Differential Androgen Deprivation Therapies with Anti-Androgens of Casodex or MDV3100 vs. Anti-Androgen Receptor of ASC-J9® Lead to Promote vs. Suppress Prostate Cancer Metastasis

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Short title: Anti-androgens promote prostate cancer metastasis

Key word: anti-androgens, prostate cancer, metastasis, MMP9, TGF-β1

Abbreviation: androgen deprivation therapy (ADT), prostate cancer (PCa), androgen receptor (AR), cryptotanshinone (CTS).
**Background:** Androgen Deprivation Therapy (ADT) suppresses prostate cancer (PCa) growth yet its effects on PCa metastasis remain unclear.

**Result:** ADT with Enzalutamide/MDV3100 or Bicalutamide/Casodex) vs. ASC-J9® or cryptotanshinone lead to enhance vs. suppress PCa metastasis.

**Conclusion:** Casodex/MDV3100 induces PCa metastasis via modulation TGF-β1/Smad3/MMP9 signaling.

**Significance:** Targeting androgen receptor with ASC-J9® or cryptotanshinone is better than targeting androgens with Casodex/MDV3100 to battle PCa metastasis.

**Abstract**

Despite the fact that androgen deprivation therapy (ADT) can effectively reduce prostate cancer (PCa) size, its effect on PCa metastasis remains unclear. We examined the existing data of PCa patients treated with ADT with anti-androgens to analyze therapy effects on primary tumors size, prostate specific antigen (PSA) values, and metastatic incidence. We found the current ADT with anti-androgens might lead to primary tumors reduction with PSA decreased yet metastases increased in some PCa patients. Using in vitro and in vivo metastasis models with human 4 PCa cell lines, we evaluated the effects of the currently used anti-androgens, Casodex and MDV3100, and the newly developed anti-AR compounds, ASC-J9® and cryptotanshinone (CTS), on PCa cell growth and invasion. In vitro results showing that 10 µM Casodex or MDV3100 treatments suppressed PCa cell growth and reduce PSA level, yet significantly enhanced PCa cells invasion. In vivo mice studies using orthotopic xenograft mouse model also confirmed these results. In contrast, ASC-J9® led to suppress PCa cell growth and cell invasion in in vitro and in vivo models. Mechanism dissection indicated these Casodex/MDV3100 treatments enhanced the TGF-β1/Smad3/MMP9 pathway, but ASC-J9® and CTS showed promising anti-invasion effects via down-regulation of MMP9 expression. These findings suggest the potential risks of using anti-androgens and provide a potential new therapy using ASC-J9® to battle PCa metastasis at the castration resistant stage.

**Introduction**

The recurrence of prostate cancer (PCa) with metastases after androgen deprivation therapy (ADT) is still a major concern (1). The newly developed anti-androgens were able to delay the recurrence (2,3). Importantly, this unwanted effect was more significant when ADT was applied to
patients in earlier stage of PCa, which was correlated with a 10 year decreased survival rate (4). These unexpected findings were in agreement with early reports showing androgen receptor (AR) might play differential roles (proliferator or suppressor) in different types of PCa cells and the loss of AR in cytokeratin 5-positive PCa epithelial basal intermediate cells might lead to enhanced PCa metastasis (5,6).

In this study, currently used antiandrogen of Casodex (Biclutamide) (7) and the newly developed anti-androgen of MDV3100 (Enzalutamide) (3) were used to evaluate their effects on PCa metastasis in various in vitro cell lines experiments and in vivo mice studies. The results showed these anti-androgens could enhance PCa cells invasion through modulation of the TGF-β1/Smad3/MMP9 pathway. In contrast, we found that the newly developed AR degradation enhancers, ASC-J9® (8-11) and cryptotanshinone (CTS) (12), could simultaneously suppress PCa cell growth and invasion, which might help us to develop a new therapy to better battle the metastatic PCa at castration resistant stage.

