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Abstract:

Small molecule inhibitors of Hsp90 show promise in the treatment of castration-resistant prostate cancer (CRPC), however these inhibitors trigger a heat shock response that attenuates drug effectiveness. Attenuation is associated with increased expression of Hsp90, Hsp70, Hsp27 and clusterin (CLU) that mediate tumor cell survival and treatment resistance. We hypothesized that preventing CLU induction in this response would enhance Hsp90 inhibitor-induced CRPC cell death in vitro and in vivo. To test this hypothesis, we treated CRPC with Hsp90 inhibitor PF-04929113 or 17-AAG in the absence or presence of OGX-011, an antisense drug that targets CLU. Treatment with either Hsp90 inhibitor alone increased nuclear translocation and transcriptional activity of the heat shock factor HSF-1, which stimulated dose- and time-dependent increases in heat shock protein expression, including especially CLU expression. Treatment-induced increases in CLU were blocked by OGX-011, which synergistically enhanced the activity of Hsp90 inhibition on CRPC cell growth and apoptosis. Accompanying these effects was a decrease in HSF-1 transcriptional activity as well as expression of HSPs, Akt, PSA and androgen receptor. In vivo evaluation of the Hsp90 inhibitors with OGX-011 in xenograft models of human CRPC demonstrated that OGX-011 markedly potentiated anti-tumor efficacy, leading to an 80% inhibition of tumor growth with prolonged survival compared to Hsp90 inhibitor monotherapy. Together, our findings indicate that Hsp90 inhibitor-induced activation of the heat shock response and CLU is attenuated by OGX-011, with synergistic effects on delaying CRPC progression.
Introduction

Prostate cancer (PCa) is the most common cancer and the third most common cause of cancer related mortality in men in the United States (1). Androgen ablation remains the standard effective therapy for patients with advanced PCa, inhibiting proliferation and inducing apoptosis in tumor cells (2). Unfortunately, after short-term remissions, surviving tumor cells recur with castrate resistant prostate cancer (CRPC) and death usually within 3 years in most men (3). CRPC progression results from mechanisms attributed to re-activation of androgen receptor axis (4), alternative mitogenic growth factor pathways (5, 6), and stress-induced pro-survival gene (7, 8) and cytoprotective chaperone networks (9, 10). To significantly improve survival in men with PCa, new therapeutic strategies to inhibit the appearance of this phenotype must be developed.

Heat shock protein 90 (Hsp90) is an ATPase-dependent molecular chaperone required for protein folding, maturation and conformational stabilization of many “client” proteins (11, 12). Hsp90 interacts with several proteins involved in CRPC, including growth factor receptors, cell cycle regulators and signaling kinases like Akt, androgen receptor (AR) or Raf-1, (13, 14). Tumor cells express higher Hsp90 levels compared with benign cells (12, 15), and Hsp90 inhibition has emerged as an exciting target in CRPC and other cancers. Many Hsp90 inhibitors were developed targeting its ATP-binding pocket, including natural compounds such as geldanamycin and its analog 17-allylamino-17-demethoxy-geldanamycin (17-AAG), or synthetic compounds including PF-04928473. These agents inhibit Hsp90 function and induce apoptosis in preclinical studies of colon, breast, PCa and other cancers (12, 16). While promising, treatment resistance emerges early due to compensatory mechanisms involving activation of heat shock factor 1 (HSF-1). Once released from Hsp90, HSF-1 translocates to the nucleus, binds to heat shock elements (HSE) of Hsp genes and increases Hsp transcription activity (13). Therefore, Hsp90 inhibition induces a heat shock response with increased expression of several Hsps including Hsp70, Hsp27 and clusterin (CLU). The up-regulation of these molecular chaperones has been reported to play a role in cellular recovery from stress by restoring protein homeostasis, promoting thermotolerance, cell survival, and treatment resistance (14, 17).

CLU is a stress-induced cytoprotective chaperone that inhibits protein aggregation in a manner analogous to small HSPs, and its promoter contains a 14-bp element recognized by
the transcription factor HSF-1 (18). In human PCa, CLU levels are low in Gleason grade 3 untreated hormone-naive tissues, but increase with higher Gleason score (19) and within weeks after androgen deprivation (20). CLU expression correlates with loss of the tumor suppressor gene Nkx3.1 during the initial stages of prostate tumorigenesis in Nkx3.1 knockout mice (21). Experimental and clinical studies associate CLU with development of treatment resistance, where CLU suppresses treatment-induced cell death in response to androgen withdrawal, chemotherapy or radiation (10, 20, 22, 23). Over-expression of CLU in human prostate LNCaP cells accelerates progression after hormone- or chemo-therapy (10, 22), identifying CLU as an anti-apoptotic gene up-regulated by treatment stress that confers therapeutic resistance. OGX-011 is a second-generation phosphorothioate antisense oligonucleotide currently in late stage clinical development that potently inhibits CLU expression and enhances the efficacy of anticancer therapies in various human cancers including PCa (17, 24). While targeting CLU synergistically enhances the cytotoxic effects of chemotherapy, a role for CLU has not been characterized in the context of Hsp90 inhibitor treatment and resistance.

