GnRH Antagonists Have Direct Inhibitory Effects On Castration-Resistant Prostate Cancer Via Intracrine Androgen and AR-V7 Expression

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Abstract

Hormone therapy is currently the mainstay in the management of locally advanced and metastatic prostate cancer. Degarelix (Firmagon), a gonadotropin-releasing hormone (GnRH) receptor antagonist differs from luteinizing hormone-releasing hormone (LHRH) agonists by avoiding “testosterone flare” and lower follicle-stimulating hormone (FSH) levels. The direct effect of degarelix and leuprolide on human prostate cancer cells was evaluated. In LNCaP, C4-2BMDDR, and CWR22Rv1 cells, degarelix significantly reduced cell viability compared with the controls (P ≤ 0.01). Leuprolide was stimulatory in the same cell lines. In C4-2B MDVR cells, degarelix alone or combined with abiraterone or enzalutamide reduced the AR-V7 protein expression compared with the control group. SCID mice bearing VCaP xenograft tumors were divided into 4 groups and treated with surgical castration, degarelix, leuprolide, or buffer alone for 4 weeks. Leuprolide slightly suppressed tumor growth compared with the vehicle control group (P > 0.05). Tumors in degarelix-treated mice were 67% of those in the leuprolide-treatment group but 170% larger than in surgically castrated ones. Measurements of intratumoral steroids in serum, tumor samples, or treated cell pellets by LC/MS confirmed that degarelix better decreased the levels of testosterone and steroidogenesis pathway intermediates, comparable to surgical castration, whereas leuprolide had no inhibitory effect. Collectively, our results suggested a selective mechanism of action of degarelix against androgen steroidogenesis and AR-variants. This study provides additional molecular insights regarding the mechanism of degarelix compared with GnRH agonist therapy, which may have clinical implications.

Introduction

Prostate cancer is the most common tumor and the second cause of cancer death in men (1). For advanced and metastatic prostate cancer, androgen deprivation therapy (ADT) using luteinizing hormone-releasing hormone (LHRH) agonists or the gonadotropin-releasing hormone (GnRH) receptor antagonist, alone or in combination with radiotherapy, is considered the best treatment option (2, 3). A new generation of androgen receptor signaling inhibitors, such as abiraterone or enzalutamide, have been approved as treatment options in castration-resistant prostate cancer (CRPC; refs. 2, 3). Different ADT strategies have been tested to achieve castration levels of testosterone (<50 ng/dL). To date, long-acting depot formulations of GnRH agonists (in combination with antiandrogens for 4 weeks to avoid the "testosterone surge") are the most commonly used agents. GnRH agonists, after a desensitization of the GnRH receptor response, determine a reduction in luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone production (2, 3). GnRH antagonists, like degarelix, are also an approved form of ADT (4). GnRH antagonists, by blocking GnRH receptors, produce a more rapid suppression of testosterone without testosterone surge or microsurge (4). Presently, international prostate cancer guidelines recommend the use of either GnRH agonists or antagonists as treatment options for ADT in patients with prostate cancer (2, 3). Although some studies have suggested differences in efficacy and disease-related outcomes (musculoskeletal and urinary events) with degarelix compared with LHRH agonists (4), a systematic review of the literature did not support the superiority of antagonists over agonists (5). All the available phase III studies included in the analysis have treatment bias, short-term follow-up and heterogeneous populations (5).