Materials and Methods

**Human patients’ data analysis**

Patients’ information was collected from the China Medical Hospital, Taiwan; the Tianjin Medical University, China; the First Affiliated Hospital of Medical School, Xi’an Jiaotong University, China; and the University Hospital in University of Occupational and Environmental Health, Japan. The samples of PCa patients before ADT were collected by Transrectal Ultrasonography of the Prostate (TRUS) guided prostate biopsy. After ADT part of the specimen were collected by palliative Transurethral Resection of the Prostate (TURP) for relieve the retention of urine. Part of samples were collected by confirmed the organ metastasis under the agreement of patients. Patient inclusion criteria: all the patients are locally advanced or metastasis PCa who were undergone by ADT therapy. The patients received the ADT combination of Luteinizing hormone-releasing hormone agonist (LHRHa) with Casodex (CASO) or Flutamide. The metastatic lesions were monitored before and after ADT. Bone scans and MRIs were used to examine metastasis lesions. The disease progression status was determined by the PSA level, primary tumor sizes, and metastatic foci.

**Cell Culture**

LNCaP, C81, C4-2, C4-2B, and CWR22Rv1 cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin), and 2 mM Glutamine (Life technology) in 5% CO₂ and 37ºC incubator.
Cell Growth Assay

The cells were seeded in 24-well tissue culture plates in RPMI medium containing 10% charcoal dextran treated FBS (CD-FBS) for 24 hr. The cells were then treated with vehicle, 10 µM Casodex, 10 µM MDV3100, 10 µM ASC-J9®, or 5 µM of CTS with/without the addition of 5 µM LY294002. The cell growth was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). The media containing MTT (0.5 µg/ml) were added into each well at indicated time points. After 2 hrs incubation at 37°C all crystals were solubilized by DMSO and the optical density of the solution was determined spectrophotometrically at 570 nm.

Cell Invasion Assay.

PCa cells were treated with anti-androgen/AR drugs and incubated for 3 days. For inhibitor studies, the appropriate inhibitors were added into the culture. Cells (1 x 10⁵) were then placed into the upper chamber of transwell plates (8 µm) with membranes pre-coated with 20% Matrigel. Each sample was assayed in triplicate. The bottom chamber contained 600 µl media supplemented with 10% fetal bovine serum (FBS). The cells invaded into the bottom were fixed, stained using 1% toluidine blue, and the numbers averaged after counting 6 randomly selected fields. Each experiment was repeated at least twice.

Orthotopic Xenograft Model.

Male 6-8 wks old nude mice were purchased from NCI. The CWR22Rv1 cells incorporated with the luciferase reporter gene were obtained by transfection and stable clone selection procedures. Cells (1 x 10⁶) mixed with matrigel (1:1, v/v) were orthotopically injected into both anterior prostates of nude mice at 8 weeks of age. When the tumors were palpable two wks after implantation, the mice were randomly assigned into 5 experimental groups and i.p. injected with drugs as follows 3 times per week for 4 weeks: Group 1 (n=20), vehicle; Group 2 (n=13), 30 mg/Kg Casodex, Group 3 (n=12), 30 mg/Kg MDV3100; Group 4 (n=12), 75 mg/Kg ASC-J9®; and Group 5 (n=12), 25 mg/Kg CTS. The mouse body weights were monitored weekly during the 4 wks of treatment. After sacrificed, the primary and metastatic tumors were evaluated by the in vivo imaging system (IVIS) and tumor tissues were removed for IHC staining.

Statistics

Data are presented as the means ± SDs for the indicated number of separate experiments. The statistical significance of differences between two groups of data was analyzed by paired t-test or fisher’s exact test and p-values <0.05 were considered significant. In the in vivo animal experiments, measurements of tumor volume and body weights among the
three groups were analyzed through one-way ANOVA (analysis of variance) coupled with the Newman-Keuls test.

Additional details of reagents, 3D invasion assay, Western blot, quantification PCR, immunohistochemistry, X-ray and CT scan, and the detection of luciferase signal by IVIS system are described in Supplementary information.

Results
ADT Reduced PCa Patients’ Primary Tumor sizes with Decreased PSA levels, yet Increased Metastasis in Some Selective Patients

We examined the available PCa patients’ information from 4 different hospitals in China, Taiwan, and Japan and found interesting results. We observed 11 patient cases showing reduced primary tumor size with low PSA reading, yet increased metastases following the ADT treatment. For example, in case of Patient 6 in Table 1, when treated with the ADT and anti-androgen (LHRH agonist plus Casodex for 10 months), there was significantly reduced primary tumor size and PSA reading (from 51 to less than 0.5 ng/ml), but metastasis unexpectedly increased despite the very low PSA reading. These clinical data from some selective PCa patients described in Table 1 raised an interesting question and the possibility that even though the current ADT with anti-androgen could reduce some selective PCa patients’ primary tumor growth and decrease their PSA reading, but might either have little effect to prevent PCa metastases or even worse, might promote PCa metastases in some selective PCa patients.

