different pathways that regulate AR stability and activity should be a promising strategy for treatment of CRPC. Heat shock proteins (HSP) are chaperones that modify stability and activity of their client proteins. HSP90, a major player in the HSP family, regulates stability of many proteins, including AR and Polo-like kinase 1 (Plk1), a critical regulator of many cell-cycle events. Further, HSP90 is overexpressed in different cancers, including prostate cancer. Herein, we show that cotreatment of prostate cancer with AR antagonist enzalutamide and HSP90 inhibitor leads to more severe cell death due to a synergistic reduction of AR protein. Interestingly, we show that overexpression of Plk1 rescued the synergistic effect and that cotargeting HSP90 and Plk1 also leads to more severe cell death. Mechanistically, we show that E3 ligase CHIP, in addition to targeting AR, is responsible for the degradation of Plk1 as well. These findings suggest that cotargeting HSP90 and some of its client proteins may be a useful strategy in treatment of CRPC.

Introduction

Androgen plays pivotal roles in the progression of prostate cancer (1), the second most commonly diagnosed cancer among men worldwide (2). Accordingly, androgen deprivation therapy (ADT), which blocks androgen production or action through either physical castration or chemical castration, is the first-line treatment for locally advanced or metastatic prostate cancer (3–5). Despite the early success of ADT, the disease eventually relapses and enters a stage called castration-resistant prostate cancer (CRPC; ref. 5). Most biologic functions of androgens are mediated by androgen receptor (AR), a ligand-dependent transcription factor that regulates gene expression of the androgen-dependent signaling components (6). AR initially localizes in the cytoplasm in a complex with heat shock proteins (HSP), cytoskeletal proteins, and co-chaperone proteins. Upon binding to androgens, AR translates into the nucleus, where it binds to the androgen response elements (ARE) in the promoter or enhancer regions of targeted genes with other coactivators and activates the androgen signaling pathway (6). It has been established that reactivation of AR signaling is responsible for relapse of prostate cancer (7, 8). Consequently, drugs targeting AR pathway such as enzalutamide have been used for CRPC patients. Although the drug provides a substantial survival benefit, patients develop enzalutamide resistance eventually due to reactivation of AR (9). Thus, new strategies targeting the AR signaling pathway are needed to overcome enzalutamide resistance.

HSPs are chaperone proteins that are expressed during stress and facilitate the stabilization, folding, and translocation of its client proteins (10). HSP90, a member of the HSP family, functions to stabilize and activate its client proteins in an ATP-dependent manner (11). HSP90 has more than 200 client proteins, which are involved in different signaling pathways and adaptive response to stress. HSP90 clients include oncogenic proteins, such as v-Src, Bcr-Abl, c-Met, and Plk1 (12, 13). Thus, inhibition of HSP90 has been considered a promising way for cancer treatment. In prostate cancer, HSP90 regulates the stability and activity of AR by forming a complex with AR in the cytoplasm, thus stabilizing AR prior to ligand binding (14). Inhibition of HSP90 leads to AR degradation and its cytoplasmic accumulation (15, 16). Most of HSP90 inhibitors developed so far target the ATP-binding domain of HSP90, leading to its inactivation and eventually degradation of its client proteins (11), with Geldanamycin (GA) as one of the such HSP90 inhibitors (16–18). Although GA has been shown to be effective in many cell lines, it did not enter clinical trials due to its severe liver cytotoxicity (11). 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a GA derivative, which also shows antitumor activity but less cytotoxicity in vivo, was the first HSP90 inhibitor that entered clinical trials (12, 19, 20). Unfortunately, due to lack of response and toxicity, the phase II studies in patents with breast cancer and melanoma were terminated early (21, 22).

Despite these, newly developed HSP90 inhibitors with higher specificity and less toxicity, such as AUY922 and STA-9090, are now in different clinical trials, and combination therapies of HSP90 inhibitors with standard chemotherapy or radiotherapy are ongoing (11, 20, 23).

