

Supplemental Material

# Androgen Receptor Antagonism Drives Efficacy of CYP17 Inhibitors in CRPC

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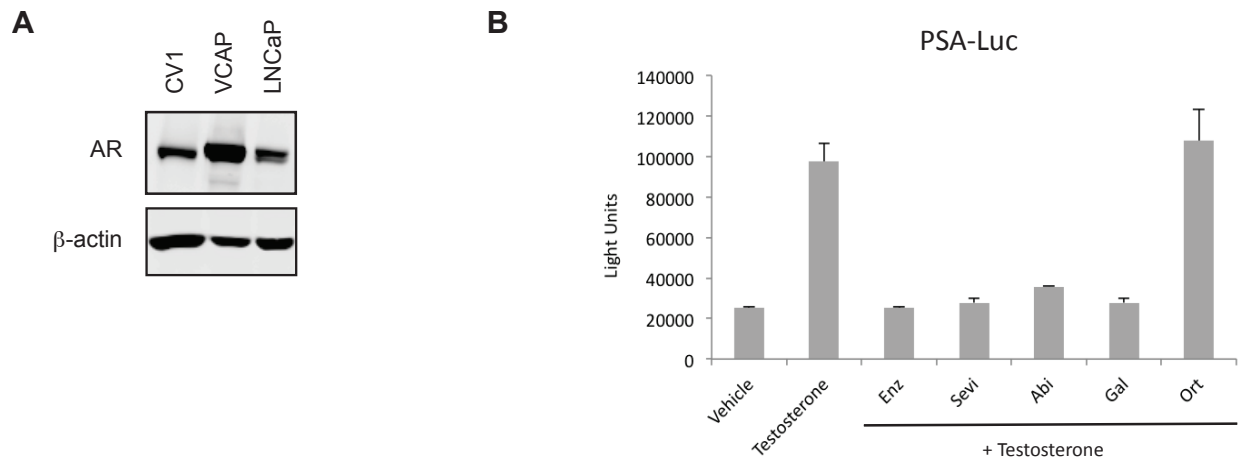
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# Contributed equally to this work

