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14. ABSTRACT

Prostate cancer is the most frequently diagnosed male cancer and second leading cause of male cancer death. Management of patients with advanced-stage disease relies on inhibiting the androgen receptor (AR) with conventional endocrine targeting therapies, and more recently with second-generation endocrine targeting therapies designed to block AR activity that re-emerges during castration. However, despite a growing armamentarium of drugs targeting the androgen/AR signaling axis, progression of castration-resistant prostate cancer (CRPC) remains a major clinical challenge that undermines survival and quality of life for prostate cancer patients. The proposed research is focused on the pre-clinical development of VPC14228, a drug-like small molecule that targets the AR:DNA interaction. During the first year of this award, we have made progress in investigating the functional effects of VPC14228 on DNA interaction and transcriptional activation mechanisms for AR, developing a definitive experimental and structural characterization of the VPC14228 interaction with the AR DBD, and conducting pre-clinical evaluation of VPC14228.

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1. INTRODUCTION:

Prostate cancer is the most frequently diagnosed male cancer and second leading cause of male cancer death. Management of patients with advanced-stage disease relies on inhibiting the androgen receptor (AR) with conventional endocrine targeting therapies, and more recently with second-generation endocrine targeting therapies designed to block AR activity that re-emerges during castration. However, despite a growing armamentarium of drugs targeting the androgen/AR signaling axis, progression of castration-resistant prostate cancer (CRPC) remains a major clinical challenge that undermines survival and quality of life for prostate cancer patients. The proposed research is focused on the pre-clinical development of VPC14228, a drug-like small molecule that targets the AR:DNA interaction. The goals of this research are to investigate the functional effects of VPC14228 on DNA interaction and transcriptional activation mechanisms for AR, develop a definitive experimental and structural characterization of the VPC14228 interaction with the AR DBD, and conduct pre-clinical evaluation of VPC14228.

2. KEYWORDS:

Androgen receptor, castration-resistant prostate cancer, DNA binding domain, androgen response element, AR inhibitor, chromatin, x-ray crystallography, pre-clinical

3. ACCOMPLISHMENTS:

a) Major goals of the project

1.1. Assay luciferase reporters in prostate cancer cell lines (months 1-12).
1.2. Determine effects of VPC14228 on AR binding to genomic AREs (months 13-24
1.3. Chromatin immunoprecipitation (ChIP) assays with lentivirus-infected cells to test AR-V binding to genomic AREs (months 13-24)
1.4.Obtain approval for mouse xenograft studies from U of M IACUC and USAMRMC ACURO (months 1-6).
1.5.Establish xenografts with prostate cancer cell lines to generate tissue for chromatin fractionation and ChIP-seq assays (months 7-36)
1.6. Perform chromatin fractionation assays with prostate cancer cell lines and xenografts (months 1-6).
1.7. Perform ChIP-seq with prostate cancer cell lines and xenografts
2.1. Form crystals of ARdbd in complex with ARE DNA (Months 1-6).
2.2. Optimize ligand soaking conditions (Months 7-18).
2.3. Collect native and Zn- or Br-anomalous X-ray diffraction data from VP14228-soaked crystals using synchrotron radiation (Months 18-21).
2.4. Obtain phases by molecular replacement and single wavelength anomalous dispersion (Months 18-21).
2.5. Identify the ligand and its binding site, build the VP14228-ARdbd-DNA model, and refine the structure (Months 21-24).
2.6. Crystallize ARdbd or ARtnd in the presence and absence of VP14228 without DNA (Months 1-20).

2.7. Soak VP14228 into apo ARdbd or ARtnd crystals (Months 12-20).
2.8. Collect native and Zn-anomalous X-ray diffraction data from co-crystallized, soaked, and apo ARdbd or ARtnd crystals, with the final data sets collected using synchrotron radiation (Months 20-24).
2.9. Obtain initial phases by molecular replacement and single wavelength anomalous dispersion (Months 24-26).
2.10. Build the model and refine the structure (Months 26-30).
3.1.Molecular modeling of derivatives of our current lead VPC-14228 (months 1-30)
3.2.Synthesis of derivatives of our lead compounds (months 6-30).
3.3.Experimental evaluation of the developed synthetic derivatives (months 4-30
3.4. Selection of several lead compounds for pharmacological development

b) Accomplishments under these goals

Task 1.1. Assay luciferase reporters in prostate cancer cell lines (Minnesota Site):