GnRH agonists and antagonists both induce castrate levels of testosterone by altering the intracellular signaling of pituitary cells, but several attempts have been made to elucidate the effect of these agents on other cells that express GnRH receptor (GnRH-R; refs. 6, 7). These studies revealed that extrapituitary tissues are affected by compounds directed toward GnRH-R (8–11). In prostate cells, GnRH-R manipulation may influence several biological processes such as cell growth, apoptosis, angiogenesis, and cell adhesion (8–11). We hypothesized that, unlike agonists, GnRH antagonists may have a direct mechanism of action on prostate cancer cells growth, by affecting the AR signaling pathway. Specifically, we investigated the role of GnRH agonists and antagonists in castration...
sensitive and castration resistant prostate cancer cell lines and xenograft variants and their possible interaction with AR and AR splice variants (AR-Vs such as AR-V7). It is already known that aberrant AR signaling and AR-Vs are able to promote the development of CRPC and may drive drug resistance (8–11). Preclinical and clinical trials have described a direct correlation between AR-V7 expression, one of the most intensively studied AR-variant, and resistance to enzalutamide and abiraterone. Moreover, AR-V7 detection has been independently associated with poor prognosis in CRPC (12, 13). AR-V7 has been proposed as a prognostic marker of PSA response, progression-free survival (PFS), and overall survival (OS) among CRPC patients treated with AR-targeted agents (abiraterone and enzalutamide) or chemotherapy (14, 15), but there are no studies that have investigated the relationship between the type of ADT (GnRH agonist or antagonist) and the expression of AR-V7.

In this study, we showed that different prostate cancer cell lines are sensitive to the antiproliferative effect of the GnRH antagonist degarelix. Furthermore, the use of degarelix, alone or in combination with enzalutamide or abiraterone, affected the expression of AR-V7. In particular, we observed a reduction of AR-V7 at both the protein and transcription levels. These insights suggest an extrapituitary activity of GnRH-R in prostate cancer tumors and may have implications regarding resistance to second generation AR pathway inhibitors.

Materials and Methods

Reagents and cell culture

LNCaP, VCaP, and CWR22Rv1 cells were obtained from the ATCC. All experiments with cell lines were performed within 6 months of receipt from ATCC or reusucitation after cryopreservation. The cells were maintained in RPMI1640 supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. VCaP cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. C4-2B MDVR (C4-2B enzalutamide resistant; ref. 16) cells were maintained in 20 µM/L enzalutamide containing medium. All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

Western blot analysis

Total protein was extracted from cultured cells and/or xenograft tumors and the concentrations were estimated using the BCA Protein Assay Reagent (Pierce). Equal amounts of denatured protein samples were loaded on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to Immobilon PVDF membrane. Immunoblotting was done by incubating membranes with primary antibodies overnight at 4°C with the indicated primary antibodies GnRH-R-2 (PA5-67876; Thermo Fisher Scientific), AR (N-20; SCBT), AR-V7 (Percision lab), -Actin was used as loading control. Following by 1 hour of secondary antibody incubation, immunoreactive proteins were visualized with SuperSignal West Pico CL (Pierce) coupled with X-ray film exposure.

Luciferase assay

LNCaP C4-2B cells were seeded in 24-well plates in regular FBS medium or CSS medium, transfected with 0.2 µg of PSA-Luc (promoter region, 63 0bp) with the internal control pTK-RL using Lipofectamine 2000 (Invitrogen). For those in CSS media, one group was treated with 10 nmol/L of R1881, one cotransfected with an AR-V7 expression vector, the last with DMSO as control. All were treated with 10 to 20 µmol/L of leuprolide or degarelix. Cells were harvested 48 hours after transfection and transactivation was examined by the dual-luciferase assay (Promega).

Proliferation assay

LNCaP, C4-2B, CWR22Rv1, VCaP, and C4-2B MDVR cells were seeded on 12-well plates at a density of 0.5 × 10^5 cells/well in media containing 10% FBS. When treated with leuprolide (20 µmol/L) and degarelix (20 µmol/L) alone or in combination with enzalutamide (20 µmol/L) and abiraterone acetate (5 µmol/L), cells were maintained in complete medium and harvested after 3 or 6 days of treatment for cell counting. A total of 20 µmol/L of leuprolide and degarelix are equivalent to 24.2 and 32.6 mg/kg if using density of water 1 g/mL.

