A novel anti-androgen, Compound 30, suppresses castration-resistant and MDV3100-resistant prostate cancer growth in vitro and in vivo

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Abstract

Resistance to anti-androgen drugs, like MDV3100, occurs in patients with castration resistant prostate cancer (CRPC). Thus, preventing or treating anti-androgen resistance is a major clinical challenge. We identified a novel anti-androgen, Compound 30, and compared its efficacy to MDV3100. We found that Compound 30 inhibits AR activity in LNCaP cells, C4-2 cells, as well as MDV3100 resistant cell lines. Compared to MDV3100, Compound 30 treatment induces greater reduction in AR, PSA, and AR transcriptional activity, and prevents AR nuclear translocation, in AR-sensitive LNCaP cells. Compound 30 has anti-proliferative effects in LNCaP cells, in castrate-resistant C4-2 cells, and those resistant to MDV3100. Compound 30 was equally as effective as MDV3100 in reducing tumor volume and PSA in vivo. More importantly, Compound 30 is effective at inhibiting AR activity in MDV3100 resistant cell lines and significantly prevented tumor growth and PSA increases in mice bearing MDV3100 resistant xenografts. Together, our data show that Compound 30 strongly inhibited AR activity and suppressed castration-resistant LNCaP growth as well as MDV3100-resistant cell growth in vitro and in vivo. These data provide a pre-clinical proof-of-principle that Compound 30 could be a promising next generation anti-AR agent, especially in the context of anti-androgens resistant tumors.
Introduction

Prostate cancer (PCa) is the most common male cancer in North America and 2nd leading cause of cancer deaths (1). Androgens drive PCa carcinogenesis and progression, regulating gene and signaling networks that promote cell survival through binding with the androgen receptor (AR), a ligand-responsive transcription factor. Androgen deprivation therapy (ADT), consisting of castration combined with an anti-androgen, blocks the growth-promoting effects of androgens and activates apoptosis in PCa tumor cells (2), prolonging survival for PCa patients (3). Despite high initial response rates, remissions following ADT are temporary due to the emergence of castration-resistant prostate cancer (CRPC), where AR reactivation occurs and tumors grow in the presence of low levels of androgens. AR activation remains a central mechanism driving CRPC progression (4), involving variable combinations of AR gene amplification, increased AR sensitivity, promiscuous AR binding mutants, altered expression of co-regulators, ligand independent activation by oncogenic signaling pathways and increases in androgen biosynthesis (5-8). These mechanisms likely work in concert to drive CRPC, hence targeting the AR remains a critical component of novel CRPC therapies (9, 10).

ADT prevents AR activation by reducing levels of androgen production through castration or by targeting the AR directly using AR inhibitors. Limited efficacy of anti-androgens like bicalutamide has led to the development of more potent AR targeting compounds. MDV3100 is a second-generation AR inhibitor that has significant anti-tumor effects both in vitro and in vivo (11). A phase I/II trial in patients with metastatic CRPC showed MDV3100 treatment decreased serum PSA by 50% and reduced or stabilized metastatic disease (12). Furthermore, the phase III AFFIRM trial (13), testing MDV3100 in post-docetaxel CRPC patients, showed an overall survival advantage and a 37% reduction in mortality risk (14). Despite these results, MDV3100 treatment does not lead to complete CRPC regression, as progression and death from drug-resistant disease occurs in most patients (14). Therefore, the development of MDV3100 resistant tumors in CRPC represents a significant challenge in the treatment of advanced PCa. As such, identifying novel compounds with activity in MDV3100 resistant tumors is critical to improve the future of PCa therapy.