Anti-androgens Casodex and MDV3100 Suppressed PCa Growth yet Enhanced PCa cells Invasion

The above unexpected clinical findings in some selective PCa patients prompted us to apply in vitro growth and invasion assays using 4 different human PCa cell lines, LNCaP (represent the stage before castration resistance), and C81, C4-2, and CWR22Rv1 (represent the stage after castration resistance) to study the potential distinct ADT effects on PCa growth vs. metastasis. We applied Casodex, the current most widely used anti-androgen (13) and MDV3100, a newly developed powerful anti-androgen that could delay the recurrence of increased PSA with extended PCa patients survival rate to 4.8 months (3), to test its effect on PCa cell growth and the MTT test results showed that 10 µM Casodex and MDV3100 significantly suppressed growth of 3 PCa cell lines, but not the CWR22Rv1 cells (Fig. 1A). However, surprisingly, we found 10 µM Casodex or 10 µM MDV3100...
promoted cells invasion of all 4 PCa cell lines tested (Fig. 1B).

To confirm these anti-androgens effects on enhanced PCa cells invasion, we performed another invasion assay with the 3D matrigel/collagen based invasion assay (14) that measures the formation of spheres with protrusions as positive indications of cells invasion. As expected, addition of 10 µM of Casodex or MDV3100 led to enhance cells invasion of the C4-2B (Fig. 1C, left) and CWR22Rv1 cells (Fig. 1C, right). Together, results from Fig. 1 demonstrated that both Casodex and MDV3100 suppressed PCa growth, yet promoted PCa cells invasion.

Combinational Therapy of Anti-Androgens with Anti-Akt Led to Better Suppress PCa Cells Growth yet Failed to Rescue PCa Invasion

Early studies demonstrated that suppression of androgen/AR signaling might enhance the Akt phosphorylation by reducing the Akt phosphatase, PHLPP (15,16). These findings suggested the combinational therapy of anti-androgens with anti-Akt might result in better efficacy to suppress PCa growth. We first examined the combinational therapy effect of using PI3K-Akt inhibitor LY294002 (5 µM) with 10 µM Casodex or MDV3100 on C4-2 and CWR22Rv1 cells growth. As expected, we found the combinational treatment further suppressed cells growth (Fig. 2A). Unfortunately, the combinational therapy still failed to prevent or reverse the cell invasion induced by anti-androgens treatment (Fig. 2B). Western blot analysis also showed that the MMP-9 expression, the cell invasion marker (17), was increased after treatment with either anti-androgens alone or combined with PI3K-Akt inhibitor LY294002 in both CWR22Rv1 and C4-2 cells (Fig. 2C), even when very low endogenous level of phosphorylated Akt was detected in CWR22Rv1 and not C4-2 cells, which could be due to the PTEN deletion in C4-2 cells and not in CWR22Rv1 cells.

Together, results from Fig. 2 demonstrated that the combinational therapy of anti-androgens and anti-Akt might further suppress PCa growth yet still fail to prevent or reverse the cell invasion induced by anti-androgens.

Anti-Androgens-Induced PCa Invasion Involved the Activation of TGF-β1/Smad3 Signaling and Enhanced MMP9 Expression

Early reports suggested that TGF-β1 signaling might have dual roles, as a suppressor of tumor growth and an enhancer for tumor metastasis at later stages (18). A recent study showed that activated AR may bind to the TGF-β1 promoter region to suppress the gene expression (19), and androgen deprivation may lead to increased expression of TGF-β1 ligand and its
downstream gene expressions, such as the TGF-β1 receptors and Smad3 (19-23). We investigated whether anti-androgens induce PCa cells invasion through TGF-β1 signaling and found addition of 10 µM Casodex or MDV3100 increased the mRNA expressions of TGF-β1, Smad3, and MMP9 in C4-2 and CWR22Rv1 cells (Fig. 3A). We then examined whether the anti-androgens-induced PCa cells invasion could be altered after adding TGF-β1 receptor kinase specific inhibitor, SB431542 (24) or the Smad3 inhibitor, Naringenin (25). As expected, we found that treatment with 5 µM SB431542 or 50 µM Naringenin for 3 days led to significant suppression of the C4-2 and CWR22Rv1 cells invasion induced by either Casodex or MDV3100 (Fig. 3B). In addition, expressions of pSmad3 and MMP9, the invasion markers, were also markedly reduced by SB431542 or Naringenin treatment (Fig. 3C) These results were similar to the previous study in breast cancer showing the activated Smad3 could directly bind to the Smad response element on the MMP9 promoter to increase the MMP9 expression (17). Then, MMP9 specific inhibitor (26) was used to further prove that anti-androgens induced PCa cells invasion is through the TGF-β1/Smad3/MMP-9 pathway. The results showed that inhibition of MMP9 activity by MMP9 inhibitor interrupted C4-2 and CWR22Rv1 cells invasion (Fig. 3D). Finally, direct treatment of CWR22Rv1 cells with 5ng/ml recombinant TGF-β1 protein also increased the cell invasion (Fig. 3E).