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Xiaoqi Liu, Purdue University, 175 S. University Street, West Lafayette, IN 47907. Phone: 765-496-3764; Fax: 765-494-7897; E-mail: liu8@purdue.edu

doi: 10.1158/1535-7163.MCT-16-0241

© 2016 American Association for Cancer Research.
Polo-like kinase 1 (Plk1) is a serine/threonine kinase that has many cell-cycle–related functions (24). Plk1, overexpressed in many human tumors, including prostate cancer, has been preclinically validated as a target for cancer treatment (25). Although inhibitors of Plk1 are in different clinical trials, we recently reported that inhibition of Plk1 potentiated enzalutamide-mediated therapy in CRPC (26). Moreover, HSP70 and HSP90 have been shown to bind to Plk1 and regulate its function (27–29).

C-terminal Hsc70-interacting protein (CHIP) is a co-chaperone protein that works with chaperone proteins (HSP70/90) to modulate the protein homeostasis, either facilitating the unfolded or damaged proteins fold properly or directing those proteins to go through the proteasome-mediated degradation (30–32). CHIP itself is an E3 ubiquitin ligase; therefore, its binding to HSPs leads to degradation of client proteins (32). While CHIP binds and regulates the degradation of AR (33–35), it is a Plk1-interacting protein during mitosis as well (36).

By combining HSP90 inhibitors with drugs targeting HSP90 client proteins like AR or Plk1 in prostate cancer, we showed that combination of HSP90 inhibitors with AR or Plk1 inhibitors led to cell death in a synergistic manner, providing a novel approach to treat CRPC. Mechanistically, CHIP-mediated degradation of AR and Plk1 leads to enhanced efficacy of HSP90 inhibitors.

Materials and Methods

Cell culture, virus infection, and drugs

HEK293T, HeLa, LNCaP, and 22RV1 cells were purchased from the American Type Culture Collection. Cells were grown and aliquots were stored in liquid nitrogen for future use. Cells were purchased more than 6 months ago and were not further tested or authenticated by authors. HEK293T and Hela cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Atlanta), 80 mmol/L L-glutamine (Sigma) and 100 units/mL penicillin, 100 units/mL streptomycin at 37°C in 5% CO2. Prostate Cancer cell line LNCaP and C4-2 were cultured in RPMI 1640 Medium (Sigma) supplemented with 10% FBS and 100 units/mL penicillin, 100 units/mL streptomycin at 37°C in 5% CO2. Enzalutamide-resistant prostate cancer cell line 22Rv1 and MR49F were also cultured in RPMI 1640 medium with 10% FBS and 100 units/mL penicillin, 100 units/mL streptomycin, with 10 mmol/L enzalutamide to maintain resistance at 37°C in 5% CO2. C4-2 and MR49F cells were used for immunoprecipitation.

Antibodies

Antibodies against Plk1 (sc-17783), HSP90 (sc-13119), and ubiquitin (sc-8017) were purchased from Santa Cruz Biotech. Antibodies against β-actin (A-5411), Cyclin B (554177), and cleaved-PARP (AB6535) were obtained from Sigma, BD Pharmingen, and EMD Millipore, respectively. All other antibodies were purchased from Cell Signaling.

Immunoblotting and immunoprecipitation

Upon harvest, cells were resuspended with TBSN buffer (20 mmol/L Tris-HCl, pH 8.0, 0.5% NP-40, 5 mmol/L EGTA, 1.5 mmol/L EDTA, 0.5 mmol/L sodium vanadate and 150 mmol/L NaCl) with protease inhibitors and phosphatase inhibitors and sonicated. After they were collected, protein concentrations were measured using Protein Assay Dye Reagent from Bio-Rad. Equal amounts of protein from each sample were mixed with SDS loading buffer and resolved by SDS-PAGE. Upon transferring to PVDF membranes, proteins were probed with indicated antibodies. For immunoprecipitation [IP], cell lysates were incubated with indicated antibodies overnight at 4°C, followed by 1 hour of incubation with protein A/G plus-Agarose beads. After supernatants were removed, beads were washed with high salt and low salt TBSN buffer, and resolved by SDS-PAGE.