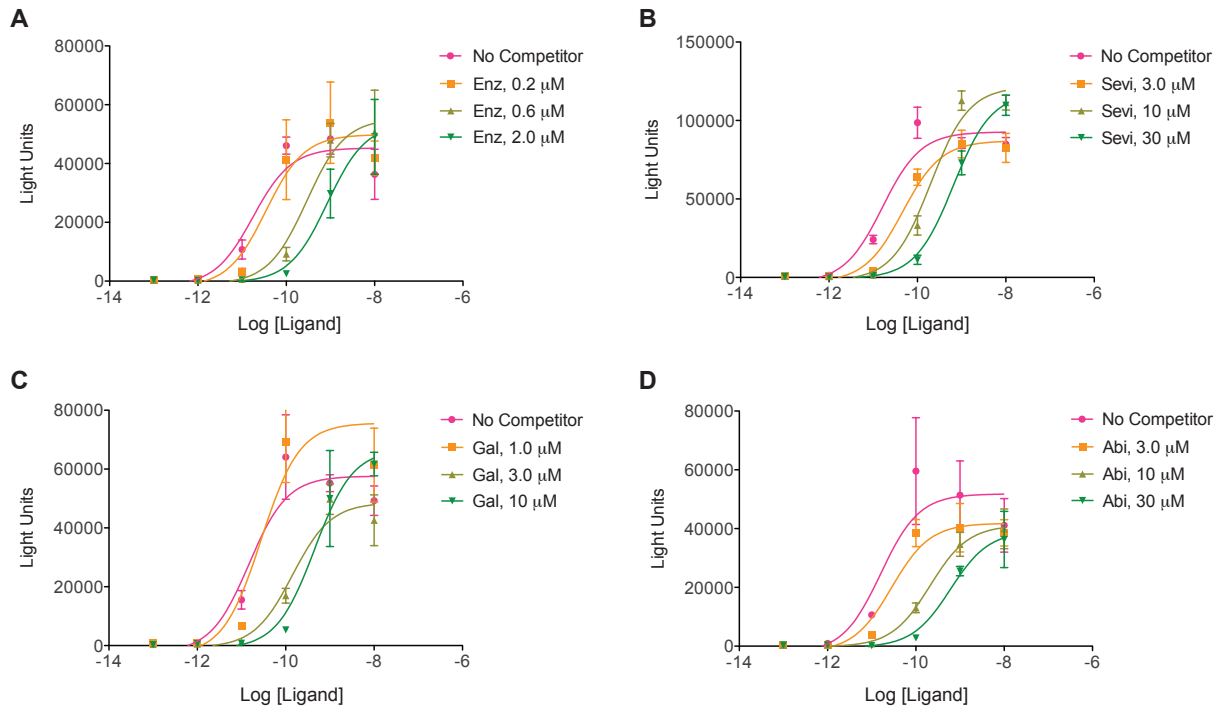
## Supplemental Tables and Figures

	<b>GR</b>	<b>PR</b>	<b>MR</b>
<b>Enzalutamide</b>	> 20 $\mu$ M	2.7 E-06	> 20 $\mu$ M
<b>Seviteronel</b>	> 20 $\mu$ M	9.0 E-06	> 20 $\mu$ M
<b>Galeterone</b>	> 20 $\mu$ M	1.9 E-06	> 20 $\mu$ M
<b>Abiraterone</b>	> 20 $\mu$ M	2.1 E-07	> 20 $\mu$ M
<b>RU 486</b>	7.9 E-09	1.0 E-11	ND
<b>Spironolactone</b>	ND	ND	3.2 E-09

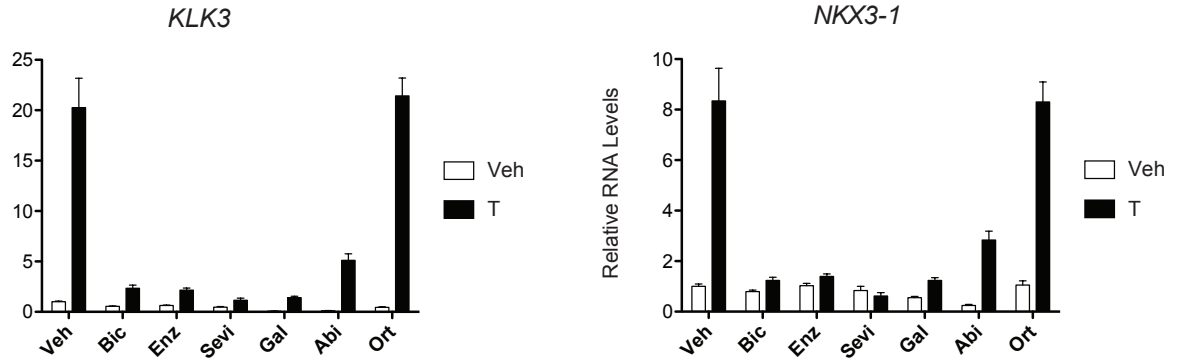
**Supplemental Table 1. Effects of CYP17 inhibitors on transcriptional activation of GR, PR, and MR.** CV1 cells were transfected with MMTV-luc, Renilla-luc, and either glucocorticoid (GR), progesterone (PR), or mineralocorticoid (MR) receptor constructs. Cells were then treated for 24 hours with increasing doses of the indicated antagonists in the presence of 0.1 nM of appropriate agonist: GR, dexamethasone; PR, R5020; MR, aldosterone. Luciferase values were measured and IC<sub>50</sub> values are shown in the table. ND, not determined.