Together, results from Fig. 3 suggested that anti-androgens-induced PCa cell invasion might go through AR→TGF-β1/Smad3/MMP-9 pathway and interruption of this pathway via either SB431542, Naringenin, or MMP9 inhibitor all suppressed these anti-androgens induced PCa cell invasion.

Anti-AR of ASC-J9® and CTS Suppressed both PCa Growth and Invasion in the PCa Cells

All above results in Fig. 1-3 concluded that the currently used anti-androgens, such as Casodex or MDV3100 might suppress PCa cells growth yet promote their invasion. We investigated the possibility that any newly developed anti-androgen/AR compounds have both anti-PCa cells growth and invasion capacity so that they can simultaneously suppress PCa growth and metastasis. We first focused on two anti-AR compounds, the AR degradation enhancer ASC-J9® (8,11,27), and CTS (12), the tanshinone extracted from the Chinese herbal Danshen. Unlike the currently used anti-androgens that reduce or prevent androgens binding to AR, the newly developed anti-AR compound, ASC-J9®, has the unique capability to degrade AR protein in selective cells. On the other hand, CTS also could suppress the AR activity by
inhibition of N terminal and C terminal interaction, or change the histone methylation pattern to reduce the AR transcriptional activity (12,28).

We found 10 µM ASC-J9® and 5 µM CTS could suppress the PCa growth (Fig. 4A) and AR down-stream target gene PSA expression (Fig. 4B) in all 4 PCa cell lines tested. Importantly, Casodex and MDV3100 induced PCa cells invasion, both ASC-J9® and CTS significantly suppressed the PCa cells invasion in chamber invasion assay (Fig. 4C) or in 3D matrigel/collagen based invasion assay (Fig. 4D). In addition, while Casodex and MDV3100 enhanced MMP9 expression (Fig. 4E), both ASC-J9® and CTS showed suppression of MMP9 expression in C4-2 and CWR22Rv1 cells (Fig. 4E). Furthermore, treatments with Casodex and MDV3100 led to increased expression of the Smad3 and p-Smad3 levels in C4-2 and CWR22Rv1 cells, whereas ASC-J9® and CTS treatment did not (Fig. 4E).

Together, results from Fig. 4 suggest that Casodex, MDV3100, ASC-J9® and CTS can promote or suppress PCa cells invasion via differential regulation of AR/TGF-β1/Smad3/MMP-9 pathway.

ASC-J9® and CTS, but not Casodex or MDV3100, Suppressed PCa Metastasis in in vivo Mouse Models

To further prove the opposite effects of these 4 anti-androgen/AR compounds using the in vivo mouse model, we exploited the CWR22Rv1 orthotopic xenografted mouse model. For monitoring metastases, the CWR22Rv1 cells were transfected with firefly luciferase reporter gene and the stable clone CWR22Rv1 (luc-CWR22Rv1) was selected, expanded, and used for the injection. When the tumors were palpable two wks after implantation, the mice were randomly assigned into 5 experimental groups and i.p. injected with drugs as follows 3 times per week for 4 wks: Group 1 (n=20), vehicle; Group 2 (n=13), Casodex, Group 3 (n=12), MDV3100; Group 4 (n=12), ASC-J9; and Group 5 (n=12), CTS. The metastasis lesions were evaluated by detection of the luciferase signals (Fig. 5A, upper panel) using IVIS system, and further confirmed by tissue sections with HE staining. Most of the metastatic tumors were found in the lumbar and mesenteric lymph nodes, while some were located in diaphragms or bones. We found 69.2% of Casodex and 67.7% of MDV3100 treated mice had significant increase in the metastatic tumors as compared to 25% of vehicle injected control mice. In contrast, the ASC-J9® (8.3%) and CTS (0%) treated mice had few or no metastatic tumor detected (Fig. 5A, lower panel). Similar results were also obtained when the metastatic foci numbers were quantified (Fig. 5B).