We hypothesized that Hsp90 inhibition induces a heat shock response with increased HSF-1 activity and CLU expression, which functions to inhibit treatment-induced apoptosis and enhance emergence of treatment resistance. It follows that knockdown of CLU using OGX-011 will potentiate the effect of Hsp90 inhibitors in CRPC.
**Materials and Methods:**

**Tumor cell lines and reagents:**

The human PCa cell line PC-3 was purchased from the American Type Culture Collection (2008, ATCC-authentication by isoenzymes analysis) and maintained in DMEM (Invitrogen-Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 2mmol/L L-glutamine. LNCaP cells were kindly provided by Dr. Leland W.K. Chung (1992, MDACC, Houston Tx) and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2009. LNCaP cells were maintained RPMI 1640 (Invitrogen Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 2mmol/L L-glutamine. All cell lines were cultured in a humidified 5% CO2/air atmosphere at 37°C. All cell lines were passaged for less than 3 months after resurrection. Western blotting and/or real time PCR was performed for AR and PSA each time when LNCaP cells were resurrected.

**Therapeutic agents:**

Hsp90 inhibitor, PF-04928473 (4-(6,6-Dimethyl-4-oxo-3-trifluoromethyl-4,5,6,7-tetrahydroindazol-1-yl)-2-(4-hydroxy-cyclohexylamino)-benzamide) and its prodrug PF-04929113 were kindly provided from Pfizer and used respectively for *in vitro* and *in vivo* studies. These compounds are novel synthetic small molecular weight inhibitors that bind the N-terminal adenosine triphosphate binding site of Hsp90 and PF-04929113 is orally bioavailable. For the *in vitro* studies, PF-04928473 was dissolved in dimethyl sulfoxide (DMSO) at 10mM stock solutions and stored at -20°C. For the *in vivo* studies, PF-04929113 was dissolved in PBS 1% carboxymethylcellulose and 0.5% Tween 80 (Invitrogen-Life Technologies, Inc.) at 15mg/ml and stored at 4°C.

17-allylamino-17-demethoxygeldanamycin (17-AAG) was kindly provided from NIH and used for *in vitro* and *in vivo* studies. For the studies, 17-AAG was dissolved in dimethyl sulfoxide (DMSO) at 10mM stock solutions and stored at -20°C.

**Clusterin siRNA and Antisense Oligonucleotides**

siRNAs were purchased from Dharmacon Research, Inc. (Lafayette, CO) using the siRNA sequence corresponding to the human CLU initiation site in exon 2 and a scramble control as
previously described (25). Second-generation antisense (OGX-011) and scrambled (ScrB) oligonucleotides with a 2′-O-(2-methoxy)ethyl modification were supplied by OncoGenex Pharmaceuticals (Vancouver, British Columbia, Canada). OGX-011 sequence (5′-CAGCAGCAGAGTCTTCAT-3′) corresponds to the initiation site in exon II of human CLU. The ScrB control sequence was 5′-CAGCGCTGACAACAGTTTCAT-3′. Prostate cells were treated with siRNA or oligonucleotides using protocols described previously (25).

**Cell proliferation and apoptosis assays:**

Prostate cancer cell lines were plated in appropriate media (DMEM or RPMI) with 5% FBS and treated with PF-04928473 or 17-AAG at indicated concentration and time and cell growth was measured using the crystal violet assay as described previously (26). Detection and quantitation of apoptotic cells were done by flow-cytometry (described below) and western blotting analysis. Each assay was repeated in triplicate.

The combination index (CI) was evaluated using CalcuSyn dose effect analysis software (Biosoft, Cambridge, UK). This method, based on the multiple drug effect equation of Chou-Talalay (27), is suitable for calculating combined drug activity over a wide range of growth inhibition: CI =1, additivity; CI >1, antagonism; CI <1, synergism. CI was calculated at ED50 and ED75.

Caspase-3 activity was assessed 3 days after treatment using the kit CaspACE Assay System, Fluorometric (Promega, Madison, WI, USA). Fifty µg of total cell lysate were incubated with caspase-3 substrate AC-DEVD-AMC at room temperature for 4h and caspase-3 activity was quantified in a fluorometer with excitation at 360nm and emission 460nm.