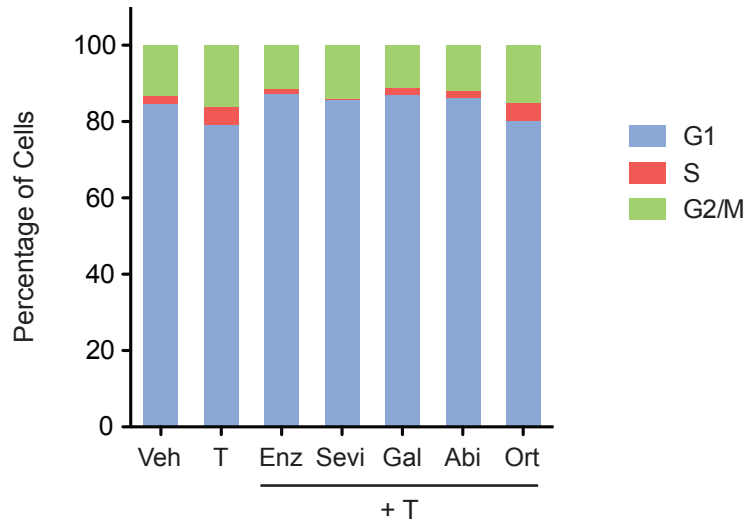
**Supplemental Figure 1. Effects of CYP17 inhibitors on PSA-Luc (A)** Protein extracts from CV1 cells transfected with wt-AR expression vector, MMTV-luc, and Renilla-luc were analyzed for AR expression using anti-AR antibody (N20). Protein extracts from VCAP and LNCaP cells are included as controls for endogenous AR expression.  $\beta$ -actin is included as a loading control. **(B)** CV1 cells were transfected with PSA-luc and treated for 24 hours with the indicated ligand in the presence of 1.0 nM testosterone. Cells were lysed and assayed for luciferase activity. Error is reported as SD from a representative experiment performed in triplicate.



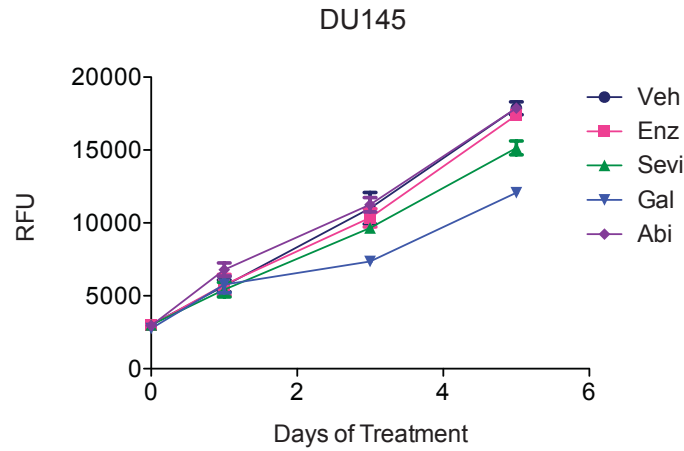
**Supplemental Figure 2. CYP17 inhibitors are competitive AR ligands.** CV1 cells were transfected with wt-AR, MMTV-luc, and Renilla-luc, followed by treatment with increasing concentrations of R1881 and the indicated dose of competitor ligand (A) enzalutamide, (B) seviteronel, (C) galeterone, or (D) abiraterone. Luciferase activity was measured after 24 hour incubation. Error bars indicate SD of triplicate samples of a representative experiment performed in duplicate. Shifts in  $EC_{50}$  but not  $V_{max}$  values indicate competitive antagonism.



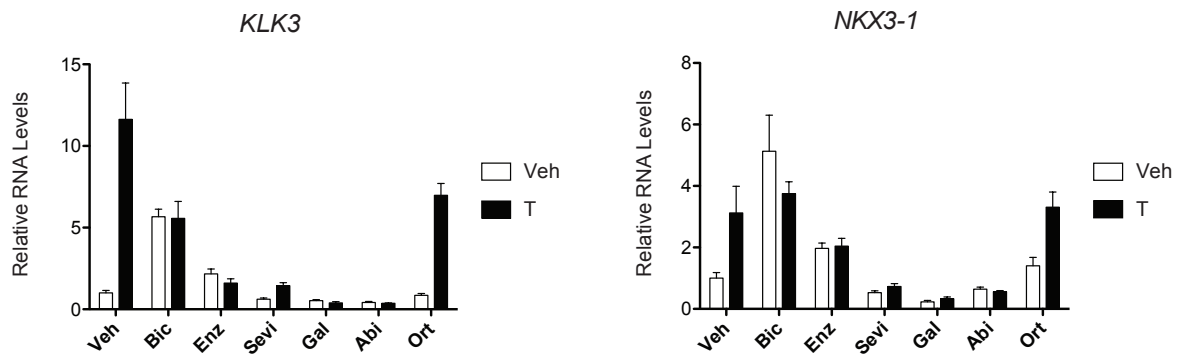
**Supplemental Figure 3. *KLK3* and *NKX3.1* expression in LNCaP cells.** LNCaP cells were treated with vehicle, 10 nM testosterone (T), and 10  $\mu$ M of the indicated ligand for 24 hours. Real-time PCR was performed to assess *KLK3* and *NKX3.1* mRNA expression. Error bars represent the SD from triplicate samples of a representative experiment performed in triplicate.