We found that VPC14228 inhibited activity of PSA-LUC and MMTV-LUC reporters in LNCaP cells (Fig. 1), but paradoxically activated a 4XARE-LUC reporter in R1-AD1 cells (Fig. 2). The Vancouver site has also observed cell line variability in luciferase reporter readouts following VPC14228 treatments. This finding indicated that luciferase-based studies should be de-emphasized because this approach is more prone to artifacts than studies with endogenous genes. This also indicated that it may be more advantageous to switch to a derivative inhibitor of the AR DBD, termed VPC14449. Therefore, based on discussion with the two collaborating sites, we collectively shifted our efforts to elucidating mechanism of action of VPC14449.

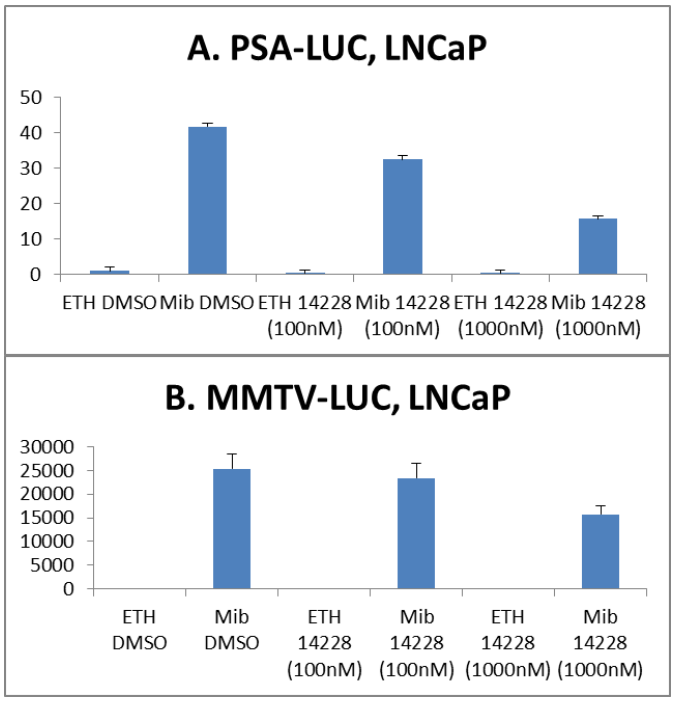


Fig. 1. LNCaP cells were transfected with A) PSA-LUC or B) MMTV-LUC reporters and treated with combinations of mibolerone (Mib) and VPC14228 at the indicated concentrations. Ethanol (ETH) and DMSO served as vehicle controls for mibolerone and VPC14228, respectively.

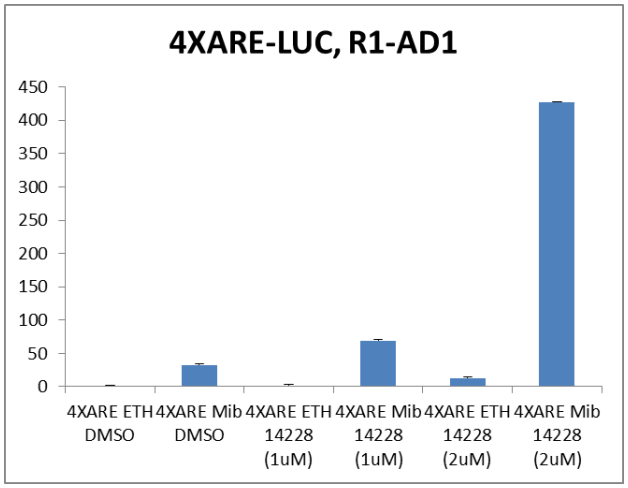


Fig. 2. R1-AD1 cells were transfected with 4XARE-LUC and treated with combinations of mibolerone (Mib) and VPC14228 at the indicated concentrations. Ethanol (ETH) and DMSO served as vehicle controls for mibolerone and VPC14228, respectively.