Real-time qRT-PCR

Total RNAs was extracted from LNCaP, C4-2B, CWR22Rv1, VCaP, and C4-2B MDVR cells using the Qiagen Rneasy Kit. qPCR analysis was performed in SsoFast EvaGreen Supermix (Bio-Rad) with specific primers for AR-FL, AR-V7, or PSA and analyzed with Bio-Rad CFX96 Real-Time PCR system (Bio-Rad). Each reaction was normalized by co-amplification of -β-actin and triplicate runs. The experiments were repeated 2 to 3 times for statistical analysis.

Primers used for real-time PCR were: AR-full length: 5’-AAG CCA GAG CTC TGC AGA TGA 3’; 5’-GCT CCT GAC ACT GGT TCA 3’; AR-V7: 5’-AAC AGA AGT ACC TGT GCG CC 3’; 3’-CCT CAC AGC CCT CGG CCC 3’. AR-V7 expression, one of the most intensively studied AR-variant, and resistance to enzalutamide and abiraterone. Moreover, AR-V7 detection has been independently associated with poor prognosis in CRPC (12, 13). AR-V7 has been proposed as a prognostic marker of PSA response, progression-free survival (PFS), and overall survival (OS) among CRPC patients treated with AR-targeted agents (abiraterone and enzalutamide) or chemotherapy (14, 15), but there are no studies that have investigated the relationship between the type of ADT (GnRH agonist or antagonist) and the expression of AR-V7.

In vivo tumorigenesis assay

Animal studies were performed based on the protocols of the Institutional Animal Care and Use Committee of the University of California at Davis (Sacramento, CA). Male SCID mice were maintained in pressurized, ventilated cages with standard rodent chow and water and a 12-hour light/dark cycle. VCaP cells (2 × 10⁶ cells) mixed with an equal amount of Matrigel (1:1) were injected subcutaneously into the flanks of 4-week-old male SCID mice. Tumor volumes were measured twice a week with calipers and tumor volumes were calculated according to the following formula: 1/2 (length × width²). After tumor size reached 100 mm³ mice were treated as follows: 3 mice: surgical castration; 3 mice: vehicle control (5% Tween 80 and 5% ethanol in PBS), 9 mice: degarelix (35 mg/kg, s.c.), 9 mice: leuprolide (6 mg/kg, s.c.). Degarelix and leuprolide human loading doses are 80 and 3.75 mg monthly, respectively, equivalent to 1.33 and 0.625 mg/kg for 60 kg adults. We used approximately 26 and 96 times higher doses of degarelix and leuprolide in mice, respectively plus weekly dosing to compensate for the fast metabolic rate of small laboratory animals. Blood samples were collected 10 days after castration and serum testosterone levels were measured by an EIA Assay Kit (Cayman). Animals were sacrificed at the end of 6 weeks from tumor implant. Tumors were collected for further analysis. IHC staining for Ki67 was performed to visualize the proliferative activity of tumors in each
treatment group, using monoclonal antibody, anti-Ki-67 (SP6; 1:200 dilution; Thermo Fisher Scientific).

**LC/MS-MS (17)**

Tissue extracts were prepared by transferring a xenograft sample to a preweighed 2 mL screw cap vial containing 10 to 20 zirconia/silica beads (2.3 mm; BioSpec) and tissue mass noted (20 to 60 mg). Following addition of 100 μL water, homogenization was carried out with a Precellys homogenizer (4 cycles, 6,000 rpm, 20 seconds each). Internal standard (IS, deuterated T & DHT) was added and samples extracted twice by 30 minutes vortexing with 1 mL 60/40 hexane/ethyl acetate (hex/EtOAc). Extracted steroids were dried (CentriVap) and reconstituted in 50 μL of 50 mM hydroxylamine/50% methanol, incubated 1 hour at 60°C and the resulting oximes analyzed using a Waters Aquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer. Separations were carried out with a 2.1 × 100 mm BEH 1.7 μmol/L C18 column, mobile phase water (A) and 0.1% formic acid in acetonitrile (B; gradient: 0.2 minutes, 25% B; 8 minutes, 70% B; 9 minutes, 100% B; 12 minutes 100% B; 12.2 minutes, 25% B; 14 minutes run length). All data were collected in ES+ by multireaction monitoring (mrm) with instrument parameters optimized for the m/z's and corresponding fragments of the oxime-steroids. Data processing were done with Quanlynx (Waters) and exported to Excel for additional normalization to weights and volumes as required. Serum extracts (50 μL) were extracted similar to above, omitting homogenizing and with a single 1.5 mL volume of hex/EtOAc. Tissue culture samples were prepared similar to tissues except that a slurry was generated from pellets by freeze/thaw cycles (3×) and vortexing prior to transfer of 100 μL to extraction tubes.