GST pull-down assay

After GST-fusion CHIP proteins were expressed and enriched using GST agarose beads, cell lysates were incubated with the GST beads carrying the proteins at 4°C overnight. After centrifugation, supernatants were removed and beads were washed three times and resolved by SDS-PAGE for Coomassie brilliant blue staining or Western blot detection.

In vitro ubiquitination assay

In vitro ubiquitination assay was performed using the CHIP Ubiquitin Ligase Kit purchased from Boston Biochem. Briefly, GST-Plk1 proteins purified from E. coli were incubated with the reaction buffer containing Mg2+–ATP and HSP70/HSP40 at 43°C for 7 minutes and kept on ice for 10 minutes. Then, addition of E1, E2, and CHIP was followed by ubiquitin to the mixture. Samples were placed at 37°C and incubated for indicated times until termination with SDS loading buffer.

Chromatin immunoprecipitation

Upon harvest with trypsin, cells were cross-linked with 1% formaldehyde and quenched with glycerol. Nuclear fraction was separated and resuspended in SDS lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% SDS, 1 mmol/L EDTA, supplemented with protease inhibitors and phosphatase inhibitors). Sonication was used to shear DNA into desired lengths. Upon centrifugation, supernatants were collected and incubated with AR antibody and protein A/G agarose beads. After several washes, chromatin complexes were eluted with elution buffer (1% SDS, 0.1 mol/L NaHCO3), and cross-linking was reserved by incubating the eluted chromatin complexes at 65°C overnight. RNase A and proteinase K were then added and incubated at 37°C for 1 hour. After DNA was isolated using a PCR purification kit from Qiagen, DNA binding to AR was measured by RT-PCR. PCR results were normalized to indicated DNA in the supernatants.

Immunofluorescence

Cells were grown on coverslips under normal culture conditions, fixed with 4% formaldehyde, and blocked with 5% BSA for

Protein purification

After indicated domains of CHIP or Plk1 were PCR amplified and subcloned into pGEX-KG, glutathione-S-transferase (GST)–tagged CHIP and GST-Plk1 proteins were expressed in Escherichia coli and purified using GST-agarose beads.
1 hour. Primary and secondary antibodies were prepared in 5% BSA and incubated on coverslips for 2 and 1 hours, respectively.

**Xenograft study**

Mice carrying LuCaP35CR tumors were obtained from Dr. Robert Vessella from University of Washington. Twenty-four mice carrying LuCaP35CR tumor were used for study. When tumors reached around 200 mm³, mice were separated into 4 groups for indicated treatments. For the first 2 weeks, enzalutamide (25 mg/kg) was gavaged twice per week and 17-AAG (20 mg/kg) was intraperitoneally injected twice per week. Starting from the third week, both drugs were administered 5 times a week. Tumor volumes were measured by formula $V = \frac{L \times W^2}{2}$ [V is volume (mm³), L is length (mm), W is width (mm)]. Mice were sacrificed when the volume of one or more tumors reached 1,000 mm³.

**Hematoxylin and eosin staining and immunohistochemistry staining**

Tumors were fixed in 10% formalin, embedded in paraffin, sectioned, and subjected to hematoxylin and eosin (H&E) staining. Immunohistochemistry (IHC) and immunofluorescence (IF) staining were performed by using the VECTASTAIN ABC Kit from Vector Laboratories.

**Cell viability assay**

Cells were seeded in 96-well plates, treated with indicated drugs for 3 days, and subjected to CellTiger-Glo luminescent cell viability assay kit from Promega.

**Subcellular fractionation**

Cytoplasmic and nuclear fractions of cells were prepared using the Nuclear Extract Kit from Active Motif. Briefly, harvested cells were resuspended in hypotonic buffer, vortexed for 10 seconds, and centrifuged for 30 minutes, vortexed for 30 seconds, and centrifuged for 10 minutes at 14,000 rpm. Supernatants were collected as cytoplasmic fraction. Nuclear pellets were resuspended using complete lysis buffer, incubated on ice for 30 minutes, vortexed for 30 seconds, and centrifuged for 10 minutes at 14,000 rpm. Supernatants were collected as nuclear fraction.