In this study, we tested the anti-cancer activity of the novel anti-androgen, Compound 30 (15), in in vitro and in vivo models of PCa. Compound 30 was developed by optimizing AR
ligand binding efficiency of aryloxy tetramethylecyclobutyl lead compounds identified in a high-throughput cell-based screen for pure, non-steroidal, AR antagonists (15). In that study, Compound 30 was shown to more potently inhibit AR activity than bicalutamide and had significant anti-tumor effects in a mouse model of CRPC (15). Likewise, we found that Compound 30 potently inhibited AR activity in LNCaP and C4-2 cells, and was as efficacious as MDV3100 in preventing tumor progression and PSA increases in a mouse model of CRPC. Importantly, using our newly developed model of MDV3100 resistance, we showed that Compound 30 has anti-proliferative and AR targeting effects in an MDV3100 resistant LNCaP cell line established from MDV3100 resistant tumors. Overall, our results suggest that Compound 30 may be a viable treatment for CRPC that is resistant to AR pathway inhibitors.
Materials and methods

Cell culture: AR positive LNCaP and C4-2 cells were provided by Dr. Leland W. K. Chung (Cedar Sinai, Los Angeles) tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2009. They were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS; Invitrogen Life Technologies). MR49F cells from MDV3100-resistant LNCaP xenografts were maintained in RPMI 1640 with 5% FBS and 10 μM MDV3100.

Reagents and antibodies: The synthetic androgen R1881 was from Perkin-Elmer (Boston, MA), Compound 30 was supplied by Pfizer Inc. (San Diego, CA), and customized synthetic MDV3100 was purchased from Shanghai Haoyuan Chemexpress (Shanghai, China). AR, PSA and cyclin D were purchased from (Santa Cruz Biotechnology, Santa Cruz, CA), PARP (Cell Signaling Technology), Vinculin (Sigma Chemical Co., St Louis, MO), and Ki67 (Lab Vision Corporation, Fremont, CA).

Cell growth assay: Cells were treated with various doses of compounds or DMSO for the indicated amount of time and growth rates were assessed with a crystal violet assay (described previously (16)). Each assay was done in triplicate.

Reverse transcription and quantitative PCR analysis. Total RNA was extracted from cultured cells after 48 hours of treatment using TRIzol reagent (Invitrogen Life Technologies, Inc.). qRT-PCR was performed as described previously (17).

Transient transfection and luciferase assay: LNCaP and MR49F cells were co-transfected with 0.6 μg of Probasin luciferase reporter and 0.6 μg of control Renilla luciferase plasmids per 10cm plate using Lipofectin (Invitrogen). Cells were treated with different compounds for one hour prior to treatment with 1 nM of R1881. Luciferase activity was measured as previously reported (18).

Immunoblotting analysis: Total proteins were extracted as previously reported using RIPA buffer (17). 30 μg of total proteins were separated by SDS-polyacrylamide gel electrophoresis.
(SDS-PAGE) and transferred to PVDF membranes. Western blots were performed as previously reported (17).

**Immunofluorescence:** LNCaP and MR49F cells were cultured in 12 well plates containing coverslips and RPMI+5% Charcoal Stripped Serum (CSS; Invitrogen Life Technologies) for 48 hours. Cells were then treated for 2 hours with indicated concentrations of compounds followed by 1 nM of R1881 treatment for 15 min. Immunofluorescence was performed as previously described (18).

**Cell cycle analysis:** Cells were incubated in the absence or the presence of different concentrations of MDV3100 or Component 30 and were stained with propidium iodide. Cell cycle population was analyzed by flow cytometry as previously reported (17).

**Assessment of in vivo tumor growth for castration-resistant LNCaP xenografts and MDV3100-resistant xenograft transplantation:** 1x10^6 LNCaP cells with 0.1 mL Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) were inoculated in bilateral flanks of 6–8 week-old male athymic nude mice (Harlan Sprague Dawley, Inc., Indianapolis, IN). Tumor volume, body weight and serum PSA levels were measured weekly. When serum PSA levels reached over 50 ng/mL, castration was performed. When PSA recovered to pre-castration levels, mice were randomized into 3 treatment groups; vehicle, 10 mg/kg of MDV3100 or 25 mg/kg of Compound 30 and treated daily.

**Generation of MDV3100 resistant tumors:** MDV3100-resistant LNCaP xenografts were excised and transplanted to castrated mice treated with 10mg/Kg/daily of MDV3100. When tumors reached 2,000 mm^3, xenografts were harvested and serially transplanted into castrated male mice receiving 10mg/Kg/daily of MDV3100. 3rd generation transplanted tumors were used as MDV3100-resistant xenografts in this study. When PSA levels reached over 50 ng/mL, mice were randomized into 3 groups; (1) stop MDV3100 dosing and switch to vehicle, (2) continuance of MDV3100, and (3) stop MDV3100 and switch to Compound 30.

