Cotargeting Androgen Receptor and Clusterin Delays Castrate-Resistant Prostate Cancer Progression by Inhibiting Adaptive Stress Response and AR Stability

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Abstract

Although androgen receptor (AR) pathway inhibitors prolong survival in castrate-resistant prostate cancer (CRPC), resistance rapidly develops and is often associated with increased stress-activated molecular chaperones like clusterin (CLU) and continued AR signaling. Because adaptive pathways activated by treatment facilitate development of acquired resistance, cotargeting the stress response, activated by AR inhibition and mediated through CLU, may create conditional lethality and improve outcomes. Here, we report that CLU is induced by AR antagonism and silencing using MDV3100 and antisense, respectively, to become highly expressed in castrate- and MDV3100-resistant tumors and cell lines. CLU, as well as AKT and mitogen-activated protein kinase (MAPK) signalosomes, increase in response to MDV3100-induced stress. Mechanistically, this stress response is coordinated by a feed-forward loop involving p90rsk (RPS6KA)-mediated phosphoactivation of YB-1 with subsequent induction of CLU. CLU inhibition repressed MDV3100-induced activation of AKT and MAPK pathways. In addition, when combined with MDV3100, CLU knockdown accelerated AR degradation and repressed AR transcriptional activity through mechanisms involving decreased YB-1–regulated expression of the AR cochaperone, FKBP52. Cotargeting the AR (with MDV3100) and CLU (with OGX-011) synergistically enhanced apoptotic rates over that seen with MDV3100 or OGX-011 monotherapy and delayed CRPC LNCaP tumor and prostate-specific antigen (PSA) progression in vivo. These data indicate that cotargeting adaptive stress pathways activated by AR pathway inhibitors, and mediated through CLU, creates conditional lethality and provides mechanistic and preclinical proof-of-principle to guide biologically rational combinatorial clinical trial design.

Introduction

Prostate cancer is the second leading cause of cancer deaths among males in western countries (1). Although early-stage disease is treated with curative surgery or radiotherapy, the mainstay of treatment for locally advanced, recurrent or metastatic prostate cancer is androgen deprivation therapy (ADT), which reduces serum testosterone to castrate levels and suppresses androgen receptor (AR) activity. Despite high initial response rates after ADT, progression to castrate-resistant prostate cancer (CRPC) occurs within 3 years (2–4). The AR is the principle driver of CRPC (5), and is supported by ADT-activated growth factor signaling pathways (6), survival genes (7), and cytoprotective chaperone networks (8). Docetaxel (9) was the first therapy to prolong survival in CRPC, stratifying the treatment landscape into pre and postchemotherapy states. More recently, the CYP17 inhibitor abiraterone (10) and the AR antagonist MDV3100 (11) have prolonged survival and are rapidly changing the CRPC treatment landscape. Despite significant responses (11, 12), these novel AR pathway inhibitors activate redundant survival pathways that adaptively drive treatment resistance and recurrent CRPC progression. Realization of the full potential of AR pathway inhibition will require characterization of these stress-activated survival responses and rational combinatorial cotargeting strategies designed to abrogate them.

Molecular chaperones play central roles in stress responses by maintaining protein homeostasis and regulating prosurvival signaling and transcriptional networks. Clusterin (CLU), a stress-activated chaperone transcriptionally regulated by HSF1 (13), YB-1 (14), and others (15), inhibits stress-induced apoptosis by suppressing protein aggregation (16), p53-activating stress signals (17), and conformationally altered Bax (17, 18) while enhancing AKT phosphorylation (19) and transactivation of NF-κB and HSF-1 (13, 20). CLU is expressed in many human cancers (21, 22), including prostate where it increases following castration to become highly expressed in...
CRPC (23). CLU overexpression confers treatment resistance (24), whereas CLU inhibition potentiates activity of most anticancer therapies in many preclinical models (25–27). The CLU inhibitor, OGX-011 (custirsen, OncoGenex Pharmaceuticals), is currently in phase III trials after a randomized phase II study in CRPC reported 7-month gain in overall survival and 50% reduced death rate (HR = 0.50) when combined with docetaxel (28).

Because CLU is induced by treatment stress, including castration (23, 24), and functions as a key mediator of the stress response (13), we hypothesized that cotargeting the AR and stress-response pathways mediated by CLU may create conditional lethality and improve cancer control. This study was set out to correlate MDV3100 treatment stress and resistance with CLU induction, identify pathways regulating CLU activation, and define mechanisms by which CLU inhibition potentiates anti-AR therapy in CRPC.