**Results**

Combination of HSP90 inhibitors with AR antagonist enzalutamide induces an increased cell death and decreased cell viability in prostate cancer cell lines. It has been shown that HSP90 inhibitor 17-AAG works synergistically with AR antagonist bicalutamide to suppress prostate cancer cell growth (38). Enzalutamide is a newly developed AR antagonist that has been approved by the FDA to treat metastatic CRPC in 2014. By preventing androgen binding to AR, enzalutamide inhibits the nuclear localization and transcriptional activity of AR (39). Despite the early clinical benefits, most patients eventually developed enzalutamide resistance due to reactivation of the AR signaling pathway by various mechanisms including AR truncation and point mutations (9). Here, three different prostate cancer cell lines were used to test whether HSP90 inhibitors (17-AAG and GA) would sensitize cells to enzalutamide treatment. Both C4-2 and 22RV1 are CRPC cells, with the later to be enzalutamide resistant as well. Like C4-2, MR49F cells are also derived from LNCaP cells but enzalutamide resistant (37). C4-2, 22RV-1, and MR49F cells were treated with enzalutamide, HSP90 inhibitor, or together, followed by various analyses. Decreased cell viability and increased cell death upon combination treatment were observed in all three cell lines (Fig. 1A–D). Enzalutamide-resistant 22RV1 and MR49F cells were also treated with increased concentrations of 17-AAG in the presence of enzalutamide (Fig. 1E and F). 17-AAG–induced apoptosis of 22RV1 and MR49F cells is apparently dose dependent, suggesting that HSP90 inhibitor is effective in treating enzalutamide-resistant CRPC. At the same time, the decrease of AR protein level was also observed as the concentration of 17-AAG is increased (Fig. 1E and F).

Cotreatment with HSP90 inhibitor and enzalutamide leads to AR protein degradation, nuclear exclusion, and decreased transcription activity. HSP90 inhibitors cause degradation of AR protein and its nuclear exclusion, whereas AR antagonist like enzalutamide also inhibits AR nuclear localization and thus its transcriptional activity. We examined the AR protein level, nuclear localization, and transcriptional activity upon cotreatment of LNCaP, C4-2, 22RV1, and MR49F cells with enzalutamide and 17-AAG.

**Combination of HSP90 inhibitors with AR antagonist enzalutamide and 17-AAG**

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>C4-2</th>
<th>22RV1</th>
<th>MR49F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Enz</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>17-AAG (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>50</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>100</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>250</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>500</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>1,000</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Figure 1.** Combination of AR antagonist enzalutamide and HSP90 inhibitors led to decreased cell viability and increased cell death. A, C4-2 cells were treated as indicated for 3 days and harvested for cell viability assay, B, C4-2 cells were treated as indicated for 24 hours, followed by IB against cleaved-PARP (Poly(ADP-ribose) polymerase-1). C and D, 22RV1 (C) and MR49F (D) cells were treated as indicated for 24 hours, followed by IB against cleaved-PARP, E and F, 22RV1 (E) and MR49F (F) cells were treated as indicated with enzalutamide (10 μmol/L) and indicated increased concentrations of 17-AAG for 24 hours, followed by IB against cleaved PARP and AR.
HSP90 inhibitor. Upon combining the two drugs, AR protein levels are further decreased, as well as the levels of prostate-specific antigen (PSA), the major AR downstream target (Fig. 2A–D). Enhanced expression of truncated version of AR (AR-V) in 22RV1 cells is one established mechanism for enzalutamide resistance (40). Of interest, combination of GA and enzalutamide almost completely abolished the expression of both full-length AR and AR-V in 22RV1 cells (Fig. 2C). We noticed that there is an increase of AR protein level in 22RV1 and MR49F cells upon enzalutamide treatment (Fig. 2B and C). Although we maintained enzalutamide resistance of the two cell lines by overexpressing AR protein, when culturing we did not add enzalutamide when we seeded the cells for treatments, so it is possible that the two cell lines overcome enzalutamide partially by overexpressing AR protein. The effects of two drug combination on AR localization were then analyzed by IF staining (Fig. 3). For LNCaP and C4-2 cells, both enzalutamide and GA treatments lead to increased AR localization in the cytoplasm, whereas enzalutamide shows less effect (Fig. 3A and B). Upon cotreatment with both enzalutamide and GA, cells showed diffused cytosolic AR pattern and reduced total AR signal as well. The effects of enzalutamide plus GA on AR localization in two enzalutamide-resistant lines were also examined. Although enzalutamide alone had little impact on AR nuclear localization in both lines, additional GA treatment clearly reduced both total AR level and its nuclear localization (Fig. 3C and D). The different responses of two lines to the combinational treatment need further experimentation. To further test the localization of AR upon different drug treatments, C4-2 cells treated with enzalutamide and 17-AAG were subjected to cell fractionation. While the cytosolic AR was reduced by the combinational treatment, a much more significant decrease was observed in the nuclear AR upon the treatment by enzalutamide plus 17-AAG (Fig. 2E). AR binding to the chromatin upon drug treatment was examined. Accordingly, C4-2 cells were treated with various conditions for 24 hours, crosslinked with formaldehyde, and harvested for chromatin isolation. Samples were subjected to anti-histone H3 IP, followed by AR immunoblotting (IB). While chromatin binding of AR was not significantly affected by two single-drug treatments, combining the two drugs leads to a reduced AR binding to chromatin (Fig. 2F). CHIP was also performed to further confirm binding of AR to AR promoter region of indicated genes.}