Osteoblastic bone metastasis may represent the majority of PCa metastases...
seen in clinics. Interestingly, the luciferase signals were detected in 2 mice treated with anti-androgens (1/13 in Casodex group and 1/12 in MDV3100 group). Using X-ray and CT-scan to examine luciferase signal in mice feet we found the bone density near the luciferase signals were increased (a typical lesion of bone metastasis in clinical PCa diseases). Meanwhile, the CT-scan also showed bone mass formation (Fig. 5C). The results suggested that our current in vivo mouse model could mimic the clinical conditions. Importantly, we found little change in mice body weights among all the mice treated with different anti-androgens (Fig. 5D), which is consistent with previous reports (19-22).

Expressions of the molecules in the TGF-β1/Smad3/MMP-9 pathway in tumor tissues of each mouse group were then evaluated by the immunohistochemistry staining. Consistent with the in vitro findings, we found expressions of TGF-β1, pSmad3, and MMP9 were higher in Casodex or MDV3100-treated tumor samples, but lower in ASC-J9® and CTS treated tumor samples when compared with the tumors in the vehicle control mice (Fig. 5E).

Together, results from in vivo mouse model studies all proved the above in vitro cell lines studies and demonstrated that Casodex and MDV3100 treatment might promote PCa metastasis, yet ASC-J9® and CTS treatment could suppress PCa metastasis, and these differential effects involved the opposite regulation of the TGF-β1/Smad3/MMP-9 pathway.

Discussion

Despite the tumor regression after ADT in advanced PCa patients, its beneficial effects on cancer specific survival is still in debate (1,4). Earlier reports showed that the PSA level may not reflect the pathological stage progression in some PCa patients, whose PSA level were below 0.5 ng/ml with nodal metastasis after ADT (29,30). Meanwhile, a recent clinical trial of abiraterone, a powerful drug that suppressed androgen biosynthesis, also found that while 79% of PCa patients have a decline in PSA level of 50% or more, 52% of PCa patients have either increased new bone lesions or intensity of existing bone lesions, which they called “bone scan flare” after 4 months of treatment, and reduced the intensity again after 7 months of treatment (31). It is interesting to note that the increase of metastasis in these PCa patients was observed at the stage when PSA level was dropped to significantly low, which might be opposite to the general concept that PSA rise during ADT is the first sign before PCa progresses to enhanced metastasis, and may suggest anti-androgen/AR signaling in PCa proliferation vs metastasis could be 2 different pathways. We found that some PCa patients who received ADT had increased metastases
even though their PSA levels dropped to less than 4 ng/ml. Therefore, continual monitoring of the new metastatic lesions formation during the ADT in PCa patients may be essential, even when the primary tumor size and the PSA levels are under control. Other studies showing the increased expression of aggressive markers after ADT in PCa patients, such as N-cadherin (32), Cadherin-11 (33), and Nestin (34) supported the similar conclusion that ADT might promote PCa metastases, and TRAMP mice studies also demonstrated the increased PCa metastasis at stage when mice serum testosterone is undetectable after ADT with castration (35,36).

Similar observation showing suppressed cancer cells growth yet promoted cancer cells invasion upon anti-angiogenesis therapy was reported. In clinical trial of melanoma treatment, anti-angiogenesis therapy led to suppress tumor cell growth through reduced blood vessel formation, yet it could simultaneously also promote the cancer metastasis (37). Nevertheless, other study also found the primary ADT in localized PCa patients might lead to lower the 10-year PCa specific survival (4), suggesting further studies may be required to determine what kind of ADT at which stages may be best for patients without leading to poor prognosis in patients.