Materials and Methods

Prostate cancer cell lines and reagents

LNCaP and C4-2 cells were kindly provided by Dr. L.W.K. Chung (1992, MD Anderson Cancer Center, Houston, Tx) and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer Iix in July 2009. PC3 cells were purchased from American Type Culture Collection (ATCC) and authenticated by ATCC’s isoenzyme analysis. CRPC and MDV3100-resistant tumors and cell lines were generated (Supplementary Fig. S1) as previously described (29). Cycloheximide and MG-132 were purchased from Calbiochem, R1881 from PerkinElmer, and MDV3100 from Haoyuan Chemexpress Co., Limited. Antibodies used are listed in Supplementary Materials and Methods.

CLU siRNA and antisense

CLU and control siRNAs were purchased from Dharmacon Research, Inc., as previously described (13). Second-generation CLU antisense (OGX-011) and scrambled (ScrB) oligodeoxynucleotides (ODN) with a 2′-O-(2-methoxy)ethyl modification were supplied by OncoGenex Pharmaceuticals, whereas AR antisense were supplied by Isis Pharmaceuticals; these are described in Supplementary Materials and Methods. Prostate cells were treated with CLU or YB-1 siRNA or oligonucleotides, using protocols described previously (13, 14).

Western blot analysis and immunoprecipitation

Total proteins were extracted using radioimmunoprecipitation assay buffer and submitted to Western blot analysis as previously described (30). For immunoprecipitation, total proteins were precleared with protein-G sepharose (Invitrogen Life Technologies), immunoprecipitated with 2 μg anti-AR, and Western blotted as previously described (30).

Quantitative reverse transcription PCR

Total RNA was extracted from cultured cells after 48 hours of treatment using TRIzol reagent (Invitrogen Life Technologies, Inc.) as previously reported (13). Primers (described in Supplementary Materials and Methods) were normalized to β-actin levels as an internal standard, and the comparative cycle threshold (Ct) method was used to calculate relative quantification of target mRNAs. Each assay was conducted in triplicate.

Immunofluorescence

LNCaP cells were grown on coverslips and transfected with CLU siRNA or ScrB control. Forty-eight hours posttransfection, cells were treated with 10 μmol/L MDV3100 ± 1 nmol/L R1881 for 6 hours. Immunofluorescence was conducted as previously reported (30). Photomicrographs were taken at ×20 magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse, Empix Imaging, Inc.).

AR transcription activity

LNCaP, CRPC-V16D, and MR49C cells (29) were treated with OGX-011 or ScrB control, CLU siRNA or Ctr siRNA and then transfected with PSA-luciferase reporter (−6,100→+12) or Probasin luciferase reporter along with Renilla plasmid as previously described (30). All experiments were carried out in triplicate wells and repeated 5 times using different preparations of plasmids.

Cell proliferation and cell-cycle assays

LNCaP, MR49C, and MR49F cells (Supplementary Fig. S1) were transfected with CLU siRNA, OGX-011, or ScrB control, and then treated with MDV3100 or dimethyl sulfoxide (DMSO) control 24 hours after transfection. After a time course exposure, cell growth was measured by crystal violet assay as previously described (30). Detection and quantitation of apoptotic cell-cycle population were analyzed by flow cytometry as previously described (30). Each assay was done in triplicate 3 times.

Crystal violet assay was used to determine synergism of combination therapy. LNCaP cells were treated with 10 nmol/L CLU or ScrB siRNA, or 500 nmol/L OGX-011 or ScrB ODN, and 1 day later combined with indicated concentration of MDV3100 or DMSO for 48 hours. CalcuSyn software was used to calculate the combination index (CI) at several effective doses (CI = 1; additive effect, CI < 1; synergy effect, CI > 1; antagonistic effect).

Protein stability and degradation

To assess the effect of combination treatment on AR protein stability, LNCaP cells were treated with OGX-011 or ScrB for 48 hours followed by RPMI +5% FBS plus 10 μmol/L cycloheximide and 10 μmol/L MDV3100 for 2 to 6 or 16 hours and Western blotted using AR and vinculin antibodies.