**Cell cycle analysis**

Prostate cancer cell lines were incubated in the absence or the presence of 1µM PF-04928473 or 17-AAG for 72h, trypsinized, washed twice and incubated in PBS containing 0.12% Triton X-100, 0.12mM EDTA and 100µg/ml ribonuclease A; 50µg/ml propidium iodide was then added to each sample for 20min at 4°C. Cell cycle distribution was analyzed by flow cytometry (Beckman Coulter Epics Elite, Beckman, Inc., Miami, FL), based on 2N and 4N DNA content. Each assay was done in triplicate.

**Western blotting analysis:**
Samples containing equal amounts of protein (depending on the antibody, 5-50µg) from lysates of cultured tumor prostate cell lines underwent electrophoresis on SDS-polyacrylamide gel and were transferred to nitrocellulose filters. The filters were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) at room temperature for 1h and blots were probed overnight at 4°C with primary antibodies (supplementary materials) to detect proteins of interests. After incubation, the filters were washed 3 times with washing buffer (PBS containing 0.1% Tween) for 5min. Filters were then incubated for 1h with 1:5,000 diluted Alexa Fluor secondary antibodies (Invitrogen) at room temperature. Specific proteins were detected using ODYSSEY IR imaging system (LI-COR Biosciences) after washing.

**Quantitative Reverse Transcription-PCR**

Total RNA was extracted from cultured cells after 48h of treatment using TRIzol reagent (Invitrogen Life Technologies, Inc.). Two µg of total RNA was reversed transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Real-time monitoring of PCR amplification of complementary DNA (cDNA) was performed using DNA primers (supplemental table S1) on ABI PRISM 7900 HT Sequence Detection System (applied Biosystems) with SYBR PCR Master Mix (Applied Biosystems). Target gene expression was normalized to GAPDH levels in respective samples as an internal standard, and the comparative cycle threshold (Ct) method was used to calculated relative quantification of target mRNAs. Each assay was performed in triplicate.

**Luciferase assay**

LNCaP and C4-2 cells (2.5x10^5) were plated on six-plates and transfected using lipofectin (6µL per well; Invitrogen Life Technologies, Inc.). The total amount HSE plasmids DNA used were normalized to 1µg per well by the addition of a control plasmid. One µM PF-04928473 or 17-AAG was added 4h after the transfection and for 48h. HSE-luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) with the aid of a microplate luminometer (EG&G Berthold). All experiments were carried out in triplicate wells and repeated 3 times using different preparations of plasmids.

**Immunofluorescence**

Tumor cells were grown on coverslips and treated with different concentration of PF-04928473 or 17-AAG for 48h. After treatment, cells were fixed in ice-cold methanol.
completed with 3% acetone for 10min at -20°C. Cells were the washed thrice with PBS and incubated with 0.2% Triton/PBS for 10min, followed by washing and 30min blocking in 3% nonfat milk before the addition of antibody overnight to detect HSF-1 (1:250). Antigens were visualized using anti-mouse antibody coupled with FITC (1:500; 30 min). Photomicrographs were taken at 20X magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse, Empix Imaging, Inc.).

**Animal Treatment**

Male athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected s.c. with 2x10^6 LNCaP cells (suspended in 0.1mL Matrigel; BD Biosciences). The mice were castrated once tumors reach between 300 and 500mm^3 or the PSA level increased above 50ng/mL. Once tumors progressed to castrate resistance, mice were randomly assigned to vehicle, PF-04929113 alone, PF-04929113+ScrB ASO or PF-04929113+OGX-011. PF-04929113 (prodrug, 25mg/kg; formulation in 0.5% CMC+0.5% Tween-80) is orally administered three times per week and OGX-011 or ScrB ASO (15mg/kg) was injected intra-peritoneally once daily for the first week and then three times per week. Each experimental group consisted of 10 mice. Tumor volume was measured twice weekly (length x width x depth x 0.5432). Serum PSA was determined weekly by enzymatic immunoassay (Abbott IMX, Montreal, Quebec, Canada). PSA doubling time (PSAdt) and velocity were calculated by the log-slope method (PSA_t = PSA_initial × e^{mt}). Data points were expressed as average tumor volume ± SEM or average PSA concentration ± SEM.

To establish PC-3 tumors, 2 x 10^6 PC-3 cells were inoculated s.c. in the flank region of 6-8 week-old male athymic mice (Harlan Sprague-Dawley, Inc.). When tumors reached 100mm^3, usually 3-4 weeks after injection, mice were randomly selected for treatment with 17-AAG (25mg/kg) + control ScrB ASO (15mg/kg) or 17AAG+OGX-011 (15mg/kg). 17-AAG was injected I.P three times per week, and OGX-011 or ScrB were injected i.p. once/day for the first week and then three times per week. For each experimental group consisted of 7 mice. Tumor volume was measured twice weekly. Data points were expressed as average tumor volume ± SEM.