**Statistical analysis**

Data are shown as the mean ± SD. All were from at least 3 independent experiments and subjected to unpaired Student t tests and 1-way ANOVA for comparison of means. P ≤ 0.05 was considered statistically significant.

**Results**

**Expression of GnRH receptors and androgen receptors in prostate cancer cell lines**

Considering the mechanism of action of GnRH agonist and antagonist in vitro, we measured the expression of GnRH type 1 and 2, AR full length (AR-FL), and its variant V7 (AR-V7) by qRT-PCR. GnRH-R type 1 mRNA levels were undetectable in 5 prostate cancer cell lines (LNCaP, C4-2B, C4-2B MDVR, VCaP, and CWR22Rv1). On the contrary, GnRH-R type 2 mRNA was present in the cell lines investigated at different expression levels and compared with LNCaP as the reference (Fig. 1A). Western blotting analysis showed the presence of GnRH-R2 in all AR-positive cells and even in PC-3 cells (Fig. 1B). We focused on the 4 AR bearing PC lines and measured their levels of AR-FL and AR-V7 side-by-side to establish the comparison and predict their response to GnRH modulators. It was validated that only VCaP and CWR22Rv1 cells have readily detectable AR-V7 (Fig. 1C and D). We then used C4-2B cells transiently transfected with the PSA-Luc plasmid to examine the action of GnRH modulators. Luciferase activity stimulated by synthetic androgen R1881 was slightly decreased by both leuprolide and degarelix, but much less than by enzalutamide. These 2 reagents also inhibited AR-V7 induced AR transcriptional activation by 30%; however, none of the inhibition registered a significant difference (Fig. 1E).

**Cell viability of prostate cancer cell lines treated with degarelix and leuprolide**

The direct effect of degarelix on human prostate cancer cell growth was evaluated. In LNCaP (androgen sensitive) and C4-2B MDVR (enzalutamide resistant with induced AR-V7 through long-term exposure) cells, degarelix significantly reduced the cell viability assayed by WST-1 compared with the control group (P ≤ 0.01) but not in CWR22Rv1 (castration resistant with truncated AR variants) cells after 6 days of treatment (Fig. 2A). Conversely, leuprolide, an LHRR agonist, had a stimulatory effect in the same cell lines, significantly promoted cell growth (P ≤ 0.01).

Based on these data, the expression of AR, AR-V7, and PSA transcripts in C4-2B MDVR cell line treated with leuprolide and degarelix for 48 hours were evaluated by qRT-PCR (Fig. 2B). Degarelix showed a significant reduction in the transcription levels of AR-V7 and PSA (P ≤ 0.05). Western blot analysis of the same cell line (C4-2B MDVR), treated at 2 different concentrations (10 and 20 μmol/L) of leuprolide and degarelix, confirmed this finding that degarelix at 20 μmol/L reduced AR-V7 at protein level compared with the control and leuprolide groups (Fig. 2C). When same treatments were applied to RV1 and VCaP cells, both harboring high levels of AR variants, AR-V7 level also reduced comparable to that in C4-2B MDVR cells. And yet, leuprolide either enhanced AR-V7 marginally in RV1 cells or maintained it around the same in VCaP cells (Supplementary Fig. S1A and S1B).