---

**Figure 2.** Combination of enzalutamide and 17-AAG led to decreased AR protein level and transcriptional activity. A and B, LNCaP (A) and C4-2 (B) cells were treated as indicated for 24 hours, followed by IB against AR, PSA, and CHIP. C and D, 22RV1 (C) and MR49F (D) cells were treated as indicated for 24 hours, followed by IB against AR and HSP90. E, C4-2 cells were treated as indicated for 24 hours, fractionated into cytoplasm and nuclear, followed by IB against AR and Plk1. F, C4-2 cells were treated as indicated for 24 hours, and crosslinked with 1% formaldehyde. The chromatin fraction was separated and subjected to anti-histone H3 IP, followed by IB against AR. G, C4-2 cells were treated as indicated for 24 hours and subjected to CHIP analysis using AR antibody. Binding of AR to the promoter region of PSA, CAMKK2, and FKBP5 was measured using RT-PCR with specific primers targeting the promoter region of indicated genes.
Combination of enzalutamide and geldanamycin led to cytoplasmic localization of AR. A–D, LNCaP (A), C4-2 (B), 22RV1 (C), and MR49F (D) cells were treated as indicated for 24 hours, and then subjected to IF staining against AR.
the promoter region of its target genes upon drug treatment. C4-2 cells were treated as indicated for 24 hours and subjected to CHIP analysis. Binding of AR to promoter region of PSA, CAMKK2, and FKBP5 was analyzed using RT-PCR with specific primers targeting the promoter region of indicated genes. Although either single-drug treatment could decrease the binding of AR to the promoter region of these target genes compared with control group, combination treatment was more effective in inhibiting AR binding to the promoter region of the three target genes than single-drug treatment (Fig. 2G).

17-AAG enhances the efficacy of enzalutamide in vivo. We next tested the effect of combination of enzalutamide and 17-AAG in the LuCaP35CR patient-derived xenograft (PDX) model. While 17-AAG alone showed a better effect than enzalutamide alone, combination of enzalutamide and 17-AAG led to a more significantly decreased tumor growth rate compared with single-drug treatments (Fig. 4A and B). Histologic analyses of the harvested tumors were performed to further characterize the drug effect. H&E staining indicated necrosis the interior tumors, consequently we mainly analyzed the exterior of the tumors where cells were
Plk1 and AR in HSP90-Targeted Therapy

still actively growing (data not shown). While single-drug treat-
ments did not lead to significant change of tumor grade and
morphology compared with the control group, combination

treatment led to increased apoptotic bodies and condensed
nuclear pyknosis (Fig. 4C, top). Combination of the two drugs
also led to a significantly decreased proliferation rate of the tumor
cells as indicated by Ki67 staining (Fig. 4C and D). The PSA level in
the serum was also decreased upon combination compared with
single-drug treatments, indicating that the transcriptional activity
of AR was inhibited (Fig. 4E). Consistent with our results using cell
lines, AR tended to be excluded from the nucleus, and total AR
signal intensity was decreased upon combination treatment in the
xenograft model (Fig. 4F).