**Generation of MDV3100-resistant MR49F cells:** 3rd generation MDV3100-resistant xenograft tumors were harvested into RPMI+10% FBS. Adherent cells were maintained in RPMI+5%FBS+10 µmol/L MDV3100.
**Immunohistochemistry:** A tissue microarray (TMA) was constructed using a manual tissue microarrayer (Beecher Instruments, Inc., Sun Prairie, WI). Immunohistochemical staining was performed as we previously reported. All comparisons of staining intensities were made at 200x magnification.

**Statistical analysis:** Data were analyzed statistically using a one-way ANOVA test, with $p<0.05$ considered to indicate significance. Student's t test (two-sided) was used to evaluate statistically significant differences in all experiments. Results are expressed as mean ± SE with at least three biological replicates. A $P$ value of $<0.05$ was considered significant.
Results

Compound 30 is more potent than MDV3100 in inhibiting AR transcriptional activity

The anti-androgen, Compound 30, is more potent than the first generation anti-androgen bicalutamide both in vitro and in vivo (15), however it has not been compared to new-generation anti-androgens. Here we explored the efficacy of Compound 30 (chemical structure is shown in Fig. 1A) compared to the new-generation anti-androgen, MDV3100, on AR pathway activity, proliferation and apoptosis in the PCa cell line LNCaP. We found that Compound 30 decreased AR transcriptional activity, measured by Probasin luciferase activity (Fig. 1B, top left panel), in a dose dependent manner and with greater potency compared to MDV3100 ([Probasin 50% effective concentration (EC50): 12.5 and 20 μmol/L, respectively] Supplemental Table 1). Similar data were observed using PSA luciferase (Fig. 1B, bottom left panel). Decreased AR activity correlated with a decrease of AR at both mRNA (Fig. 1B, right upper panel) and protein (Fig. 1C) levels, and these decreases were exaggerated in comparison to those induced by MDV3100, especially at the 10 μM dose. In accordance with our PSA luciferase data, we observed a dose dependent decrease in PSA protein expression in LNCaP cells treated with Compound 30 (Fig. 1C) and Compound 30 induced an approximate 70% decrease in PSA mRNA levels at 0.1μM, compared to an approximate 30% decrease induced by MDV3100 at the same dose (Fig. 1B right lower panel). Lastly, we found that like MDV3100, Compound 30 also inhibited AR nuclear translocation, as shown by fluorescence microscopy (Fig. 1D). Interestingly, we observed that while treatment of LNCaP cells with MDV3100 also reduced AR protein expression, AR mRNA levels were dramatically increased by treatment with 1 or 10 μM MDV3100. This may be a compensatory mechanism to upregulate AR protein production after MDV3100 treatment that was not observed with Compound 30.

Compound 30 potently inhibits cell proliferation and induces apoptosis

Compound 30 was able to inhibit AR activity more potently than MDV3100 as measured by decreases in PSA and AR protein and mRNA expression. We found that Compound 30 was significantly more effective than MDV3100 in suppressing cell growth (Fig. 2A) in AR-positive
LNCaP and its castration-resistant subline, C4-2 cells. More importantly, Compound 30 was equally as efficacious in suppressing growth in both cell lines ([IC₅₀ = 1.86 μmol/L for LNCaP and 2.02 μmol/L for C4-2]), while MDV3100 had a stronger anti-proliferative effect in androgen sensitive LNCaP cells compared to androgen resistant C4-2 cells ([IC₅₀ = 12.52 μmol/L for LNCaP and 34.90 μmol/L for C4-2]) (Fig. 2A, Supplemental Table 2). Reduction in cellular proliferation induced by MDV3100 and Compound 30 was associated with dose-dependent arrest in the sub-G0 phase of cell cycle as assessed by flow cytometry (Fig. 2B) and these data showed that at the 10μM dose, Compound 30 had a more substantial effect on cell cycle arrest than MDV3100. Furthermore, since MDV3100 has been shown to induce PARP cleavage in VCaP cells, we investigated effects of Cpd30 treatment on PARP cleavage in LNCaP cells compared to MDV3100. Importantly, only Compound 30 was able to induce PARP cleavage in LNCaP cells in a dose dependent fashion (Fig. 2C).