We noticed that while both Casodex and MDV3100 have significant anti-growth effects in LNCaP, C81 and C4-2 cells, both anti-androgen drugs showed less effect on CWR22Rv1 cell growth, which could be due to the existence of the AR variant (27,38). Recent reports documented that Casodex failed to suppress AR transactivation and this is probably due to lack of androgen-binding domain (27,38,39). Moreover, Hu et al also found in both in vitro and in vivo studies that MDV3100 or abiraterone treatments increased AR3 expression, which might then enhance cell cycle related genes to promote PCa progression (39). In contrast, ASC-J9® could degrade both full length AR and AR variant AR3 (27) provided another explanation why ASC-J9® showed better therapeutic effects to battle both PCa cells growth and metastasis.

Recent reports of LNCaP cells studies suggested that the ADT promoted Akt signaling might explain the PCa cells survival under ADT condition (40,41), and therefore, the ADT combined with anti-Akt could lead to better efficacy to suppress PCa growth (Fig. 2A). However, this therapy still failed to interrupt the PCa cell invasion induced by these anti-androgens treatments, suggesting that Akt signaling might play differential roles in different types of PCa cells that might lead to differential effects on cells growth vs. metastasis (42). Our finding that TGF-β1/Smad3 signaling was activated during ADT to enhance the cell invasion through activation of MMP9 pathway might provide
another new potential therapeutic approach that combines ADT with inhibitor of TGF-β1/Smad3/MMP9 signaling to suppress both PCa cell growth and metastasis.

Early studies found ASC-J9 could target the interaction of AR with selective co-regulators, such as ARA55 and ARA70, which resulted in degradation of the AR protein through the ubiquitination pathway (8-11). The pre-clinical toxicity tests on mice also showed little toxicity with little influence of serum testosterone, and mice still have sexual activity and fertility (8-11). Since AR co-regulators can determine specific AR targets in different types of cells (43), regulation of AR co-regulators interaction may also change the gene expression profile regarding the metastasis related genes. A previous study showed CTS can reverse the demethylation process mediated by LSD1, and inhibit AR transactivation at the epigenetic level (28). It would be interesting to identify the potential regulation of PCa metastasis abilities via epigenetic markers.

In conclusion, the current studies point out that suppression of androgens binding to AR using Casodex and MDV3100 in PCa might have the potential risk to increase cancer metastasis in some PCa patients. A novel therapy via targeting AR and its downstream signaling of TGF-β1/Smad3/MMP-9 may be developed to better battle metastatic PCa at the castration resistant stage.

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Conflict of Interest
ASC-J9 was patented by the University of Rochester, University of North Carolina, and AndroScience, and then licensed to AndroScience. Both the University of Rochester and C.C. own royalties and equity in AndroScience.
References:


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**Table and Figure legends**

**Table 1 ADT Reduced PCa Patients’ Primary Tumors with Decreased PSA, yet Increased Metastasis.** Patients’ information was collected from 4 different hospitals in Asia countries as described in Material and Methods section. The metastatic foci were shown as the size of largest meta-tumor x the number of meta-foci.