Animal treatment

Male athymic mice (Harlan Sprague-Dawley, Inc.) were injected subcutaneously with 1 × 107 LNCaP cells suspended in Matrigel. When tumors reached 150 mm3, and serum prostate-specific antigen (PSA) was more than 50 ng/mL, mice were castrated. Once tumors progressed to CRPC, mice were randomly assigned to 10 mg/kg MDV3100 daily plus either 10 mg/kg OGX-011 or ScrB ODN intraaperitoneally once daily for 7 days and then 3 times per week thereafter. Each
The experimental group consisted of 12 mice. Tumor volume and serum PSA were measured as previously described (13). All animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care.

### Statistical analysis

All results are expressed as the average ± SE. Two-tailed t tests, one-way ANOVA, or Wilcoxon matched pairs tests were used for statistical analysis. Synergism was calculated by CalcuSyn software. The differences between single treatment and combination treatment was analysis by Freidman test and done with JMP version 4. *P* < 0.05, **P* < 0.01, and ***P* < 0.001 were considered significant.

### Results

**CLU is highly expressed in MDV3100-resistant xenografts and cell lines**

To understand the mechanisms of MDV3100 recurrence, we developed LNCaP-derived MDV3100-resistant tumors and cell lines (Kuruma and colleagues; Supplementary Fig. S1) and confirmed that CLU levels were increased by Western blot analysis (Fig. 1A, left) and immunohistochemistry (Fig. 1A, middle and right). Elevated CLU was also observed in most MDV3100-resistant cell lines derived from these xenografts (Fig. 1B). These data indicate that CLU increases with MDV3100 resistance, similar to comparisons between castrate sensitive and CRPC tumors (24).

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**Figure 1.** MDV3100 induces upregulation of CLU. A, CLU is highly expressed in MDV3100-resistant xenografts. Western blot analysis was conducted from MDV3100-resistant or control CRPC tumors using CLU and vinculin (as a loading control) antibodies (left). Tumors were evaluated by immunohistochemistry using CLU antibody on MDV3100-resistant tumors and control CRPC tumors (middle, right). B, CLU is highly expressed in MDV3100-resistant cell lines. Western blot analysis was conducted from cell lines generated from CRPC and MDV3100-resistant tumors using CLU and vinculin antibodies. C, CLU is induced by MDV3100 in time- and dose-dependent manner. LNCaP cells were treated with MDV3100 at indicated time (left) or concentration (right) and Western blot analysis was conducted with CLU, AR, and PSA antibodies. D, CLU was induced after AR knockdown LNCaP cells were treated with AR-ASO targeting AR exon 1 or intron 1, or control ASO and Western blotted for CLU, AR, and PSA antibodies.
**AR pathway inhibition induces CLU**

We used MDV3100 and AR antisense approaches to assess effects of AR pathway inhibition on CLU induction in MDV3100-sensitive and -resistant LNCaP cells. Similar to bicalutamide and ADT using charcoal stripped serum (Supplementary Fig. S2), MDV3100 induces CLU in both a time- and dose-dependent manner in castrate-sensitive LNCaP (Fig. 1C) and castrate-resistant C4-2 (Supplementary Fig. S3) cells, in parallel with reduced AR activity as indicated by decreased PSA expression. These data suggest that MDV3100 induces activation of CLU, similar to that seen with first-line ADT in castrate-sensitive tumors (24). To determine whether CLU is also induced as a consequence of AR inhibition even in MDV3100-resistant cells, AR-ASO targeting exon 1 and intron 1 of the AR were used. Both AR-ASO potently downregulate AR in a dose-dependent and sequence-specific manner in MR49F cells, with parallel induction of CLU (Fig. 1D). These data suggest that AR pathway inhibition by ADT, AR antagonism, or antisense knockdown induces CLU in castrate-sensitive and -resistant cells, possibly as an early adaptive response to AR-inhibited stress.