**Compound 30 elicits anti-cancer activity in a castration-resistant in vivo model**

Since Compound 30 showed potent anti-proliferative activity *in vitro*, we sought to test its efficacy compared to MDV3100 in an LNCaP CRPC model *in vivo*. LNCaP cells were xenografted into athymic male nude mice and mice were castrated when serum PSA reached ~50ng/ml. Treatment was initiated at the time when serum PSA reached pre-castration levels, which represents the hallmark of CRPC in the LNCaP model (19). Mice with CRPC tumors were randomized to three treatment groups: vehicle (8 mice), 10 mg/kg/day MDV3100 (13 mice) and 25 mg/kg/day Compound 30 (13 mice). As shown in Figure 3A, both compounds significantly suppressed tumor growth (left panel) and decreased serum PSA (right panel) compared to vehicle controls. After 5-weeks of treatment, there were no significant differences between Compound 30 and MDV3100 treated tumor volumes or PSA (change from baseline was +488% in vehicle, +19 % in MDV3100 and +7 % in Compound 30, and serum PSA change from baseline was +619% in vehicle, -32% in MDV3100 and -27% in Compound 30). Waterfall plots of individual responses in the MDV3100-treatment group showed 5 (38%) animals had reduced tumor volume from baseline (Fig B, left panel) and 9 (69%) animals had reduced PSA levels from baseline (Fig. 3B, right panel). In the Compound 30-treated group, 6 (46%) animals had reduced tumor volume from baseline (Fig 3B, left panel) and 10 (77%) mice had reduced PSA.
levels from baseline (Fig. 3B, right panel). Furthermore, staining for the cellular proliferation marker Ki67 in tumors from MDV3100 and Compound 30 treated mice showed similar reduction in Ki67 expression compared to vehicle control (Fig. 3D). However, because our *in vitro* data showed that Compound 30 had a greater effect on cell cycle repartition than MDV3100 (Figure 2C), we further investigated effects on cell cycle progression by analyzing the expression of cyclin D protein Cyclin D which is known as a G1 phase cyclins that regulate the entry of cells into G1, in Compound 30 and MDV3100 treated tumors. Consistent with our *in vitro* results, we observed lower Cyclin D protein expression in 2 out of 4 randomly selected Compound 30 treated tumors compared to those from MDV3100 treated mice (Fig. 3C).

**Compound 30 affects cell growth and AR transcriptional activity in vitro in MDV3100-resistant MR49F cells**

Our *in vivo* experiment confirmed that both MDV3100 and Compound 30 had potent anti-cancer activity in CRPC tumors. However, we also observed that tumors treated with MDV3100 for 8 weeks or longer eventually resumed growth, which we interpreted as the establishment of MDV3100 resistance. Resistance to anticancer therapies is a common phenomenon observed in patients, therefore we sought to develop a pre-clinical model of MDV3100-resistance. LNCaP-CRPC xenografts were treated with 10 mg/kg/day MDV3100 until tumor volume and PSA necessitated euthanasia and tumors were harvested and re-implanted in pre-castrated male nude mice subcutaneously (1st generation transplant). Mice were continuously treated with MDV3100 to ensure maintenance of the resistant phenotype. This procedure was repeated to obtain 2nd and 3rd transplant generations. 3rd generation transplanted MDV3100-resistant xenograft tumors were harvested and single cell suspensions grown in *in vitro* culture under the constant pressure of 10uM MDV3100 to generate MDV3100-resistant MR49F cells (Supplemental material, Fig. 1). Results from immunoblotting analyses showed that MR49F cells expressed both AR and PSA protein, and their expression was not affected by MDV3100 treatment (Supplemental material, Fig. 2).