**Supplemental Figure 4. CYP17 inhibitors prevent entry of prostate cancer cells into S phase.** LNCaP cells were treated with vehicle, 10 nM testosterone (T), and 10  $\mu$ M of the indicated ligand for 24 hours. DNA was stained with propidium iodide and cell cycle analysis was performed using flow cytometry. Data are presented as percentage of cells in G1, S, or G2/M phase. Representative data are shown from an experiment performed in triplicate.

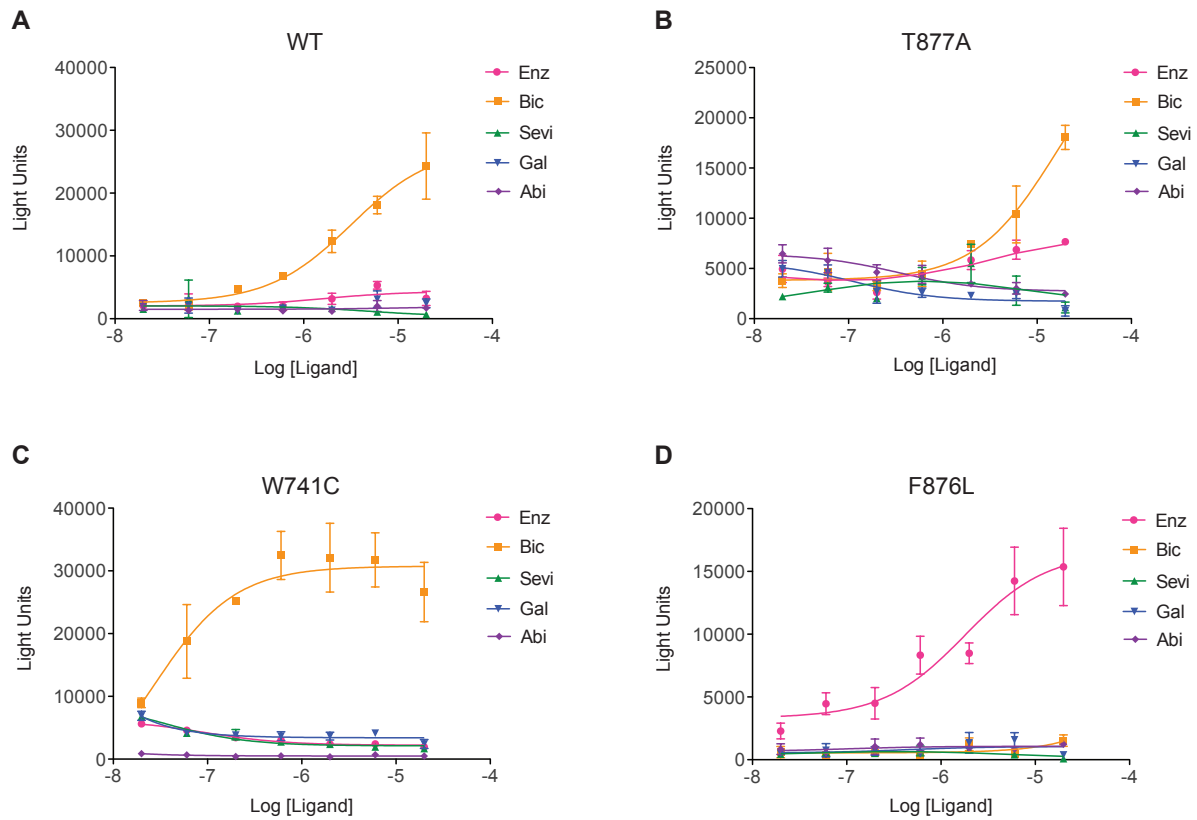


**Supplemental Figure 5. CYP17 inhibitors have minimal effects on AR-negative DU145 cell growth.** DU145 cells were treated with 10  $\mu$ M of the indicated compound and analyzed for cell growth by Hoechst dye (DNA content) on 1, 3, and 5 days post-treatment. Error bars represent SD of triplicate samples of a representative experiment performed in duplicate.