**Fig. 1 ADT with Anti-Androgens Suppressed PCa Cell Growth but Enhanced Cell Invasion. A.** The effect of anti-androgens on PCa cell growth. The PCa cells (LNCap, C81, C4-2, and
CWR22Rv1) were treated with anti-androgens 10 µM Casodex (CASO), 10 µM MDV3100 (MDV), or vehicle control, and incubated in 10% CD-FBS RPMI medium at 10 nM DHT, and the cell growth was analyzed by MTT assay at indicated time points. **B. The effect of anti-androgens on PCa cell invasion.** The cell lines were treated with 10 µM CASO, 10 µM MDV or vehicle control and incubated in 10% CD-FBS RPMI medium at 10 nM DHT for 3 days. Invasion assays were then performed using matrigel pre-coated transwell plates for 48 hrs. The invaded cell numbers were shown as fold change after counting 6 randomly selected fields (400X). **C. The 3D invasion assay in C4-2B (middle) and CWR22Rv1 (right) cells.** As illustrated at top, the matrigel/collagen mixture coated the bottom of the 48-well plates. After incubation at 37°C for 1 hr, 1 x 10⁴ PCa cells were plated onto the plate in media containing 1% matrigel. The media were replaced 24 hrs later and the anti-androgens or vehicle control were added. After 2 wks, the spheres with/without protrusion (upper panel) were counted and shown as percentage (%) compared to the control (bottom panel). All the experiments were repeated at least twice and each experiment was triplicated. All the experiments were repeated at least twice and each experiment was triplicated. *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 2 Inhibition of Akt Activity Failed to Reverse the Anti-androgens-Induced PCa Cells Invasion and MMP9 Expression.** **A. Anti-androgen plus Anti-Akt further inhibited PCa cells growth.** C4-2 and CWR22Rv1 cells were treated with 10 µM Casodex (CASO), 10 µM MDV3100 (MDV3100), or vehicle control, ± 5 µM LY294002 (LY, PI3K/Akt inhibitor), incubated in 10% CD-FBS RPMI plus 10 nM DHT, and cell growth analyzed by MTT at indicated time points. **B. Anti-Akt treatments failed to reverse anti-androgens/promoted PCa invasion.** C4-2 and CWR22Rv1 cells were treated with 10 µM CASO, 10 µM MDV, vehicle control, ± 5 µM LY for 3 days for invasion assays. After 48 hrs, invaded cell numbers were shown as fold change from 6 randomly selected fields (400X). **C. Anti-Akt failed to reverse the anti-androgens/promoted MMP9 expression.** After 3 days inhibitor treatments, cell extracts were obtained for Western blot analysis of Akt, p-Akt, MMP9, and GAPDH. All the experiments have been repeated twice independently. *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 3 Activation of TGF-β1/Smad3 Signaling Contributed to the Anti-Androgens-Induced PCa Cells Invasion and MMP9 Expression.** **A. Anti-androgens promote TGF-β1/Smad3/MMP9 expression in PCa cells.** C4-2 and CWR22Rv1 cells treated with 10 µM Casodex (CASO), 10 µM MDV3100 (MDV), or vehicle control (NC), incubated in 10% CD-FBS RPMI at 10 nM DHT for 24 hrs, then gene expression determined by qPCR. **B. Blocking TGF-β1/Smad3 signals reversed...**
the CASO or MDV-promoted PCa invasion. C4-2 and CWR22Rv1 cells treated with 10 µM Casodex (CASO), 10 µM MDV3100 (MDV), or vehicle control (NC), and co-treated with 5 µM SB431542 (SB) or 50 µM Naringenin (NAR), incubated in 10% CD-FBS RPMI media at 10 nM DHT, and then invasion assays performed. After 48 hrs, the invaded cell numbers were shown in folds change after counting 6 randomly selected fields. C. Blocking TGF-β1/Smad3 signals reversed the anti-androgen increased MMP9 expression. The effect of CASO/MDV and SB/NAR on the expression of TGF-β1, p-Smad3, Smad3, MMP9, and GAPDH were analyzed by Western blotting. The TGF-β1 receptor kinase inhibitor, 10 µM SB431542, and the 50 µM Smad3 specific inhibitor, Naringenin, were used to block TGF-β1 receptor kinase and Smad3 signals, respectively, that were induced by anti-androgen treatment. D. Blocking MMP9 activity reversed the CASO or MDV3100-promoted PCa invasion. C4-2 and CWR22Rv1 cells were treated for 3 days with the combination of MMP9 inhibitors and anti-androgens and then invasion assays performed. E. Direct treatment of TGF-β1 recombinant protein increased PCa invasion. CWR22Rv1 cells were treated for 3 days with the 5ng/ml human recombinant TGF-β1 protein and then invasion assay performed. All the experiments have been repeated twice independently. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 4 Alternative Anti-Androgens Suppressed Growth and Invasion of PCa Cells through Distinct Mechanism. (A-B) The anti-androgen/AR compounds inhibited (A) PCa cell growth and (B) PSA expression. LNCaP, C81, C4-2, and CWR22Rv1 cells were treated with 10 µM Casodex (CASO), 10 µM MDV3100 (MDV), 10 µM ASC-J9® (ASC), 5 µM CTS, or vehicle (NC), incubated in 10% CD-FBS RPMI at 10 nM DHT, (A) cell growth was analyzed by MTT assay at indicated time points, and (B) PSA expression in the PCa cells treated with various anti-androgen/AR compounds was determined by quantitative PCR after 24 hrs treatment. C. ASC-J9®/CTS, but not CASO/MDV, inhibit PCa invasion. The PCa cells were treated with 10 µM CASO, 10 µM MDV, 10 µM ASC, 5 µM CTS, or vehicle control (NC) for 3 days then invasion assays were performed. The invaded cell numbers were shown in fold change after counting 6 randomly selected fields. D. 3D invasion assay in C4-2B and CWR22Rv1 cells treated with various anti-androgen/AR compounds. The 3D invasion assay was performed in C4-2B and CWR22Rv1 cells treated with 4 different compounds or vehicle control. After 2 wks, the spheres with/without protrusion were counted and shown in percentage (%) compared to the control. E. Comparison of different anti-androgen/AR compounds on TGF-β1/Smad3/MMP9 signaling in PCa cells. C4-2 and CWR22Rv1 cells treated with 10 µM CASO, 10 µM MDV, 10 µM ASC, 5 µM CTS, or vehicle for 3 days were harvested. The expressions of TGF-β1, p-Smad3, Smad3, MMP9,
and GAPDH were analyzed by Western blot analysis. All the experiments have been repeated twice independently. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).