As expected, MR49F cells showed a significant decrease in their response to MDV3100 treatment when compared to LNCaP parental cells, (IC50 = 12.52 μmol/L in LNCaP versus 33.89 μmol/L in MR49F) (Fig. 4A, left, Supplemental Table 3). In addition, MDV3100 was no
longer able to reduce PSA expression, measured by western blot (Fig 4C) or inhibit AR translocation from cytoplasm to nucleus upon androgen stimulation (Fig. 4D). Taken together, these results suggest that MR49F cells, which still express AR and PSA, resisted the effects of MDV3100 on AR activity.

Once MDV3100 resistant cells were established, we assessed the anti-cancer activity of Compound 30 on these cells. Importantly, although Compound 30 did not decrease AR protein expression in MDV3100-resistant tumors to the same extent as in LNCaP cells, reduction in AR expression was observed at higher treatment doses, and it profoundly reduced the expression of PSA in MR49F cells at as low a dose as 1µM (Fig 4C). In addition, Compound 30 suppressed AR transcriptional activity in MR49F cells with greater potency to that obtained from LNCaP parental cells (EC50 = 0.23µmol/L in MR49F and 2.0 µmol/L LNCaP cells) (Fig. 4B, Supplemental Tables 1 and 4) and, more importantly, it was effective in its anti-proliferative effects in MR49F cells (Fig. 4A right, Supplemental Table 5). Lastly, AR nuclear translocation after androgen stimulation was inhibited by Compound 30 (Fig. 4D). Together, these results indicated that in the context of MDV3100-resistance modeled in MR49F cells, Compound 30 retained its ability to function as a potent AR antagonist and cell growth inhibitor.

**Compound 30 has anti-cancer activity of in MDV3100-resistant tumors in vivo**

As described above, our *in vitro* studies indicated that Compound 30 was still effective in inhibiting AR activity in the context of MDV3100 resistance. Therefore, in order to test the efficacy of Compound 30 in suppressing MDV3100 tumor growth *in vivo*, we implanted 3rd generation MDV3100-resistant tumors into 34 mice under continuous treatment with 10mg/kg/day MDV3100. When tumors reached 300 mm³ or ~ 50ng/ml PSA, mice were randomly divided into 3 groups. At this time, MDV3100 treatment ceased, except for those animals assigned to MDV3100 treated group (11 animals). The other two groups received treatment with either vehicle (10 mice) or 25 mg/kg/day Compound 30 (13 mice) (Fig 5A). Consistent with the resistant nature of MR49F cells, we found that MDV3100-resistant tumors grew rapidly in the absence or presence of MDV3100, while those treated with Compound 30 showed significantly lower tumor volume and PSA. After 3 weeks of treatment, tumor volume change from baseline was 324% in vehicle-, 389% in MDV3100- and 138% Compound 30-
treated groups (Fig 5A, left panel) and serum PSA change from baseline was +708% in vehicle-, +1,013% in MDV3100- and +485%, in Compound 30-groups (Fig. 5A, right panel). Mice were euthanized when tumor volume exceeded 10% of their body weight and therefore at 3-weeks of treatment 1 mouse in vehicle-, 5 mice in MDV3100- and 2 mice in Compound 30-treated groups required euthanasia (Fig. 5B). Nevertheless, the group treated with Compound 30 survived significantly longer than vehicle and MDV3100 treatment groups (Fig. 5C). In addition, results from tumor IHC showed a marginal decrease Ki67 staining in Compound 30 treated tumors compared to vehicle and MDV3100-treated tumors (Fig. 5D) suggesting that Compound 30 had an impact on cell proliferation in vivo.
Discussion

Androgen ablation remains the most effective therapy for patients with advanced PCa. While most patients initially respond, progression to CRPC frequently occurs within 18 to 36 months. Blocking ligand-binding to the AR is a complimentary approach that has been used clinically for nearly three decades. While first generation anti-androgens, such as bicalutamide, do inhibit AR activity, they can induce mutated or overexpressed AR which limits their clinical application in CRPC, where AR variants and overexpression occur frequently (20). In fact, even after castration, over 80% of CRPC express AR and androgen-responsive genes (4, 21-23). Furthermore, it is well known that AR activity is a key driver of CRPC progression (24-26). Hence, identifying more effective AR pathway inhibitors in CRPC should enhance survival and slow disease progression.