**MDV3100-induced CLU is mediated via p90Rsk-YB-1 signaling pathway**

We next set out to define transcriptional regulation of CLU induction following MDV3100 treatment stress. We recently reported that CLU is upregulated via YB-1 after treatment stress (14); YB-1 is stress-activated via phosphorylation by AKT and p90RSK (a RPS6KA multigene family member; refs. 31, 32), stimulating its nuclear translocation. Although MDV3100 has been reported to activate AKT (33), we found that MDV3100 also induces a downstream effector of extracellular signal-
regulated kinase (ERK), p90Rsk (Fig. 2A); because both AKT and ERK pathways can activate YB-1 (31, 32), we hypothesized that MDV3100 induces CLU upregulation via phosphoactivation of YB-1. Figure 2A confirms that MDV3100 increases downstream effectors of AKT (pS6K) and Erk (p90Rsk), accompanied by increased phospho-YB-1 levels. YB-1 silencing abrogates MDV3100-induced CLU expression at both the protein and mRNA levels (Fig. 2B), suggesting that MDV3100-induced CLU is mediated by YB-1. Because YB-1 can be phosphoactivated by both AKT and p90Rsk (31, 32), we next defined which pathway mediated MDV3100-induced upregulation of CLU using inhibitors of AKT (LY294002) and p90Rsk (SL0101). MDV3100-induced upregulation of CLU was abrogated by p90Rsk (Fig. 2C, left), but not AKT inhibition (Fig. 2C, right), indicating that p90Rsk-YB-1 pathway mediates MDV3100-induced CLU expression.

CLU knockdown synergetically enhances MDV3100-induced inhibition of LNCaP cell growth

Because AR pathway inhibition induces CLU (Fig. 1), which has antiapoptotic functions and a role in treatment resistance, we evaluated whether CLU knockdown potentiates the activity of MDV3100. LNCaP cells were treated with the CLU antisense drug, OGX-011, followed by indicated concentrations of MDV3100. OGX-011 significantly enhanced MDV3100 activity, reducing cell viability in both a time- (Fig. 3A, left) and dose- (Fig. 3A, right) dependent manner. To determine whether this
Effect was additive or synergistic, dose-dependent effects with constant ratio design and CI values were calculated according to the Chou and Talalay median effect principal (34). OGX-011 synergistically enhances the inhibitory effect of MDV3100 on cell growth in LNCaP (Fig. 3B) as well as C4-2 cells (Supplementary Fig. S4, top), but not in AR-negative PC3 cells (Supplementary Fig. S4, bottom). Flow cytometric analysis indicates that OGX-011 combined with MDV3100 significantly ($P < 0.001$) increases the sub-G1 apoptotic fraction compared with monotherapy (Fig. 3C, left). In addition, combination OGX-011 plus MDV3100 increased caspase-dependent apoptosis, as shown by cleaved PARP and caspase-3 activity (Fig. 3C, right). Collectively, these data indicate that OGX-011 synergistically enhances MDV3100-induced apoptosis.

**Combined MDV3100 plus OGX-011 synergistically delays CRPC LNCaP tumor growth**

We next evaluated the *in vivo* effects of cotargeting the AR and stress response using combined OGX-011 and MDV3100 in CRPC LNCaP model. Male nude mice bearing LNCaP xenografts were castrated when serum PSA reached 75 ng/mL and followed until serum PSA and tumor growth rates increased back to precastrate levels, indicating progression to castration resistance. Mice were then randomly assigned for treatment with MDV3100 plus either control ($n = 12$) or OGX-011 ($n = 12$). At baseline, mean LNCaP tumor volume and serum PSA levels were similar in both groups. OGX-011 significantly enhanced the antitumor effect of MDV3100, reducing mean tumor volume from 1,600 to 650 mm$^3$ by 12 weeks ($^{++}, P < 0.05$), compared with control (Fig. 4A and C). Mean serum PSA levels were also significantly lower ($\sim$4-fold) in the OGX-011+MDV-3100 group compared with MDV3100+control group ($P < 0.001$; Fig. 4B and D). Waterfall plots (Fig. 4C and D) illustrate more frequent tumor volume, and serum PSA decreases in the combination group. Overall survival (defined as euthanasia for tumor volume exceeding 10% of body weight) was significantly prolonged with combined MDV-3100+OGX-011 compared with MDV3100+control (90% vs. 30% at week 16, respectively; $P < 0.05$). These data show that cotargeting the AR and the induced CLU-mediated stress response potentiates MDV3100 activity in a human CRPC xenograft model.