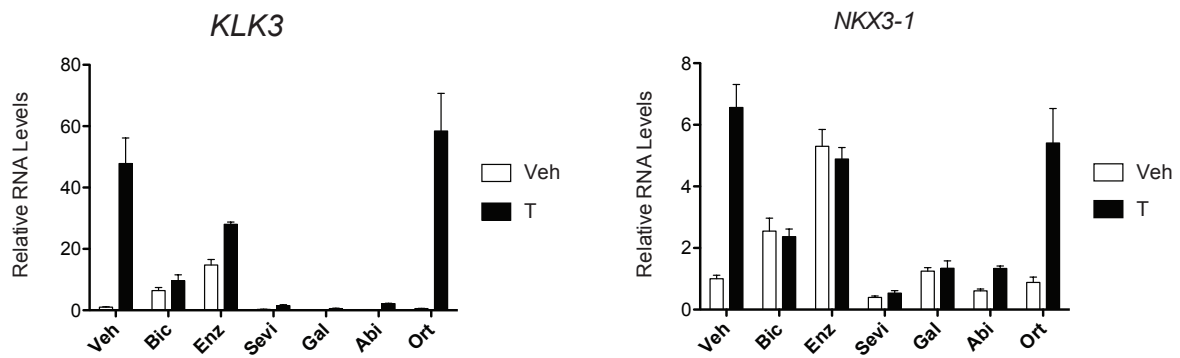


**Supplemental Figure 6. *KLK3* and *NKX3.1* expression in LNCaP-AR cells.** LNCaP-AR cells were treated with vehicle, 1.0 nM testosterone (T), and 10  $\mu$ M of the indicated ligand for 24 hours. Real-time PCR was performed to assess *KLK3* and *NKX3.1* mRNA expression. Error bars represent the SD from triplicate samples of a representative experiment performed in triplicate.