When tumor volume reached ≥10% of body weight, mice were sacrificed and tumors harvested for evaluation of protein expression by western blotting analyses and
immunohistochemistry. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and appropriate institutional certification.

**Immunohistochemistry**

Immunohistochemical stains were performed on formalin-fixed and paraffin-embedded 4µm sections of tumor samples using adequate primary antibody (supplementary materials), and the Ventana autostainer Discover XT (Ventana Medical System) with enzyme labeled biotin streptavidin system and solvent resistant 3,3'-diaminobenzidine Map kit. All comparisons of staining intensities were made at 200x magnifications.

**Statistical analysis**

All *in vitro* data were assessed using the Student t test and Mann-Whitney test. Tumor volumes of mice were compared using Kruskal-Wallis test. Overall survival was analyzed using Kaplan-Meier curves and statistical significance between the groups was assessed with the log-rank test (Graphpad Prism). Levels of statistical significance were set at $P<0.05$. 
Results:

Hsp90 inhibitors induce expression of HSPs in PCa cells in vitro and in vivo.

Dose- and time-dependent effects of 17-AAG or PF-04928473 on the expression of CLU, Hsp90, Hsp70 and Akt protein and mRNA levels were evaluated in LNCaP and PC-3 cells. Both 17-AAG and PF-04928473 increased Hsp70 and CLU protein levels 3-fold in a dose- and time-dependent manner (Fig. 1A, B and C). Hsp90 inhibition induced a dose- and a time-dependent decline of Akt expression as previously reported (28). mRNA levels of CLU, Hsp70 and Hsp90 also increased after Hsp90 inhibitor treatment (Fig. 1D).

Next we assessed the effects of PF-04928473 treatment on CLU expression in vivo in CRPC LNCaP xenografts using immunohistochemistry and western blot (Fig. 2). CLU expression increased 4-fold after treatment with PF-04929113 (***, p<0.001) compared with vehicle treated tumor (Fig. 2A, B). Similarly, Hsp70, considered a pharmacodynamic measure of Hsp90 inhibition (16, 29), increased 2.3-fold after treatment with PF-04929113 (***, p<0.001) (Fig. 2A).

Treatment-induced feed forward loop involving CLU and HSF-1 activity

Since HSF-1 is the pre-dominant regulator of the heat shock response (30, 31), we evaluated the effect of Hsp90 inhibition on HSF-1-activity and expression of HSPs. As expected, 17-AAG or PF-04928473 significantly induced CLU (Fig. 1) as well as HSF-1 activity in a dose-dependent manner (***, p<0.001; Fig. 3A). CLU overexpression protected PC-3 tumor cells from PF-04928473-induced apoptosis (**, p<0.01; Fig. S1A). Moreover, HSF-1 knockdown using siRNA decreases CLU expression, sensitizing tumor cells to apoptosis-induced by PF-04928473 (Fig. S1B), confirming that the protective effect of CLU is mediated by HSF-1. Surprisingly, overexpression of CLU also increased HSF-1 activity (***, p<0.001, Fig. 3B), while CLU knockdown using siRNA or OGX-011 significantly decreased HSF-1 activity (*, p<0.05; ***, p<0.001; Fig. 3C), identifying novel feed-forward regulation of HSF-1 by CLU. Indeed, silencing of CLU inhibited HSF-1 transcriptional activity-induced by 17-AAG or PF-04928473 (Fig. 3C), as well as HSF-1 regulated genes such as Hsp27 and Hsp70 (Fig. 3D). CLU knockdown also sequesters HSF-1 in the cytoplasm (Fig. S1C), suggesting that CLU plays a role in HSF-1 nuclear translocation and transactivation.

OGX-011 enhances Hsp90 inhibitor induced apoptosis in prostate cancer cell lines
Since Hsp90 inhibitors induce up-regulation of CLU and CLU functions as a mediator in treatment resistance (17, 24, 32), we next evaluated if CLU knockdown potentiated the effect of Hsp90 inhibition. LNCaP cells were treated with OGX-011 and subsequently treated with indicated concentrations of 17-AAG or PF-04928473. OGX-011 significantly enhanced 17-AAG or PF-04928473 activity, reducing cell viability by an additional 20% at 100nM and 1000nM (*, p<0.05) compared with cells treated with control ScrB and Hsp90 inhibitor (Fig. 4A). To determine whether this effect was additive or synergistic, the dose-dependent effects with constant ratio design and the combination index (CI) values was performed and calculated according to the Chou and Talalay median effect principal (27). Figure 4B shows the dose response curve (combination treatment, OGX-011 or PF-04928473 monotherapy) and the combination index plots, indicating that OGX-011 synergistically enhances the effect of Hsp90 inhibitor on tumor cell growth.