Task 1.2. Determine effects of VPC14228 on AR binding to genomic AREs (Minnesota Site):

We used electrophoretic mobility shift assays to test the effect of VPC14449 on AR binding to DNA *in vitro*. In nuclear extracts prepared from COS7 cells transfected with an AR expression construct, VPC14449 has no effect on AR binding to an androgen response element (ARE) (Fig. 3). This indicated that a chromatin environment may be required for effects of VPC14449. Therefore, we focused our efforts on chromatin-based assays.

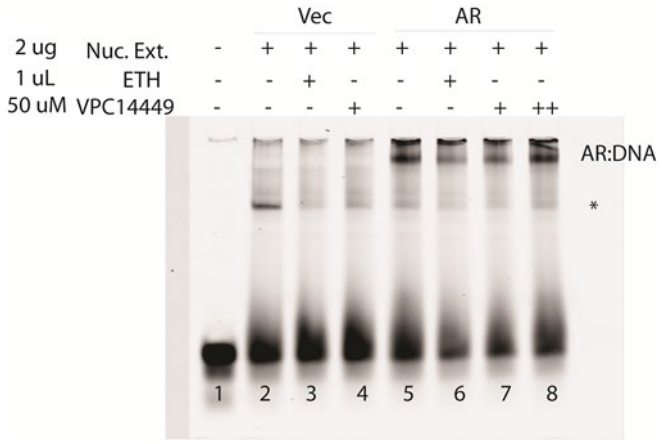


Fig. 3. COS7 cells were transfected with an AR expression construct (AR) or vector control (Vec) 48h prior to preparation of nuclear extracts. Nuclear extracts were incubated with a labeled DNA probe containing a consensus androgen response element in the presence or absence of VPC14449 as indicated. * denotes non-specific binding. AR:DNA indicates the complex between AR and the labeled DNA

Task 1.3. Chromatin immunoprecipitation (ChIP) assays with lentivirus-infected cells to test AR-V binding to genomic AREs (Minnesota Site):

To test the effects of VPC14449 on the ability of AR and AR-Vs to bind discrete AREs, we performed chromatin immunoprecipitation (ChIP) with antibodies specific for the AR NH2-terminal domain (NTD). VPC14449 inhibited full-length AR binding to AREs in the FASN, FKBP5, and TSC2 loci (Fig. 4A). VPC14449 also inhibited ARv567es binding to AREs in the FASN and FKBP5 loci, but had no effect on ARv567es binding to the TSC2 ARE (Fig. 4B). This

aligns with chromatin fractionation data (Fig. 2), supporting the concept that VPC14449 inhibits binding of AR-Vs to select AREs *in vivo*.

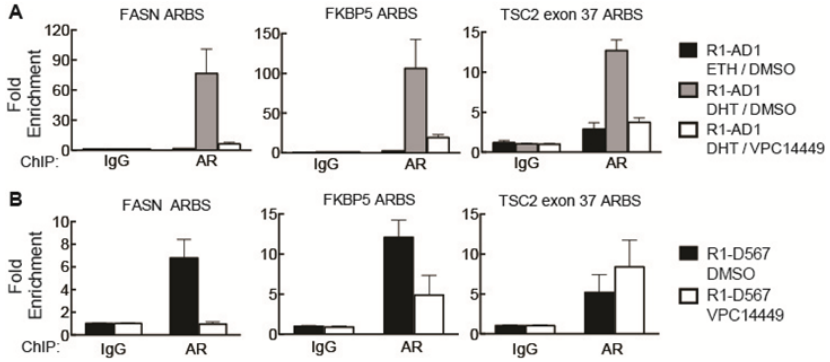


Fig. 4. (A) R1-AD1 cells expressing full-length AR were subjected to ChIP to test the impact of VPC14449 on AR binding to AREs in the FASN, FKBP5, and TSC2 loci. **(B)** R1-D567 cells expressing ARv567es were subjected to ChIP to test the impact of VPC14449 on ARv567es binding to AREs in the FASN, FKBP5, and TSC2 loci.

Task 1.4.Obtain approval for mouse xenograft studies from U of M IACUC and USAMRMC ACURO (Minnesota Site):

U of M IACUC approval was obtained Feb 27, 2012. USAMRMC ACURA approval was obtained Dec. 14, 2014.