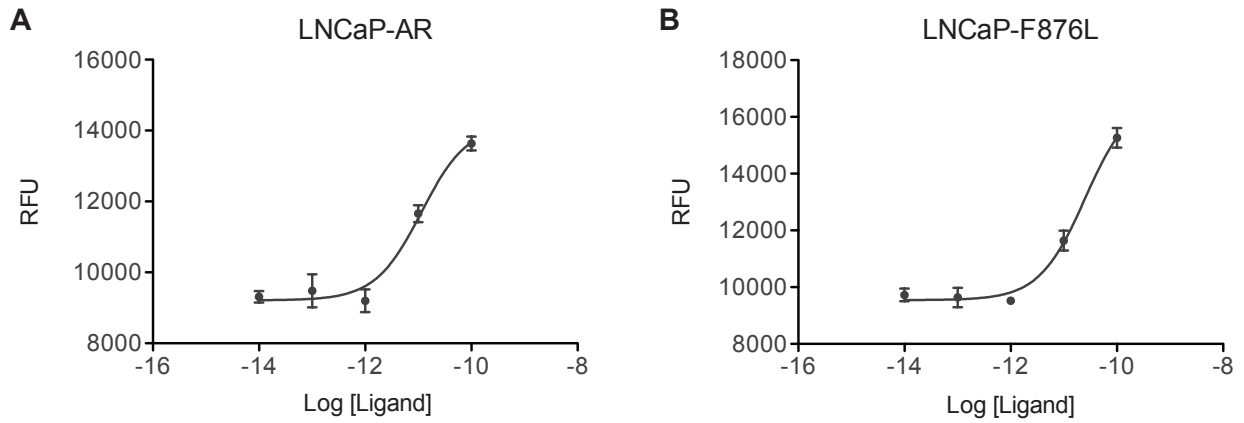




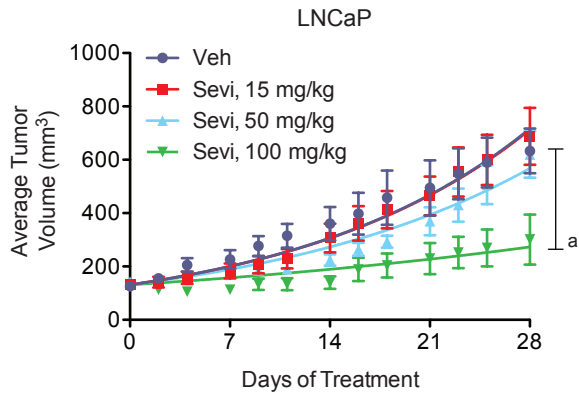
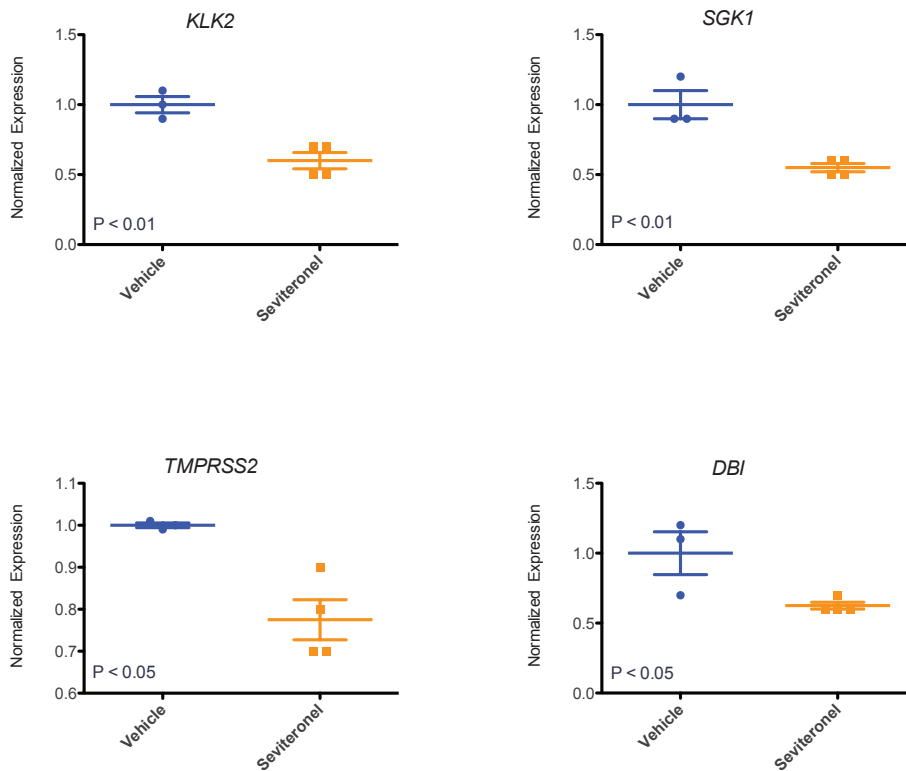
**Supplemental Figure 7. CYP17 inhibitors do not display agonist activity on wild-type or mutant AR.** CV1 cells were transfected with MMTV-luc, Renilla-luc, and either (A) wt-AR, (B) AR-T877A, (C) AR-W741C, or (D) AR-F876L expression plasmids. Cells were then treated for 24 hours with increasing doses of indicated ligands in the absence of R1881 and luciferase activity was measured. Error bars represent SD of triplicate samples for a representative experiment performed in duplicate.



**Supplemental Figure 8. *KLK3* and *NKX3.1* expression in LNCaP AR-F876L cells.** LNCaP AR F876L cells were treated with vehicle, 3.0 nM testosterone (T), and 10  $\mu$ M of the indicated ligand for 24 hours. Real-time PCR was performed to assess *KLK3* and *NKX3.1* mRNA expression. Error bars represent the SD from triplicate samples of a representative experiment performed in triplicate.