**Fig. 5 Anti-Androgen/Anti-AR Compounds treatments in CWR22Rv1 Orthotopic Xenograft Mouse Model Exhibited Differential Effects on PCa Growth and Metastases.** The luc-CWR22Rv1 cells were implanted into nude mice. Cancer cells (\(1 \times 10^6\)) mixed with matrigel and injected into both anterior prostates of nude mice. Two wks after inoculation, different anti-androgen/AR compounds (30 mg/Kg Casodex (CASO), 30 mg/Kg MDV3100 (MDV), 75 mg/Kg ASC-J9® (ASC), 25 mg/Kg CTS) or vehicle were i.p. injected three times per wk for 4 wks. The mice were sacrificed and reporter gene signal detected by IVIS imaging system. **A. The metastasis tumors detected by IVIS system.** The mice were anesthetized by isoflurane, and injected with D-luciferin (150 mg/Kg) for 10 mins before detection. The metastasis tumor was monitored by IVIS system. After sacrificing, primary tumors were removed, and IVIS system used again to confirm the existence of metastases. **B. Quantification of metastatic lesions in mice treated with different anti-androgen/anti-AR compounds.** After euthanizing, metastatic tumors were confirmed by IVIS detection and the numbers of metastatic foci in each mouse was then quantified. The numbers of mice used in each group were indicated (mice with metastasis/total mice). **C. The metastatic tumors in bone.** Metastatic signals in the bone found in MDV3100 treated mice were confirmed by X-ray and CT scan. White arrows indicate increased bone density and osteoblastic lesion formation. **D. Mice body weight change with various compounds treatment.** Mice body weights were checked weekly starting at first injection of compounds. **E. Comparisons of TGF-β1/Smad3/MMP9 signals in in vivo xenografted PCa tissue.** Tumor tissue sections were stained using IHC for TGF-β1, p-Smad3, MMP9, and PSA expressions (400x), the quantification results were shown at right. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
Table 1

<table>
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<tr>
<th>Age</th>
<th>ADT drug applied</th>
<th>PSA (ng/ml)</th>
<th>Primary tumor size</th>
<th>Metastasis</th>
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<tr>
<td></td>
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<td>Before</td>
<td>After</td>
<td>Before</td>
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<tr>
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</table>

*These patients’ data were from over 1,300 PCa patients received ADT in 4 different Hospitals in China, Taiwan, and Japan.

**Casodex was withdrawn 9 months after complete androgen blockage (CAB) initiation, because of adverse event (liver dysfunction). Thereafter, LH-RHa alone was administered. Biopsy specimens of the local recurrence lesion revealed neuroendocrine cancer (small cell carcinoma) and Gleason grade was not determined.

*** Local recurrence after prostatectomy. Thus, primary tumor sizes before and after ADT were not determined; and the PSA level is low before ADT
Fig. 1

A

B

C

Supply with different anti-androgens
Replace medium every 4 days

5% Matrigel + cells
Matrigel + collagen

Protrusion
No protrusion

Protrusion
No protrusion

Protrusion
No protrusion
Differential Androgen Deprivation Therapies with Anti-Androgens of Casodex or MDV3100 vs Anti-Androgen Receptor of ASC-J9 Lead to Promote vs Suppress Prostate Cancer Metastasis

Tzu-Hua Lin, Soo Ok Lee, Yuanjie Niu, Defeng Xu, Liang Liang, Lei Li, Shauh-Der Yeh, Naohiro Fujimoto, Shuyuan Yeh and Chawnshang Chang

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