Moreover, OGX-011 potentiates the effect of Hsp90 inhibitor to induce apoptosis (Fig. 4C and D). Flow cytometric analysis shows that apoptotic rates (subG1 fraction) increased significantly (p<0.001) when OGX-011 is combined with 17-AAG (53%) or PF-04928473 (65.4%), compared to control ScrB (4.2%), OGX-011 (17.4%), control ScrB ASO+17-AAG (18.3%) or control ScrB +PF-04928473 (24.8%; Fig. 4D). Moreover, the combination OGX-011 with 17-AAG or PF-04928473 increased caspase-dependent apoptosis compared to Hsp90 inhibitor- or OGX-011 monotherapy, as shown by cleaved PARP and caspase-3 expression (Fig 4C). The significant increase of caspase-3 activity confirms that OGX-011 sensitizes cells to Hsp90 inhibition with increased apoptotic rates (Fig. 4D). Reduced cell viability from combined CLU plus Hsp90 inhibition results, in part, from decreases in p-Akt levels in both PC-3 and LNCaP cells, as well as AR (and PSA) expression in LNCaP cells (Fig. 4C).

**OGX-011 potentiates 17-AAG activity in PC-3 xenografts in vivo.**

We next evaluated effects of combining OGX-011 with 17-AAG in PC-3 tumors in vivo. Male nude mice bearing PC-3 xenografts were randomly assigned for treatment with OGX-011+17-AAG (n=7) vs control ScrB +17-AAG (n=7). OGX-011 significantly enhanced the antitumor effect of 17-AAG in vivo, reducing mean tumor volume from 2935.3 mm³ to 1176.9 mm³ after 68 days (**; p≤0.01), compared to control ScrB (Fig. 5A). Cancer specific survival was significantly prolonged with combined OGX-011 + 17-AAG compared with
controls (71.4% vs 14.3% at day 72, respectively; *, p≤0.05; Fig. 5B. Immunohistochemical analysis reveals decreased CLU, Ki67, and Akt expression after treatment with OGX-011 + 17-AAG compared to other groups (Fig 5C). Additionally, OGX-011+17-AAG-treated tumors had higher apoptotic rates as shown by increased TUNEL staining, compared with other groups (Fig 5C).

OGX-011 potentiates PF-04929113 activity in CRPC LNCaP xenografts in vivo

We next assessed the effects of combined treatment with OGX-011 and PF-04929113 in castrate resistant LNCaP tumors. Mice bearing LNCaP tumors were castrated when PSA values exceeded 50ng/ml. Once PSA levels relapsed above pre-castration levels mice were randomly assigned to vehicle control, PF-04929113 alone, PF-04929113+control ScrB, or PF-04929113+OGX-011 (n=10 in each group). Mice treated with OGX-011+PF-04929113 had significant delays in tumor growth compared with all other groups (Fig 6A). By 7 weeks post treatment, all mice in the control had been euthanized; tumor volume in the OGX-011+PF-04929113 group was 517.4mm$^3$ compared to 2483.6mm$^3$ for PF-04929113 alone and 2176.4mm$^3$ for PF-04929113+control ScrB; ***, p<0.001; Fig. 6A).

Serum PSA levels were also significantly lower (~4-fold) in the OGX-011+PF-04929113 group compared with other groups (***, p<0.001; Fig 6B). The combination OGX-011+PF-04929113 group had a mean PSA level of 120ng/ml after 42 days compared to 418.7ng/ml in vehicle group, 527ng/ml in PF-04929113 alone, or 480.3ng/ml in scrB +PF-4929113 groups. The combination OGX-011+PF-04929113 group had a significantly prolonged PSA doubling time (33.6 weeks; *, p<0.05) and decreased PSA velocity (13.78ng/mL/week; *, p<0.05) compared with other groups (PSA doubling time: ~2.4weeks; velocity: ~85ng/mL/week; Fig. 6C).

Overall survival was significantly prolonged in mice treated with combined OGX-011+PF-04929113 (Fig 6D). By day 57, all mice died or were euthanized due to high tumor burden in control, PF-04929113 alone, or control ScrB+PF-04929113 groups compared with the combined OGX-011+PF-04929113 group, where all mice were still alive (p<0.001) after 62 days. These data demonstrate that targeting CLU using OGX-011 potentiates the effects of PF-04929113 to significantly inhibit tumor growth and prolong survival in human CRPC xenograft models.
Consistent with in vitro findings, immunohistochemical analysis reveals decreased CLU, Ki67, Akt, and AR expression after treatment with combined OGX-011 + PF-04929113 compared with other groups (Fig 7A). The immunostaining results were corroborated by western blots (Fig 7B). Additionally, tumors treated with combination OGX-011 + PF-04929113 had higher apoptosis rates compared with other groups as shown by increased TUNEL staining (Fig 7A). These data suggest that delays in tumor progression in OGX-011+PF-04929113 treated mice result from both reduced proliferation rates as well as increased apoptosis rates.
Discussion:

Development of treatment resistance is a common feature of most malignancies and the underlying basis for most cancer deaths. Treatment resistance evolves, in part, from selective pressures of treatment that collectively increase the apoptotic rheostat of cancer cells. Survival proteins up-regulated after treatment stress include anti-apoptotic members of the bcl-2 protein family, survivin, and molecular chaperones like CLU and other HSPs (33).