**Combination MDV3100 plus CLU silencing reduces AR nuclear translocation and transcriptional activity**

Analysis of *in vivo* studies suggest that combination MDV3100+OGX-011 led to PSA decreases disproportionate to
changes in tumor volume compared with MDV3100 monotherapy tumors; we therefore explored effects of CLU inhibition on AR signaling under conditions of MDV3100 treatment. Castrate-sensitive LNCaP and CRPC LNCaP-derived (V16D) cells were treated with MDV3100 and evaluated for changes in R1881-stimulated PSA transactivation. As expected, MDV3100 reduced R1881-induced AR transcription as measured by PSA luciferase transactivation assay (Fig. 5A); interestingly, CLU siRNA also reduced AR activity and this effect was enhanced by MDV3100, suggesting that CLU knockdown potentiates MDV3100 inhibition of AR activity. Similar data were obtained using CLU siRNA in androgen-independent AR+ LAPC4 and 22RV1 cells, CRPC and MDV3100-resistant LNCaP cells (Supplementary Fig. S5). MDV3100 and CLU silencing also inhibited AR-regulated gene expression, which was maximally reduced by combination therapy (Fig. 5B). In keeping with these observations, immunofluorescence (Fig. 5C) and subcellular fractionation studies (Fig. 5D) confirm that MDV3100 inhibition of R1881-induced AR nuclear translocation was significantly enhanced in combination with CLU siRNA.

**CLU knockdown combined with MDV3100 accelerates proteasome-mediated AR degradation**

We found that CLU silencing decreases AR protein levels in parental LNCaP (Fig. 6A), CRPC LNCaP-derived V16D (Supplementary Fig. S6, left), and MDV-resistant LNCaP-derived
Figure 6. CLU knockdown combined with MDV3100 accelerates AR proteasomal degradation. A, CLU knockdown in combination with MDV3100 decreases AR protein levels. LNCaP cells were treated with either OGX-011 (left) or CLU siRNA (right) and treated with 10 μmol/L MDV3100 for 24 hours and Western blot analyses was conducted for AR and CLU (top). Vinculin was used as a loading control. Quantitative reverse transcription PCR (bottom) was used to evaluate expression of AR. B, CLU plus MDV3100 alters AR stability. LNCaP cells were treated with OGX-011 or ScrB and followed by 10 μmol/L cycloheximide treatment. Proteins were extracted and Western blot analyses conducted for AR. C, CLU knockdown reduces MDV3100-induced AR/Hsp90 heterocomplex levels. LNCaP cells were treated with CLU or control siRNA with 10 μmol/L MDV3100 for 24 hours and then MG-132 for 6 hours. Proteins were extracted for Western blot analysis using AR (left). LNCaP cells were transfected with His ubiquitin and AR and followed by siRNA CLU or control siRNA treatment with 10 μmol/L MDV3100 for 24 hours and then MG-132 for 6 hours. Proteins were immunoprecipitated using AR and Western blot analysis conducted using AR (middle). LNCaP cells were treated with His ubiquitin and AR and followed by siRNA CLU or control siRNA treatment with 10 μmol/L MDV3100 for 24 hours and then MG-132 for 6 hours. Proteins were immunoprecipitated using ubiquitin and Western blot analysis conducted using AR, ubiquitin antibodies (right). IP, immunoprecipitation; IgG, immunoglobulin G.
cells (Supplementary Fig. S6, right) as well as in LAPC4 and 22R1 cells (Supplementary Fig. S7); these changes are seen at the protein, but not mRNA levels (Fig. 6A, bottom) and enhanced by MDV3100, suggesting that CLU knockdown in combination with MDV3100 alters AR protein stability. The effect of CLU silencing −/+MDV3100 on AR biosynthesis using cycloheximide was next evaluated. Both MDV3100 and CLU siRNA induced rapid and profound decrease of AR 6 hours after cycloheximide, whereas this effect was seen after only 2 hours when MDV3100 was combined with CLU knockdown (Fig. 6B). These data suggest that CLU silencing and MDV3100, both decrease AR protein turnover, but this effect is potentiated when combined.

Protein ubiquitination and acetylation are important determinants of AR transcriptional activation (35, 36). AR forms a heterodimer complex with Hsp90 to provide stability for ligand-unbound AR; without Hsp90 binding, the AR is ubiquitinated and degraded by the proteasome (37, 38). We found that MDV3100 increases AR-Hsp90 heterocomplex levels (Fig. 6C), consistent with prior reports that MDV3100 sequesters AR in the cytoplasm (11), with decreased AR acetylation (Fig. 6C) and AR transcriptional activity (Fig. 5A). Interestingly, CLU knockdown profoundly decreases AR/Hsp90 heterocomplex and AR acetylation levels (Fig. 6C). These data are consistent with a view that the MDV3100-AR-Hsp90 heterocomplex becomes more vulnerable to degradation under conditions of CLU silencing. The proteasome inhibitor, MG132, abrogated AR degradation under conditions of CLU knockdown plus MDV3100 (Fig. 6D, left) with increased ubiquitinated AR levels (Fig. 6D, middle and right). Collectively, these data suggest that when CLU knockdown is combined with MDV3100, the AR is deacetylated and ubiquitinated with accelerated proteasome-mediated degradation.