Several new classes of AR pathway inhibitors are now in development and or approved for treatment of CRPC, including more potent AR antagonists (e.g., MDV3100) and inhibitors of intratumoral steroidogenesis (e.g., abiraterone) (13, 20, 27), as well as AR chaperone inhibitors (28-31). MDV3100 is a potent, second-generation AR antagonist that binds to the ligand binding domain of the AR with ~8-fold higher affinity than bicalutamide and impairs AR nuclear translocation and DNA binding (11). MDV3100 inhibits growth of CRPC xenografts and has efficacy in phase III clinical trials (32) and has been recently approved. However, despite its promising effects in some CRPC patients, resistance to MDV3100 eventually occurred. The heterogeneity of responses of CRPC patients to MDV3100 treatment and the eventual tumor progression, indicating MDV3100 resistance, suggest that AR targeting agents that are effective in MDV3100 resistant tumors will be required to treat CRPC.

In this study we report for the first time that a new AR antagonist, Compound 30 (15), exhibits potent AR inhibition not only in CRPC, but also in MDV3100-resistant LNCaP derived cells in vitro and in vivo. In LNCaP cells, Compound 30 suppressed AR transcriptional activity and PSA expression levels in a dose-dependent manner. Similar to MDV3100 (11), Compound 30 prevented AR translocation to the nucleus, highlighting an important level of control over AR activity. However, Compound 30 also enhanced the reduction in AR protein expression compared to MDV3100, providing an alternative mechanism for reducing AR activity. The more modest reduction in AR protein expression induced by MDV3100 may be hindered by...
compensatory synthesis of AR, as evidenced by dramatically increased AR mRNA expression after MDV3100 treatment. Interestingly, this was not observed after treatment with Compound 30, possibly indicating an advantage of this drug over MDV3100. One possible mechanism for the downregulation of both AR mRNA and protein levels in Cpd30 treated cells may be a feed forward loop, whereby reduced levels of AR protein expression and translocation to the nucleus inhibit AR dependent transcription factors in promoting the transcription and translation of AR.

Importantly, our in vivo studies pointed to an unmet application for the use of Compound 30 in the treatment of anti-AR resistant CRPC. When we compared the anti-tumor activity of Compound 30 and MDV3100 in mice bearing castration resistant xenograft tumors, we found both compounds significantly prevented increases in tumor volume and PSA. However, after long term treatment with MDV3100 or Compound 30 (≥ 8 weeks), even well-controlled tumors recurred and PSA recovered, suggesting that resistance to both anti-androgens occurs. Indeed, despite efficacy of MDV3100 and its imminent approval for the treatment of CRPC (32), most patients treated with MDV3100 will eventually recur, providing clinical evidence for anti-AR resistance. In light of our data showing tumor recurrence with MDV3100, we developed a model of MDV3100 resistance in vivo and in vitro and were able to generate LNCaP-derived MDV3100 resistant xenograft tumors and cell lines that still expressed AR and PSA. We used these resistant cells to test the efficacy of Compound 30. Importantly, although our in vivo studies showed similar anti-cancer activity between Compound 30 and MDV3100 in CRPC xenografts, our in vitro results showed significant anti-proliferative effects of Compound 30 in MDV3100 resistant cells. Accordingly, we found Compound 30 suppressed progression of MDV3100 resistant tumors in castrated mice and significantly prolonged survival in mice bearing MDV3100 resistant tumors. Furthermore, in vitro studies using the MR49F MDV3100 resistant cell line showed that Compound 30 effectively inhibited AR transcriptional activity and AR translocation to the nucleus, and potently suppressed cell proliferation.