AR protein level upon drug treatment is partially dependent on
CHIP. CHIP, a co-chaperone protein that works with chaperone
proteins like HSPs to regulate protein hemostasis, is also an E3
ubiquitin ligase. It has been established that proteins with critical
roles in cell signaling and cancer progression, such as p53, HIF1α,

roles in cell signaling and cancer progression, such as p53, HIF1α,

roles in cell signaling and cancer progression, such as p53, HIF1α,

roles in cell signaling and cancer progression, such as p53, HIF1α,

roles in cell signaling and cancer progression, such as p53, HIF1α,
needed to identify new targets and develop novel approaches to increase the efficiency of enzalutamide. HSPs have important roles in regulating the homeostasis of its client proteins, including AR and Plk1. Indeed, a co-IP experiment indicated a complex formation among HSP90, AR, and Plk1, and inhibition of HSP90 led to dissociation of AR and Plk1 from HSP90 (Fig. 7A). Inhibition of HSP90 leads to protein degradation and nuclear exclusion of AR, thus targeting HSP90 is one promising approach in treating prostate cancer. Besides AR, inhibition of HSP90 may also lead to degradation of its other client proteins, eventually contributing to inhibition of cancer cells by providing a broader spectrum of inhibition.

Figure 5. Plk1 is involved in the combination effect of enzalutamide and HSP90 inhibitors. A, 22RV1 cells were depleted of CHIP using lentivirus-based shRNA, treated with geldanamycin (1 μmol/L) for indicated times, and subjected to IB against AR. Arrow indicates the position of AR-V. B, MR49F cells were depleted of CHIP using lentivirus-based shRNA, treated with geldanamycin (1 μmol/L) for indicated times, and subjected to IB against AR. C, 22RV1 cells were depleted of CHIP, treated as indicated for 24 hours, followed by IB against cleaved PARP, Plk1, and CHIP. D, LNCaP cells were infected with lentivirus to overexpress Plk1, treated as indicated for 24 hours. E–G, LNCaP (E), 22RV1(F), and MR49F (G) cells were treated with 17-AAG (100 nmol/L) for 24 hours, followed by IB against cleaved PARP. H–J, LNCaP (H), C4-2 (I), and MR49F (J) cells were treated with 17-AAG (100 nmol/L) for 24 hours and subjected to IB against Plk1. K, HEK293T cells were cotransfected with Flag-CHIP, His-ubiquitin, and GFP-Plk1, treated as indicated for 24 hours, subjected to IP against Plk1, followed by IB against ubiquitin and Plk1. L and M, HeLa cells were depleted of CHIP using lentivirus-based shRNA, treated with (M) or without (L) nocodazole (100 nmol/L), subjected to IB against Plk1. N and O, HeLa (N) and MR49F (O) cells were depleted of CHIP using lentivirus-based shRNA and subjected to FACS analysis. Percentages of cells in different cell-cycle stages were calculated. P, HEK293T cells were transfected with Flag-CHIP, treated with MG132 (10 μmol/L) for 6 hours, and subjected to IB against Plk1.
In this study, we first tested combining HSP90 inhibitors GA or 17-AAG with AR inhibitor enzalutamide in cultured prostate cancer cells. Cotargeting HSP90 and AR led to more severe cell death in different prostate cancer cell lines, including the androgen-dependent LNCaP, androgen-independent C4-2, and enzalutamide-resistant 22RV1 and MR49F cells (Figs. 1 and 2). The combination led to increased AR protein degradation, decreased AR nuclear localization, and reduced AR transcriptional activity (Fig. 2 and 3). It is of clinical significance for 22RV1 and MR49F cells to respond to the combination treatment, as these cell lines have acquired enzalutamide resistance via different mechanisms such as increased expression of AR-V or point mutations. Although inhibition of HSP90 is expected to lead to degradation of its client proteins, it is not clear why combination of AR antagonist with HSP90 inhibitor would lead to further degradation of AR, especially in those resistant cell lines. Importantly, we found combination of enzalutamide and 17-AAG led to inhibition of tumor growth of a PDX model LuCaP35CR (Fig. 4). We also noticed that upon single-drug treatment, 17-AAG is more effective in causing cancer cell death and tumor volume reduction, whereas it is not as effective as enzalutamide in downregulating AR protein level (Figs. 1, 2, 4).