**Leuprolide and degarelix combined with AR pathway inhibitor therapies**

We then examined the benefit of combinations of ARSI with these GnRH modulators in growth inhibition of enzalutamide resistant C4-2B MDVR and CWR22Rv1 cells with the trypan-blue cell counting method. With cell proliferation not affected by enzalutamide, 20 μmol/L of leuprolide alone or in combination did not affect the cell growth to a statistical significance. However, 20 μmol/L of degarelix alone significantly increased proliferation by 75% and slightly further reduced viable cell counts when combined with enzalutamide (Fig. 2D). The MDVR cells were cross-resistant to abiraterone. Nevertheless, degarelix combined with abiraterone showed benefit with significant inhibition compared with control in both MDVR and RV1 cells. Western blot analysis of treated C4-2B MDVR lysates (Fig. 2E) revealed that, although the protein levels of AR-FL remained similar, AR-V7 levels were significantly reduced in degarelix-treated samples, alone (40% of the control) or in combinations with enzalutamide or abiraterone (26% of control). Conversely, leuprolide slightly increases AR-V7 protein level when used alone, but significantly when combined with enzalutamide or abiraterone.

To scrutinize whether this reduction of AR-V7 by degarelix treatments was via downregulation of its transcript or protein degradation, we performed qRT-PCR assays of C4-2B MDVR cells treated with leuprolide, degarelix, and enzalutamide, alone or in combination (Fig. 2F and G). Indeed, quantification of the mRNA levels of AF-FL, AR-V7, and PSA (Fig. 2G) further confirms that degarelix targets AR-V7 specifically at the transcript level whereas leuprolide shows stimulation when combined with enzalutamide.
In vitro analysis of androgens and androgen precursors levels in prostate cancer cell lines

In parallel to the in vivo study, we treated 2 CRPC (C4-2B and VCaP) lines with leuprolide and degarelix in androgen-deprived conditions to investigate the concentrations of androgens and androgen precursors under GnRH agonist and antagonist treatments. We measured steroids in these cell pellets using a validated LC/MS-MS assay (Fig. 3). When maintained in CS medium, the basal level of testosterone detected in these cells was very low. Degarelix further decreases testosterone significantly in both lines whereas leuprolide displays no difference ($P < 0.01$ and $0.05$, respectively). The same result was observed for the other intracellular androgen, DHEA. As for DHT, the effect of degarelix was only observed in VCaP cells ($P < 0.05$).

Inhibition of growth of VCaP xenografts by GnRH agonist and antagonists

The common concept how GnRHR agonist or antagonist works is through regulating the pituitary gland of males to suppress testicular androgen synthesis. Although we observed direct effect of degarelix on PC cells in vitro, we sought to test both drugs in vivo to demonstrate potential differences in treating prostate tumors. It
has been demonstrated that both GnRHR agonist and antagonist are functional in mouse studies (18, 19). We chose VCaP cells because of their CRPC characteristics and yet still demonstrating a response to castration. SCID mice bearing VCaP xenograft tumors (average volume of 136 mm$^3$) were treated with degarelix (35 mg/Kg weekly), leuprolide (6 mg/kg weekly), surgical castration, or vehicle for 4 weeks (Fig. 4A). Surgical castration effectively controlled the tumor growth, with slight relapse 2 weeks after the surgery. Leuprolide only slightly suppressed tumor growth compared with the vehicle control group ($P > 0.05$). However, tumors in the degarelix-treated group were half the size of those from control group ($P < 0.05$) and 67% of those in leuprolide group (Fig. 4B). There were no differences in mouse body weights among the treatment groups. After 4 weeks of treatment, we measured testosterone levels in the mouse xenografts. Because of a considerable variability especially in the control group, testosterone levels did not show any significant differences among degarelix, leuprolide, and surgical castration.
groups (Fig. 4C). All 3 treatments effectively decreased serum testosterone levels. Analysis of tumor lysates from representative tumor samples from each group showed that AR-V7 was below detection in control and with light exposure in degarelix but strongly enhanced in both castration and Leuprolide groups (Fig. 4D). A comprehensive blot with more tumors samples under longer exposure was shown in Supplementary Fig. S2. This is in agreement with the in vitro studies that degarelix alone down-regulates AR-V7. To estimate growth fraction, IHC staining for anti-Ki-67 antibody was used to determine the percentage of Ki-67-positive tumor cells on slide-mounted, paraffin-embedded tumor sections (Fig. 4E and F). Surgical castration and degarelix groups were associated with significantly lower Ki-67 positive cells compared with the leuprolide or control groups (P < 0.05), demonstrating a greater reduction of cell proliferation after surgical castration or degarelix treatment.