Despite the positive effect of Compound 30 on MDV3100 resistant cells, we did observe indications of resistance to Compound 30 in both LNCaP and MR49F xenografts. This is not surprising, as the likelihood that a patient will become resistant to various treatments can be very high, depending on the therapy and type of cancer. Furthermore, we observed that in MDV3100 resistant cell lines, resistance to Compound 30 emerged 4-6 weeks after treatment. While our
data indicates that AR is still active, the emergence of tumors that are resistant to both Compound 30 and MDV3100 suggests that targeting AR with a drug against ligand binding domain provides only a short responding period before tumors become refractory to treatment. Possibly, targeting the AR with similar inhibitors, as in the case of MDV3100 and Compound 30, activates a resistance “memory” program, allowing cells to respond faster with survival mechanisms that were effective in protecting them from second line therapy. However, given the maintained anti-cancer effects on MDV3100 resistant cells \textit{in vitro} and significant increase in survival \textit{in vivo} in Compound 30 treated mice with MDV3100 resistant tumors, Compound 30 may still be a viable alternative treatment offered to patients who fail MDV3100 therapy. In addition, our results do not exclude the possibility that Compound 30 may be more effective in some CRPC patients as a second-line hormone therapy, or improve the efficacy of MDV3100 if used in combination with this drug. Future studies investigating the sequential and combinatorial use of these drugs are necessary.

In summary, our results suggest that Compound 30 is able to inhibit the continued AR activity that is associated with anti-AR resistance and may be a viable treatment strategy for MDV3100 resistant tumors \textit{in vivo}. Furthermore, our data provide a pre-clinical proof of principle that novel anti-androgens, while they may have similar efficacy against CRPC as other drugs, may be developed as an alternative in patients resistant to MDV3100.

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References
Figure legends

**Figure 1:** Compound 30 inhibits activity and expression of the AR. **A:** Chemical structure of Compound 30. **B (left)** 24h post-transfection LNCaP cells transfected with Probasin (left top) or PSA (left bottom) luciferase and control renilla plasmids were treated for 24h with various concentrations of Compound 30 or MDV3100. Cells were harvested and luciferase activity was determined. Data represent means of at least three independent experiments done in triplicate. **(B right)** LNCaP cells were treated with Compound 30 or MDV3100 for 24h and RNA was extracted and qRT-PCR was performed to evaluate the expression of AR (right top) and PSA (right bottom). **C** LNCaP cells were treated with Compound 30 or MDV3100 for 24h, AR and PSA were analyzed by western blot, vinculin was used as a loading control. **D** LNCaP cells were maintained in androgen deprived conditions for 24h and treated with 10 μM Compound 30 or MDV3100 for 2h followed by addition of 1 nM of R1881. After 15 minutes incubation, cells were fixed and AR localization was assessed by immunofluorescence imaging.

**Figure 2:** Compound 30 inhibits cell growth and induces apoptosis. **A**) LNCaP (left panel) and C4-2 cells (right panel) were treated with Compound 30 or MDV3100 for 72h and cell growth was evaluated using crystal violet. **B** LNCaP cells were treated with of Compound 30 or MDV3100 for 72h. Cells were stained with propidium iodide and cell cycle populations were analyzed by flow cytometry. **C** LNCaP cells were treated with Compound 30 or MDV3100 for 72h and total proteins were analyzed for the expression of cleaved PARP; vinculin was used as a loading control.

**Figure 3:** Compound 30 prevents in vivo tumor growth and PSA increases in castration-resistant LNCaP xenografts. LNCaP cells were xenografted into athymic male nude mice, followed by castration when serum PSA reached 50 ng/ml. Treatments were initiated at the time when serum PSA reached pre-castration levels. Mice with CRPC tumors were randomized into three groups receiving vehicle control (8 mice), 10mg/kg MDV3100 daily (13 mice) or 25mg/kg Compound 30 daily (13 mice). **A** Mean tumor volume (right) and circulating PSA (left) for each treatment group are reported. **B** Waterfall plots represent percent change in tumor volume (left panel) and PSA (right panel) after 5 weeks of treatment for individual mice in each treatment
group. C) Total proteins were extracted from tumors from 4 randomly mice and analyzed for the expression of Cyclin D; vinculin was used as a loading control. D) Tumors were collected at the end of the treatment and Ki67 positivity was evaluated by Immunohistochemistry (IHC).