**CLU knockdown decreases levels of the molecular cochaperone FKBP52**

The preceding data indicate that MDV3100-sequestered, AR-Hsp90 cytoplasmic heterocomplexes, when combined with CLU knockdown, are destabilized and lead to AR ubiquitination and degradation with reduced AR nuclear translocation and activity. One explanation is that CLU inhibition may lower Hsp90 levels through its regulation of HSF-1 activity (13); however, MDV3100 did not induce HSF-1 activity (Supplementary Fig. S8), and combination therapy did not lower the levels of the HSF-1-regulated gene, Hsp90 (Fig. 6C, input). These observations, along with data in Fig. 2, implicate YB-1, rather than HSF-1, as the key stress-activated transcription factor mediating MDV3100 increases of CLU. Because Hsp90 cooperates with other cochaperones to stabilize client proteins, we initially used an unbiased approach to identify AR-Hsp90 cochaperones regulated by CLU. Gene profiling analysis from LNCaP and PC-3 cells treated with control versus CLU siRNA indicated that CLU expression correlated with the Hsp90 cochaperone FKBP52 (Hsp56) and that CLU knockdown reduces FKBP52, but not FKBP51 or Hsp90, protein levels while abrogating MDV3100-induced phosphorylation of YB-1, PAKT, and p90Rsk (Fig. 7A). To define how FKBP52 levels decrease under conditions of MDV3100-induced stress and CLU knockdown, we mined public databases and found that YB-1 binds to FKBP52 DNA with a high stringency of 12 in chromatin immunoprecipitation (ChIP) on ChIP analysis (39). Western blotting confirmed that YB-1 knockdown decreases FKBP52 expression levels (Fig. 7B). To ascertain the role of FKBP52 in AR stability under conditions of CLU silencing, FKBP52 was overexpressed after CLU knockdown and AR expression was evaluated. Figure 7C shows that FKBP52 partially rescues AR from degradation induced by CLU knockdown. FKBP52 overexpression also partially restores PSA expression, indicating increased AR activity when FKBP52 levels are restored under conditions of CLU knockdown. These data suggest that CLU regulates the stability of the AR–cochaperone complex by affecting FKBP52 levels. Although FKBP52 rescue can partially reverse CLU knockdown effects on AR stability in the absence of MDV3100, AR levels were not rescued when MDV3100 was present (Fig. 7C). These data define an MDV3100-induced feed-forward loop involving pYB-1, p90Rsk, CLU, and the AR cochaperone FKBP52 that collectively support AR stability, nuclear translocation, and activation that may be cotargeted using OGX-011.

**Discussion**

Many strategies used to kill cancer cells induce stress and redundant survival responses that promote survival and emergence of treatment resistance, which is the underlying basis for most cancer deaths. This therapeutic resistance results from the Darwinian interplay of innate and adaptive survival pathways activated by selective pressures of treatment. In prostate cancer, castration induces apoptosis and clinical responses in most patients but also triggers progression to CRPC (2, 3). Similarly, newer AR pathway inhibitors like abiraterone and MDV3100 prolong survival but resistance develops in many initial responders (11, 12, 40). Disease progression frequently correlates with rising PSA levels, indicating continued AR signaling and highlighting need for additional therapies targeting the molecular basis of treatment-resistant CRPC. Defining interactions between the AR and redundant survival pathways will build new combinatorial strategies that control progression and improve outcomes.

Persistent AR signaling in CRPC can occur via AR amplification or mutations, as well as altered levels of AR coactivators or cochaperones (41–44). Another more dynamic mechanism involves reciprocal feedback regulation between AR and phosphoinositide 3-kinase (PI3K) pathways whereby AR inhibition activates AKT signaling by reducing the levels of the AKT phosphatase PHLPP, and PI3K inhibition activates AR signaling by relieving feedback inhibition of HER kinases; inhibition of one activates the other, thereby enhancing survival (33). These insights guide design of combinatorial regimens targeting the AR pathway and AKT or HSP chaperones Hsp90 and Hsp27 (45).