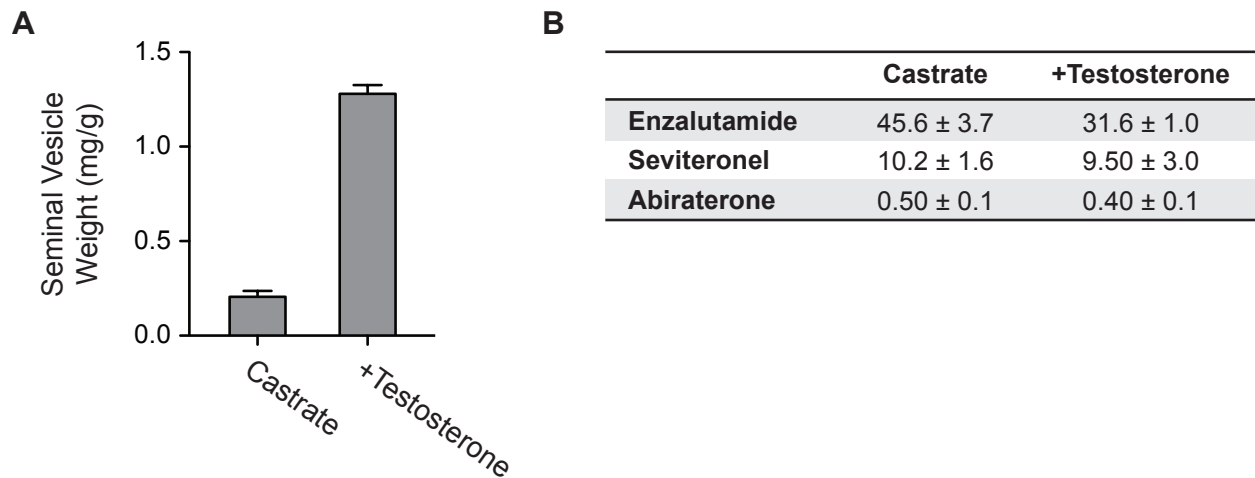


**Supplemental Figure 9. R1881 promotes growth of AR overexpression and enzalutamide-resistant CRPC models. (A) LNCaP-AR or (B) LNCaP-F876L cells were treated with increasing doses of R1881. Following seven-day incubation, cell growth was measured by assessing DNA content using Hoechst dye. Error bars represent SD of triplicate samples from a representative experiment performed in duplicate.**

**A****B**

**Supplemental Figure 10. Effects of seviteronel in LNCaP xenografts.** (A) LNCaP cells were implanted in flanks of intact male SCID mice, grown to ~0.15 cm<sup>3</sup>, and randomized (n=5-6) to treatment with vehicle or increasing seviteronel doses, twice daily. Only the average tumor volume of 100 mg/kg seviteronel differed significantly from vehicle (two-way ANOVA; a,

p<0.0005). **(B)** LNCaP cells were implanted in the flanks of castrated male NSG mice receiving testosterone supplementation (5 mg/90 day sustained release pellet, ~1.9 mg/kg/d). When tumors reached ~0.25 cm<sup>3</sup> volume, animals were randomized (n=5) to 100 mg/kg seviteronel p.o. b.i.d. for 8 days. Animals were then euthanized and the expression of AR target genes in tumor tissues was analyzed by quantitative RT-PCR. Data is presented as fold change in the expression of AR target genes as plotted by grouped (three to five animals per group) analysis (GraphPad Prism 6.0) and evaluated by unpaired non-parametric t-test using two-tailed analysis of significance values. Error bars represent SEM.



**Supplemental Figure 11. Drug exposure in LNCaP-F876L xenograft study.** (A) Seminal vesicle weight was measured after euthanasia in each arm of the LNCaP-F876L xenograft study to verify substantial exposure to testosterone (castrate, n=9; +testosterone, n=6). Error bars indicate SEM. (B) Levels of enzalutamide, seviteronel, and abiraterone in plasma of mice in castrated and castrated + testosterone arms of the LNCaP-F876L study. Error represents SEM.

## **Supplemental Methods**

**NR Reporter Gene Assay.** CV1 cells were seeded in 96-well plates and transfected using Lipofectin reagent (Invitrogen) as described in the manufacturer's instructions. The DNA mixture for each 96 well plate consisted of either 100 ng pRST7-PR, 100 ng pRST7-GR, 100 ng pRST7-MR plus 2400 ng MMTV-luc, and 500 ng Renilla-luc. After overnight incubation, cells were treated with hormone for 24 hours. Cells were then lysed and quantified for luciferase activity using a Dual Luciferase Reagent.