**Figure 4: Compound 30 affects cell growth and AR transcriptional activity in vitro in MDV3100-resistant MR49F cells.** A) LNCaP and MDV3100 resistant cells (MR49F) were treated with MDV3100 (Left panel) or compound 30 (Right panel) for 72h and cell growth was evaluated using crystal violet (left panel). B) 24 hours post-transfection, MR49F cells transfected with Probasin luciferase and control renilla plasmids, were treated for 24h with Compound 30 or MDV3100. Cells were harvested and luciferase activity was determined. Data represent mean of at least three independent experiments done in triplicate. C) MR49F cells were treated with Compound 30 or MDV3100 for 24h. Total proteins were extracted analyzed for the expression of AR and PSA; vinculin was used as a loading control. D) MR49F cells were maintained in androgen deprived conditions for 24h and treated with 10 μM Compound 30 or MDV3100 for 2h followed by addition of 1 nmol/L of R1881. After 15 minutes incubation, cells were fixed and AR localization was assessed by immunofluorescence.

**Figure 5: Compound 30 has anti-cancer activity of in MDV3100-resistant tumors in vivo.** A) MDV3100 resistant tumors were transplanted to castrated mice and maintained under MDV3100 pressure (10mg/Kg). When PSA reached 50-75 ng/ml, mice were randomly divided into three groups and treated with vehicle (10 mice), 10 mg/kg/day MDV3100 (11 mice), and 25 mg/kg/day Compound 30 (13 mice). A) Mean tumor volume (right) and circulating PSA (left) for each treatment group are reported. B) Waterfall plots represent percent change in tumor volume (left panel) and PSA (right panel) after 3 weeks of treatment for individual mice in each treatment group. C) Cancer-specific survival was compared between the 3 groups over the period of the treatment and is represented by a Kaplan-Meier curve. D) Tumors were collected at the end of the treatment and Ki67 positivity was evaluated between different groups by immunohistochemistry.
Figure 1:

A

Compound 30

B

Probasin reporter assay

PSA reporter assay

C

Cpd 30 MDV3100

D

R1881

MDV3100

Cpd 30
Figure 2:

A

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\begin{tabular}{c}
\textbf{LNCaP} \\
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\textbf{Cpd 30} \\
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\textbf{C4-2} \\
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B

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\textbf{Cell cycle} \\
\textbf{Sub G0/G1 G1 G1 S G2/M} \\
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\textbf{1 μmol/L} \\
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\textbf{10 μmol/L} \\
\textbf{MDV3100} \\
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C

\begin{center}
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\textbf{cPARP} \\
\textbf{vinculin} \\
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Figure 3:

A

Tumor Volume

Serum PSA

B

Change in Tumor Volume-5 Weeks

Change in PSA-5 Weeks

C

vehicle MDV3100 Cpd 30

Cyclin D1

D

vehicle MDV3100 Cpd 30

Ki 67
Figure 4:

A. MDV3100 IC50 in LNCap and MR49F cells

B. Probasin reporter assay

C. MDV3100 vs. Compound 30 IC50 in 49F Cells

D. Table:

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D1881

AR

DAPI

merge
Figure 5:

A

Tumor Volume

Vehicle

MDV3100

Cpd 30

Serum PSA

Vehicle

MDV3100

Cpd 30

B

Change in Tumor Volume-3 Weeks

% Volume change

Vehicle

MDV3100

Cpd 30

Change in PSA-3 Weeks

% PSA change

Vehicle

MDV3100

Cpd 30

C

Survival

Percent survival

Vehicle

MDV3100

Cpd 30

Weeks of treatment

log rank test:
Vehicle vs. Cpd 30, p=0.0088,
MDV3100 vs. Cpd 30, p=0.036
A novel anti-androgen, Compound 30, suppresses castration-resistant and MDV3100-resistant prostate cancer growth in vitro and in vivo

Hidetoshi Kuruma, Hiroaki Matsumoto, Masaki Shiota, et al.

*Mol Cancer Ther* Published OnlineFirst March 14, 2013.

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