Because AR pathway inhibition is known to induce stress and CLU with reciprocal pathway activation of AKT (34), targeting the stress response activated by AR pathway inhibitors is another combinatorial strategy. This study defines feed-forward links between stress-induced YB-1 to CLU activation in parallel with enhanced AKT and mitogen-activated protein kinase (MAPK) pathways.
kinase (MAPK) signaling, which collectively support AR stability and activity during MDV3100 treatment. YB-1 and CLU are both stress-activated survival chaperones functionally associated with cancer treatment resistance (14). Under stress conditions, YB-1 is phosphoactivated by AKT (31) and p90RSK (32), stimulating its nuclear translocation and binding to target promoters. Our findings identify p90RSK as the predominant pathway increasing CLU expression after MDV3100 treatment, whereas YB-1 knockdown abrogates MDV3100-induced CLU.

We previously reported that YB-1 transcriptionally enhances CLU following treatment stress and CLU functions as a key mediator of paclitaxel resistance in prostate cancer (14). Together, these data suggest that MDV3100-induced CLU occurs via p90RSK and YB-1 signaling pathway.

This stress-activated antiapoptotic function for CLU can lead to resistance to many anticancer therapies. The CLU antisense inhibitor, OGX-011, enhances cancer cell death in combination with therapeutic stressors in many preclinical cancer models (15). Phase III trials evaluating combined docetaxel plus OGX-011 are underway in men with metastatic CRPC after randomized phase II studies reported a survival benefit when OGX-011 was added to docetaxel (15). Although CLU inhibition can enhance castration and delay time to CRPC in castrate-sensitive xenografts (24, 46), MDV3100 now enables investigation of AR pathway and CLU inhibition in CRPC models. In this study, we show that CLU is induced by MDV3100 and AR knockdown in MDV-sensitive and -resistant LNCaP cells, respectively, and that CLU remained highly expressed in most MDV3100-resistant xenografts and cell lines. Mechanistically, MDV3100-induced cross-talk activation of AKT and MAPK pathways was repressed when combined with OGX-011, and cotargeting AR and CLU synergistically enhanced apoptotic rates over monotherapy. Unexpectedly, we found that AR ubiquitination and proteasome-mediated degradation rates were accelerated when MDV3100 was combined with CLU knockdown. Stress activation of YB-1 and MAPK was blunted with combined therapy, resulting in decreased YB-1–regulated levels of FKBP52 (Hsp56), which led to AR ubiquitination, proteasomal degradation and decreased transcriptional activity beyond that observed with MDV3100 monotherapy.

These results highlight a role for CLU in supporting YB-1–mediated expression of other molecular chaperones under context-dependent stress conditions, analogous to CLU-enhanced HSF-1–mediated transactivation of Hsp70 and Hsp27 after Hsp90 inhibition (13). HSF-1 and YB-1 orchestrate expression of other molecular chaperones involved in processes of folding, trafficking, and transcriptional activation of the AR. In the absence of ligand, AR is predominately cytoplasmic, maintained in an inactive, but highly responsive state by a large dynamic heterocomplex composed of Hsp90 and Hsp70, and chaperones like FKBP52. These Hsp-AR chaperones support AR stability and activation (47, 48). Compared with the first-generation AR antagonist bicalutamide, which leads to AR dissociation from its Hsp heterocomplex and does not inhibit
AR nuclear transport, MDV3100-bound AR remains cytoplasmic and complexed with Hsp90, which may increase AR susceptibility to degradation under conditions of stress andCLU suppression. MDV3100-induced changes in AR acetylation and/or conformation may render it dependent to other co-factors in addition to FKBP52.

In summary, we define a stress-induced feed-forward loop involving MDV3100-induced Akt and p90rsk, phosphoactivation of YB-1, and increased expression of CLU. CLU knockdown abrogates MDV3100-induced activation of both Akt and p90rsk, defining another mechanism by which CLU inhibition potentiates anti-AR therapy. Hence, cotargeting adaptive stress pathways activated by AR pathway inhibitors, and mediated through CLU, potentiate anti-AR activity by decreasing AR expression levels and activity, as well as activation of cross-talk signaling pathways induced by MDV3100. These results provide mechanistic and preclinical proof-of-principle to support combinatorial clinical studies with MDV3100 and OGX-011.

Disclosure of Potential Conflicts of Interest
M.E. Gleave is employed as an advisor, has commercial research grant and ownership interest (including patents) in OncoGenex, and is a consultant/advisory board member of Teva and Astellas. No potential conflicts of interest were disclosed by the other authors.

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