**PSA-Luc Reporter Gene Assay.** CV1 cells were seeded in 96-well plates and transfected using Lipofectin reagent (Invitrogen) as described in the manufacturer's instructions. The DNA mixture for each 96 well plate consisted of 100 ng pcDNA-AR, 2400 ng PSA-Luc, and 500 ng Renilla-Luc. After overnight incubation, cells were treated with hormone for 24 hours. Cells were then lysed and quantified for luciferase activity using a Dual Luciferase Reagent.

**Cell Cycle Analysis (Propidium Iodide Staining).** LNCaP cells were treated with hormone for 24 hours and then resuspended in ice cold PBS. Ethanol was added dropwise to a final concentration of 80% and incubated overnight. Cells were washed in PBS and resuspended in 500  $\mu$ L PBS supplemented with 100  $\mu$ g DNaseI-free RNaseA and 50  $\mu$ g propidium iodide. Cell cycle analysis was performed after collecting ten thousand events using the Accuri C6 flow cytometer (BD Biosciences). Data were analyzed using the CFlow Plus program software.

**LNCaP Xenograft for Seviteronel Dose.** The National Cancer Institute (NCI) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and follows the Public Health Service (PHS) Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the Guide for the Care and

Use of Laboratory Animals. The study protocol was approved by the NCI Animal Care and Use Committee. Six-week-old, male, severe combined immunodeficiency (SCID) mice were obtained from the NCI-Frederick Animal Production Area.  $3 \times 10^6$  LNCaP cells were injected subcutaneously into the rear flank of each SCID mouse. Mice were monitored and weighed daily. Mice were randomized to treatment groups when tumor size reached  $\sim 100 \text{ mm}^3$ . The treatment groups were: vehicle bid, seviteronel 15 mg/kg bid, seviteronel 50 mg/kg bid, and seviteronel 100 mg/kg bid (n=7). Fresh stock solutions of test articles were prepared in 0.5% CMC in Millipore water on a more than weekly basis and stored at 4°C. The stock solutions were diluted with vehicle (0.5% CMC) prior to oral dosing to maintain a consistent dosing volume (100  $\mu\text{L}$ /20 g body weight). An oral bid dosing interval of 10-14 hours was maintained throughout the study. Tumor measurements were taken three times a week for four weeks, with tumor volume being calculated by  $V = (\pi/6)*l*w*h$ .

**LNCaP Xenograft for Seviteronel Pharmacodynamic Response.** Duke University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and follows the Public Health Service (PHS) Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the Guide for the Care and Use of Laboratory Animals, 8<sup>th</sup> ed. The study protocol was approved by the Duke University Animal Care and Use Committee. Six-week-old, male NSG (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ) mice were obtained from Jackson Laboratories. Animals were orchietomized and testosterone treatment pellets (5 mg/90 d, Innovative Research of America) were implanted sc in the scapular region during the same surgery. 24 hours later,  $3 \times 10^6$  LNCaP cells mixed 1:1 with Matrigel (BD Biosciences) were injected subcutaneously into the rear flank of each mouse. Mice were monitored and weighed three times weekly. When the average tumor size reached  $\sim 0.25 \text{ cm}^3$  (0.17-0.29  $\text{cm}^3$ ), mice were randomized (n=5, average tumor volume/group = 0.22 or 0.24  $\text{cm}^3$ ) to treatment with vehicle (0.5% CMC) or seviteronel (100 mg/kg). Fresh stock solutions of



test articles were prepared in 0.5% CMC in Millipore water on a more than weekly basis and stored at 4°C with constant agitation. An oral bid dosing interval of 8-10 hours was maintained throughout the study. Tumor measurements were taken three times weekly for one week, with tumor volume being calculated by  $V = (l \times w^2)/2$ . On the final day of treatment, animals were euthanized 2 hours after the final dose and tumor tissues immediately preserved in LN<sub>2</sub>. Frozen tissues were pulverized and RNA was extracted as described.

**Measurement of Seminal Vesicle Weight.** Seminal vesicles were removed and weighed at necropsy, and seminal vesicle weight/body weight prior to necropsy (mg/g) was calculated.

**Drug Plasma Levels.** Plasma levels of seviteronel, abiraterone, and enzalutamide from the F876L xenograft study were determined using validated LC-MS assay developed at OpAns (Durham, NC).