Molecular chaperones help cells cope with stress-induced protein aggregation, and play prominent roles in cell signaling and transcriptional regulatory networks. Chaperones act as genetic buffers stabilizing the phenotype of various cells and organisms at times of environmental stress, and enhance Darwinian fitness of cells during cancer progression and treatment resistance (13). Heat shock chaperones are key components of the heat shock response, a highly conserved stress-activated protective mechanism also associated with oncogenic transformation and thermo-tolerance (34). Chaperones are particularly important in regulating misfolded protein and endoplasmic reticular (ER) stress responses, an emerging area of interest in treatment stress and resistance. A growing enthusiasm for therapeutic modulation of this proteostasis network highlights Hsp’s and CLU as rational targets because of their multifunctional roles in signaling and transcriptional networks associated with cancer progression and treatment resistance. Cancer cells express higher levels of molecular chaperones and pirate the protective functions of HSF1 to support their transformation (34). Indeed, inhibitors of Hsp90, Hsp70, Hsp27 or CLU have all been reported to induce cancer cell death and sensitize chemotherapy (28, 35).

Several Hsp90 inhibitors including PF04928473 have potent anti-tumor activity in various preclinical models (28, 36, 37) and are in clinical trials (28, 38). Consistent with prior reports (28, 39), here we report that Hsp90 inhibitors induce a stress response with activation of the transcription factor HSF-1 and subsequent increased levels of Hsp90 itself, Hsp70 and CLU. This heat shock response likely enhance emergence of treatment resistance, as inhibition of transcription using Actinomycin D attenuates 17-AAG-mediated Hsp70 and Hsp27 expression and potentiates the effect of 17-AAG in vitro (39). Additionally, inhibition of the stress response by silencing HSF-1 also increases the activity of Hsp90 inhibitors (40). In this study, we set out to evaluate the role of CLU in this heat shock response since CLU is
dramatically induced by Hsp90 inhibitor treatment and CLU inhibitors are in late stage clinical development.

CLU is associated with many varied patho-physiological processes including reproduction, lipid transport, complement regulation and apoptosis (17, 41). CLU expression is rapidly up-regulated in various tissues undergoing apoptosis, including normal and malignant prostate and breast tissues following hormone withdrawal (2, 42). Previous studies have also linked CLU expression with induction and progression of many cancers, including CRPC (17). Furthermore, CLU up-regulation following androgen ablation in xenograft tumor models accelerates progression to castrate resistance and renders cells resistant to other apoptotic stimuli, including taxane chemotherapy (10, 43). Consistent with these accumulated findings (43), inhibition of CLU using OGX-011 synergistically enhances conventional as well as molecular targeted therapies in PCa preclinical models (25). Indeed, OGX-011 is now in Phase III trials as Phase II studies reported >90% inhibition of CLU in human prostate cancer tissues (44), and 7 months prolonged survival when OGX-011 is combined with docetaxel in CRPC (45, 46).