Figure 6. CHIP ubiquitinates Plk1 in vitro and in cells. A, Plk1 interacted with CHIP in Hela cells. HeLa cells were treated with or without nocodazole (100 nmol/L) and subjected to IP against Plk1 or CHIP, followed by IB against Plk1 and CHIP. B, purified GST-CHIP interacted with Plk1 in Hela lysates. N-terminal, C-terminal, and full-length GST-CHIP proteins were purified with GST beads, incubated with HeLa cell lysates, followed by Coomassie blue staining (CBB) or IB against Plk1. C, CHIP ubiquitinated Plk1 in vitro. D, GST-Plk1 (wild-type or kinase-dead K82M mutant), purified from insect cells, was subjected to in vitro ubiquitination assay as in C for 30 minutes. E, GST-Plk1 fragments were subjected to in vitro ubiquitination assay as in C for 30 minutes. F and G, overexpression of CHIP led to increased ubiquitination of Plk1 in cells. F, HEK293T cells were cotransfected with Flag-CHIP, GFP-Plk1, and His-ubiquitin, treated with nocodazole (100 nmol/L) for 24 hours and MG132 (10 μmol/L) for 6 hours, subjected to IP against Plk1, followed by IB against Plk1 and ubiquitin. G, HeLa cells were transfected with Flag-CHIP and His-ubiquitin, treated with nocodazole (100 nmol/L) for 24 hours and MG132 (10 μmol/L) for 6 hours, subjected to anti-Plk1 IP, followed by IB against Plk1 and ubiquitin.
Because inhibition of HSP90 could lead to degradation of its multiple client proteins, AR is one of many HSP90 clients that are degraded upon 17-AAG treatment. Other HSP90 client proteins, including Plk1, EGFR, and HIF1a, are also degraded upon 17-AAG treatment (29, 44, 45), resulting in even severe cancer cell death and tumor volume reduction. Although we found that downregulation of AR upon combination of HSP90 inhibitor and enzalutamide is one of the reasons for increased cancer cell death, it is possible that modulation of other signaling pathways due to HSP90 inhibition also synergizes with inhibition of AR pathway, eventually resulting in increased cell death. This possibility needs further investigation as it might provide evidence for new combination therapy.

We further found that the HSP90 inhibition-induced AR degradation is partially dependent on CHIP, an E3 ligase that works with chaperone proteins like HSPs to regulate the homeostasis of client proteins (Fig. 5A). Acting as a co-chaperone, CHIP directs client proteins of HSPs for proteasome degradation. However, one report showed binding of CHIP to different HSPs might lead to different destination of client proteins. When CHIP binds to HSP70, it leads to protein degradation. In striking contrast, CHIP can stabilize the client protein when it binds to HSP90 (32, 46). Thus, it is possible that inhibition of HSP90 leads to increased binding of client proteins with HSP70/CHIP complex thus resulting in their degradation.

Interestingly, the protein level of Plk1 was also increased upon CHIP depletion, suggesting that Plk1 might be a CHIP substrate. We then demonstrated this important finding by a series of in vitro and in cell ubiquitination assays (Fig. 6). It has been reported that Plk1 can be ubiquitinated by other E3 ubiquitin ligases. E3 ligase Chfr ubiquitination of Plk1 results in a delay of cells entering mitosis in the presence of mitotic stress through negative regulation of Cdc2 activation (47). Ubiquitination of Plk1 by Cullin 3 ligase complex with the BTB adaptor KLHL22 directs Plk1 dissociation from the kinetochore without affecting Plk1 stability (48, 49). Although CHIP depletion did not affect cell-cycle progression during nonstress condition (Fig. 5N and O), HSP90 and CHIP are chaperone proteins that are responsible for protein fate during stress. Therefore, it is possible that CHIP-dependent Plk1 ubiquitination would affect cell-cycle progression when cells are under stress, similar to Chfr. We hypothesize that when cells are depleted of CHIP, Plk1 cannot be efficiently ubiquitinated under stress, consequently cells continue to cycle with unrepair DNA damage, leading to severe cell death (Fig. 5C).