Tissue androgen and androgen precursor analysis
We assessed whether mean intratumoral testosterone androgen precursors levels differed among the four treatment arms in SCID mice bearing VCaP xenograft tumors. There are significant differences in tumor testosterone levels between control and surgical castration (6.345 vs. 0.189 ng/g tumor, P < 0.001) and control and degarelix groups (6.345 vs. 1.059 ng/g tumor, P < 0.001), as well as degarelix versus Leuprolide (1.059 vs. 6.812 ng/g tumor, P < 0.001; Fig. 5). Most of other intermediates in steroidogenesis followed the same pattern as testosterone with significant differences between control and castration, and control and degarelix groups. In serum samples, testosterone levels are much higher in surgical castration or degarelix treatment. The magnitude of change did not register statistical significance. Although GnRH agonists are associated with an initial testosterone surge in patients with metastasis, they ultimately decrease serum testosterone levels to castrate and are considered a standard of care (20). GnRH antagonists result in castrate testosterone levels without testosterone flare (21). Arguably, some data have suggested degarelix to have better disease control and, in particular, a superior PSA progression-free survival (22) and a more favorable control of serum alkaline phosphatase (23). A recent pooled analysis of data from 5 randomized trials of degarelix versus GnRH agonists showed higher overall survival during the first year of treatment for men receiving degarelix (4). Data also showed that, in patients with a history of cardiovascular disease, there was a significantly lower risk (>50%) of a subsequent cardiovascular event or death over 1 year of treatment with degarelix versus GnRH agonists (24).

Our study sought to identify any direct effect of GnRH antagonists on prostate cancer tumors. Although it has been previously described that prostate and other periferal tissues express GnRH-R (26, 27), we needed to confirm these results to investigate the direct effects of degarelix on prostate cancer cell lines. We corroborated that all the human prostate cancer cell lines express GnRH receptor type II, but not GnRH receptor type I.

Finding GnRH-R in LNCAp, VCaP, CWR22-Rv1, C4-2B, C4-2B MDVR cells led to their use in our studies. Indeed, prostate cancer cells respond to both agonist and antagonist in cell proliferation but ambivalently. The stimulatory effect of leuprolide on cell growth is not predicted. Upon binding to GnRH-R on cell surface, leuprolide might turn on some unknown signaling pathway to produce a short-term enhancement. Inhibition of tumor growth by leuprolide was moderate but more in agreement of its efficacy. Both leuprolide and degarelix affect AR transactivation activity. AR-driven PSA-Luciferase activity changes upon leuprolide and degarelix treatments when induced by exogenous AR-V7 in C4-2B cells. The magnitude of change did not register statistical significance probably due to the overexpression of AR-V7 through transfection.

Cell proliferation assays showed a lower cell viability after degarelix exposure compared with leuprolide treatment. This
suggests a direct antitumorigenic role of degarelix on prostate cancer cell growth and it is consistent with the previous report that degarelix determines the activation of caspase 3/7 in prostate cancer cell lines (6). Moreover, another GnRH antagonist, cetorelix, revealed a direct inhibitory effect on prostate cell line growth with a mechanism involving cell-cycle arrest and a change in proinflammatory cytokines (28, 29). In our experiments, we did not find that the GnRH agonist leuprolide inhibits prostate cells viability, even when tested at higher concentrations.