Here, we show that Hsp90 inhibitors increase CLU levels both in vitro and in vivo, while OGX-011 inhibits PF-04928473 or 17-AAG induced CLU. As expected (39, 40), PF-04928473 or 17-AAG induces HSF-1 transcriptional activity leading to up-regulation of HSPs expression. Surprisingly, we found that CLU silencing abrogates, while CLU overexpression enhances, Hsp90 inhibitor-induced HSF-1 transcription activity, identifying a role for CLU in the regulation of HSF-1 and the heat shock response itself. CLU knockdown blocks the translocation to HSF-1 to the nucleus following treatment with Hsp90 inhibitors. This effect of CLU on HSF-1 activity is biologically relevant since CLU overexpression protects, while CLU silencing enhances, cytotoxicity of Hsp90 inhibitors. Consistent with these in vitro results, synergistic effects were also observed in vivo in PC-3 and LNCaP models when OGX-011 was combined with Hsp90 inhibitors. Combination OGX-011 plus Hsp90 inhibitor significantly delay CRPC tumor growth and prolonged survival in PC-3 and LNCaP models. Increased apoptotic rates with combined Hsp90 and CLU inhibition suggests that delayed tumor progression resulted from enhanced treatment-induced apoptosis. Collectively, these results highlight, for the first time, a biologically relevant feed-forward regulation loop of CLU on HSF-1 and the heat shock response.
In addition to the effects of CLU inhibition on the heat shock response, observations in the castrate-sensitive, AR-positive LNCaP model highlight another possible benefit of combined CLU and Hsp90 suppression involving AR activity. Hsp90 inhibition is known to destabilize and degrade the AR with decreased PSA expression (16, 47). *In vivo*, serum PSA levels as well as PSA doubling time and velocity, were significantly reduced with combination OGX-011 therapy compared with PF-04929113 monotherapy. Serum PSA level is an established and useful AR-regulated biomarker (48) and a valuable tool in assessing efficacy of chemotherapy. Interestingly, at the low doses of Hsp90 inhibitor used in this in vivo study, no effect on serum PSA level was apparent. Lower PSA levels with combination therapy correlated with lower AR levels. This correlation between CLU inhibition and lower AR levels may involve the regulation loop of CLU on HSF-1 and the role of HSF-1 in regulating expression of other AR chaperones (eg. Hsp27, Hsp70, Hsp90, FKBP5.2) and we are actively exploring the molecular basis in ongoing experiments. While CLU is known to be transcriptionally activated by HSF-1 (17), in this study we also show that CLU exerts a feed forward loop that in turn activates HSF-1. CLU knockdown decreases HSF-1 transcriptional activity and abrogates its nuclear translocation, which subsequently leads to decreased Hsp27, Hsp70 and Hsp90 expression, similar to that observed after HSF-1 knockdown (49). Consequently, AR stability is reduced because of lowered chaperone levels.

In addition to synergistically enhancing anti-tumor activity, combination therapy may also allow dose reduction strategies to reduce toxicity that has been associated with Hsp90 inhibitors in clinical trials. For example, 17-AAG induced hepatotoxicity as monotherapy at 60mg/kg/day (50), while PF-04929113 caused body weight loss at 50mg/kg/day. In a previous study, 50mg/kg PF-04929113 as monotherapy inhibited LNCaP CRPC tumor progression (28). At sub-therapeutic doses of 25mg/kg/day used in the present study, PF-04929113 monotherapy showed marginal, non-significant decreases in tumor volume and no effect on serum PSA levels; however, significant delays in tumor progression were seen at this lower dose when PF-04929113 was combined with OGX-011, with no toxicity observed.

In summary, this paper helps define how stress induced by Hsp90 inhibitors regulates CLU by induction of HSF-1 activity and, in turn, how CLU regulates HSF-1 activity, cell survival, and treatment resistance. We demonstrate, for the first time, that CLU inhibition abrogates the heat shock response induced Hsp90 inhibitors. These observations are clinically relevant since CLU inhibitors are in phase III clinical trials, and provide a framework for building new
drug combinations based on mechanism-based interventions to overcome drug resistance. The present study supports for the first time the development of targeted strategies employing OGX-011 in combination with Hsp90 inhibitors to improve patient outcome in CRPC.

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Conflict of interest

The University of British Columbia has submitted patent applications, listing Dr. Gleave as inventor, on the antisense sequence described in this paper. This IP has been licensed to OncoGenex Technologies, a Vancouver-based biotechnology company that Dr. Gleave has founding shares in. M.-J. Yin is an employee of Pfizer.

Abbreviations

PCa prostate cancer
CRPC castrate resistant prostate cancer
HSP heat shock proteins
CLU clusterin
PSA prostate specific antigen
17-AAG 17-allylamino-17-demethoxygeldanamycin
Aso antisense oligonucleotide
Bibliography


Legends

Figure 1. PF-04929113 and 17-AAG induce HSPs and CLU expression in PCa cells in vitro. PC-3 and LNCaP cells were treated with 1μM PF-04928473 (A) or 1 μM 17-AAG (C) for the indicated time points. In parallel, PC-3 and LNCaP cells were treated for 48h with PF-04928473 for the indicated doses (B). Protein extracts were analyzed for CLU, Hsp70, Akt and vinculin. Tumor cells were treated for 24h with 1μM PF-04928473 or 1μM 17-AAG (D). mRNA extracts were analyzed by real-time PCR for CLU, Hsp90 and Hsp70. ***, p<0.001.

Figure 2. PF-04928473 induces HSP and CLU expression in PCa xenografts. Mice bearing LNCaP or PC-3 tumors were treated for 6 weeks with 50mg/kg PF-04929113 or vehicle (Control). A, tumors were collected and CLU and Hsp70 were evaluated by immunohistochemical analysis. B, total proteins were extracted from the xenograft tumors and CLU expression was analyzed by western blotting. The relative levels were normalized with GAPDH and estimated in densitometric units. ***, p<0.001.