Whether CHIP-dependent Plk1 ubiquitination would affect cell-cycle progression under different stress and the underlying mechanism needs further careful investigation. Because Plk1 is also an HSP90 client protein, we tested whether Plk1 inhibition affects the efficacy of HSP90 inhibitor as well. As described, a combined inhibition of Plk1 and HSP90 enhanced apoptosis in a synergistic manner in multiple prostate cancer cell lines (Fig. 5). Accumulating evidence supports the notion that Plk1 is a valid target for overcoming therapy resistance in prostate cancer. For example, inhibition of Plk1 enhances the efficacy of enzalutamide in both cultured cells and PDX tumors, as Plk1 inhibition prevents activation of the PI3K/AKT/mTOR pathway, which acts upstream of cholesterol biosynthesis, a mechanism to drive AR pathway reactivation (26). Inhibition of Plk1 also overcomes the resistance to metformin, a promising drug for CRPC treatment (50), as Plk1 clearly promotes aerobic glycolysis (51). Finally, we recently showed that combination of inhibition of Plk1 and the WNT/β-catenin pathway is a valid approach to treat CRPC, as Plk1 phosphorylation of Axin2 contributes to inactivation of the WNT/β-catenin pathway (52).

In summary, HSP90 and Plk1 are two promising targets to treat enzalutamide-resistant CRPC. In the absence of HSP90 inhibitor 17-AAG, CHIP-associated ubiquitin ligase activity is inhibited, a stable AR/HSP90/Plk1 complex will lead to stabilized AR and Plk1, thus contributing to prostate cancer progression. Upon 17-AAG treatment, the AR/HSP90/Plk1 complex is likely to be destabilized, free AR and Plk1 will go through CHIP-dependent ubiquitination, followed by protein degradation. Altogether, a combination of enzalutamide, HSP90 inhibitor 17-AAG, and Plk1 inhibitor BI2536 will likely achieve the best therapeutic outcome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L. Chen, S. Sarkar, N. Ahmad, J. Lanier, X. Liu
Development of methodology: E. Farah
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Chen, J. Li, S. Sarkar
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Chen, J. Li, X. Liu
Writing, review, and/or revision of the manuscript: L. Chen, S. Sarkar, N. Ahmad, S. Gupta, X. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Chen, E. Farah, X. Liu
Study supervision: X. Liu

Figure 7.
CHIP-mediated degradation of AR and Plk1 regulates the efficacy of HSP90 inhibitor. A, LNCaP cells were treated with or without 17-AAG for 24 hours, subjected to IP against HSP90, followed by IB against HSP90, Plk1, and AR. B, a working model of inhibition of HSP90 signaling pathways.

Downloaded from mct.aacrjournals.org on December 25, 2021. © 2016 American Association for Cancer Research.
Acknowledgments
The authors thank Sandra Torregrosa-Allen and Benjamin Ramsey for their help with xenograft study.

Grant Support
This work was supported by NIH grants R01 CA157429 (X. Liu), R01 CA192894 (X. Liu), R01 AR059130 (N. Ahmad), and R01 CA176748 (N. Ahmad).

References

Received April 21, 2016; revised June 9, 2016; accepted June 26, 2016; published OnlineFirst July 7, 2016.
Molecular Cancer Therapeutics

Cotargeting HSP90 and Its Client Proteins for Treatment of Prostate Cancer

Long Chen, Jie Li, Elia Farah, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-16-0241

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2016/07/07/1535-7163.MCT-16-0241.DC1

Cited articles
This article cites 52 articles, 16 of which you can access for free at:
http://mct.aacrjournals.org/content/15/9/2107.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/15/9/2107.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/15/9/2107.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.