We have repeated observed the effect of degarelix on the level of AR-V7, both at mRNA and protein levels. The occurrence of AR variants is mostly through gene rearrangement and RNA
splicing (30–32). ADT with prolonged GnRH-R agonist or antagonist induces the emergence of AR-V7 (31). Indeed, both cell and tumor lysates from the leuprolide groups reveal upregulation of AR-V7, mimicking what have been observed in some patients with CRPC after long-term ADT (12, 33, 34). However, in our hands, both in vitro and in vivo treatments of degarelix reduced AR-V7. It is not clear whether degarelix has any effect on molecules regulating splicing such as U2AF65, ASF/SF2, JMJD1A, HoxB13, or hnRNPA1 (31, 35–37). With its higher impact on the protein level, it is possible that degarelix causes some protein degradation through the ubiquitin proteasome system (38, 39). The exact mechanisms behind this down-regulation remain to be investigated.

As intratumoral androgens are known to drive CRPC (40, 41), we measured androgen levels using LC/MS-MS (17) in prostate cancer cell lines and xenograft tumor samples. Overall, the measurements using LC/MS-MS from both the in vitro and in vivo studies are consistent with the trend in proliferation assays. Degarelix alone significantly reduced the intracellular levels of testosterone compared with leuprolide. Degarelix also reduces the levels of most of the intermediates in steroidogenesis, comparable to those detected in the surgical castration group. The canonical effect of degarelix on testosterone is through pituitary cells. Although it may be the rationale how tumor inhibition by degarelix was accomplished in the xenograft model, there is no collaboration of pituitary tissue in tissue flasks. Inhibition of intracrine steroidogenesis via the backdoor pathway is a plausible explanation. It is not known whether degarelix imposes any inhibitory effect on the steroid biosynthetic enzymes such as CYP17A1, HSD3B2/3, AKR1C1/2/3, etc. We observed an additive effect on cell proliferation and reduction in AR-V7 when using abiraterone that targets CYP17A1 in combination with degarelix. Therefore, CYP17A1 is unlikely the target. Knockdown of AKR1C3 with shRNA restores sensitivity to

![Figure 5](image-url)

**Figure 5.**
LC/MS measurements of steroids: testosterone, DHEA, pregnenolone, androsterone, androstenedione, and 5-pregnan-3-ol-20-one from tumors in all 4 treatment groups. Error bars represent SE. *P ≤ 0.05; ****, P ≤ 0.001.
enzalutamide in C4-2B MDVR cells (16). Inhibition of AKR1C3 with shRNA or the small molecule inhibitor directly down-regulates AR-V7. Whether degarelix targets intracrine androgen synthesis and AR-V7 directly through AKR1C3 remains to be explored.

Above all, this study provides insight regarding a direct anti-prostate cancer tumor effect of degarelix. In our hands, the GnRHR antagonist degarelix inhibits CRPC cell growth in vitro and tumor progression in vivo possibly through down-regulation AR-V7, superior to the receptor agonist. It may suggest a biological rationale to consider antagonists rather than agonists, especially in combination with ARSI drugs to prolong the onset of CRPC. However, this is hypothesis generating and demands further investigation into the interplay between androgen deprivation therapies and resistance mechanisms in CRPC.

Figure 6.
LC/MS measurements of steroids: testosterone, androsterone, androstenedione, progestosterone, pregnan-3,20-dione, and 5-pregn-3-ol-20-one from blood samples collected from all 4 treatment groups. Error bars represent SE. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005.

Disclosure of Potential Conflicts of Interest
C.P. Evans reports receiving a commercial research grant from Ferring Pharmaceuticals. J.C. Yang reports receiving a commercial research grant from Ferring Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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