Figure 3. CLU induction following Hsp90 inhibitor treatment is cytoprotective via an increase of HSF-1 activity. A, LNCaP cells were treated with indicated concentrations of 17-AAG or PF-04928473 for 48h. B, LNCaP cells were transiently transfected with indicated concentrations of CLU-plasmid for 48h. Total amount of plasmid DNA transfected was normalized to 2μg per well by the addition of an empty vector. C (Top), LNCaP cells were transfected with 20nM CLU siRNA or control siScr, followed of 17-AAG or PF-04928473 treatment (1μM) for 48h. C (bottom), LNCaP cells were treated twice with 300nM OGX-011 or control ScrB ASO. D, LNCaP and PC-3 cells were treated twice with 300nM OGX-011 or control ScrB ASO, followed by 1μM of 17-AAG or PF-04928473 for 48h. Cells were harvested, and HSE-luciferase activity or western blotting analyses were performed. Means of at least three independent experiments done in triplicate. ***, p<0.001; *, p<0.05; ns, not significant.

Figure 4. CLU knockdown enhances effects of Hsp90 inhibitor treatment in PCa cells. A, LNCaP cells were treated twice with 300nM OGX-011 or control ScrB, followed by the indicated concentration of 17-AAG or PF-0492873 for 24h. Cell growth was determined by crystal violet and compared with control. B, dose dependent effects and combination index (CI) values calculated by CalcuSyn software were assessed in LNCap cells treated for 48h with OGX-011 alone, PF-04928473 alone or combined treatment at indicated concentration.
with constant ratio design between both drugs. The CI for ED$_{50}$ and ED$_{75}$ was 0.4 and 0.75, respectively, indicative of a synergistic effect of this combined treatment. C and D, LNCaP cells were treated twice with 300nM OGX-011 or control ScrB, followed by 1µM 17-AAG or PF-04928473 for 48h. Cells were harvested, and western blotting analyses were performed (C). The proportion of cells in subG$_1$, G$_0$-G$_1$, S, G$_2$-M was determined by propidium iodide staining and caspase-3 activity was determined on the cell lysates and the results are expressed in arbitrary units and corrected for protein content (D). All experiments were repeated at least thrice.

**Figure 5. OGX-011 potentiates 17-AAG activity in PC-3 xenograft model.** Mice were treated IP with 25mg/kg 17-AAG and 15mg/kg OGX-011 starting when tumors reached 300mm$^3$ as described in Materials and Methods (M&M). A, The mean tumor volume of mice treated with OGX-011+17-AAG was compared with control ScrB+17-AAG ± SEM (n=7). **, p<0.01. B, in Kaplan-Meier curve, cancer-specific survival was compared between mice treated with OGX-011+17-AAG and control ScrB+17-AAG over a 72-d period. *, p<0.05. C, tumors were collected after 72-d and CLU, Ki67 and TUNEL were evaluated by immunohistochemical analysis (original magnification: x200).

**Figure 6. OGX-011 potentiates PF-04929113 activity in LNCaP CRPC xenograft model.** Castrated mice were treated with 25mg/kg PF-04929113 and 15mg/kg OGX-011 starting when serum PSA values relapsed to pre-castration levels as described in M&M. The mean tumor volume (A) and the serum PSA level (B) were compared between the 4 groups ± SEM (n=10). ***, p<0.001. C, PSA doubling time and velocity were calculated as described M&M. *, p<0.05. D, in Kaplan-Meier curve, cancer-specific survival was compared between the 4 groups over a 62-d period. ***, p<0.001. Progression-free survival was defined as time for the first tumor volume doubling.

**Figure 7. OGX-011 potentiates PF-04929113-induced apoptosis in CRPC LNCaP tumors.** A, tumors were collected after 57 days and CLU, Ki67, AR, AKT and TUNEL were evaluated by immunohistochemical analysis (original magnification: x200). B, total proteins were extracted from the xenograft tumors and CLU, AR, Akt and PSA were analyzed by western blotting. The relative levels were normalized with vinculin and estimated in densitometric units ±SEM.
Figure 4

(A) Graph showing cell proliferation (% of control) for different concentrations of PF-04928473 and 17-AAG.

(B) Graph showing the effect of various doses on cell proliferation.

(C) Protein expression analysis using Western blotting with different markers and treatments.

(D) Graph showing the percentage of cell cycle repartition in subG1, G0/G1, S, and G2/M phases for different treatments.

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Figure 5

(A) Tumor volume (mm³) over time for different treatment groups.

(B) Cancer specific survival over time for different treatment groups.

(C) Immunohistochemical staining for Clusterin, K67, and TUNEL in various treatment groups.

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