Task 1.5.Establish xenografts with prostate cancer cell lines to generate tissue for chromatin fractionation and ChIP-seq assays (Minnesota Site):

We were unable to obtain xenografts from mice treated with AR DBD inhibitors because none of the compounds developed for the study had adequate drug-like properties that would result in appropriate tumor tissue exposure following treatment of tumor-bearing mice. Therefore, all studies performed at the University of Minnesota site were conducted in prostate cancer cell lines grown in vitro.

Task 1.6. Chromatin immunoprecipitation (ChIP) assays with lentivirus-infected cells to test AR-V binding to genomic AREs (Minnesota Site):

We next used subcellular fractionation techniques to test the effects of VPC14449 on AR and AR variant (AR-V) expression, subcellular localization, and binding to chromatin AREs *in vivo*. VPC14449 did not inhibit expression levels of AR or AR-Vs, and did not affect the ability of these species to translocate to the nucleus (Fig. 5A). However, VPC14449 inhibited chromatin binding of full-length AR and AR-Vs, although the effect on full-length AR was more pronounced (Fig. 5A). Similarly, VPC14449 inhibited chromatin binding of AR and AR-Vs in additional models of full-length AR expression (Fig. 5B), AR-V expression (Fig. 5C), or mixed AR/AR-V expression (Fig. 5D).

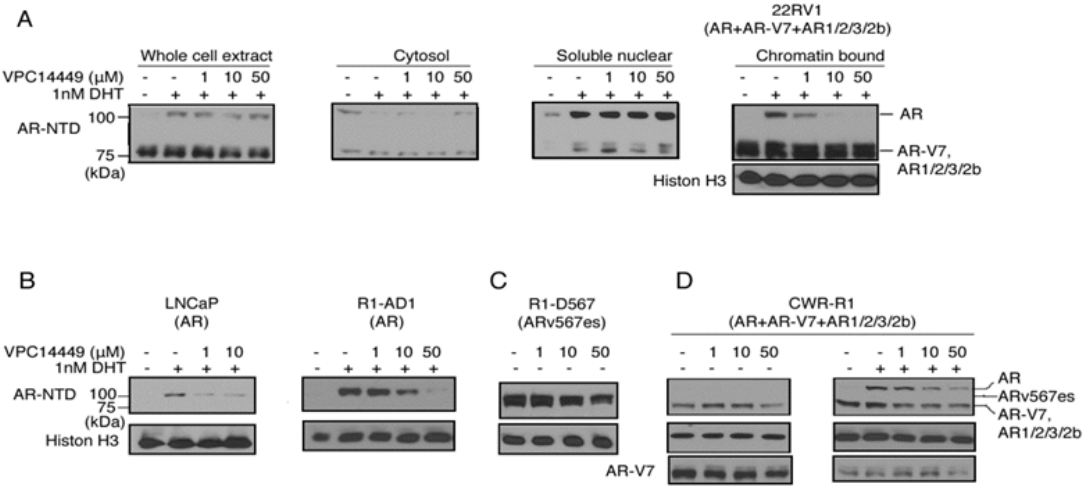


Fig. 5. (A) 22Rv1 cells were treated with dihydrotestosterone (DHT) and VPC14449 as indicated, and subjected to subcellular fractionation to prepare whole cell extracts as well as cytosolic, soluble nuclear, and insoluble nuclear (chromatin) fractions. Cell fractions were subjected to western blot with an antibody specific for the AR NTD. Chromatin fractions from **(B)** LNCaP and R1-AD1 cells expressing full-length AR, **(C)** R1-D567 cells expressing AR v567es, and **(D)** CWR-R1 cells expressing AR, AR-V7 and AR 1/2/3/2b were subjected to western blot with an antibody specific for the AR NTD as in A

Task 1.7. Perform ChIP-seq with prostate cancer cell lines and xenografts (Minnesota Site):

We used a ChIP-seq approach to determine the changes to genome wide interactions of the full-length AR and Arv567es (a truncated variant related to ARV7) by VPC-14449 treatment in two isogenic cell-lines (R1-AD1 and R1-d567). We found that VPC14449 provided a robust blockade of full length AR binding to chromatin in R1-AD1 cells. However, VPC14449 had a less pronounced effect on inhibiting AR-V binding in R1-D567c cells **(Fig. 6)**.

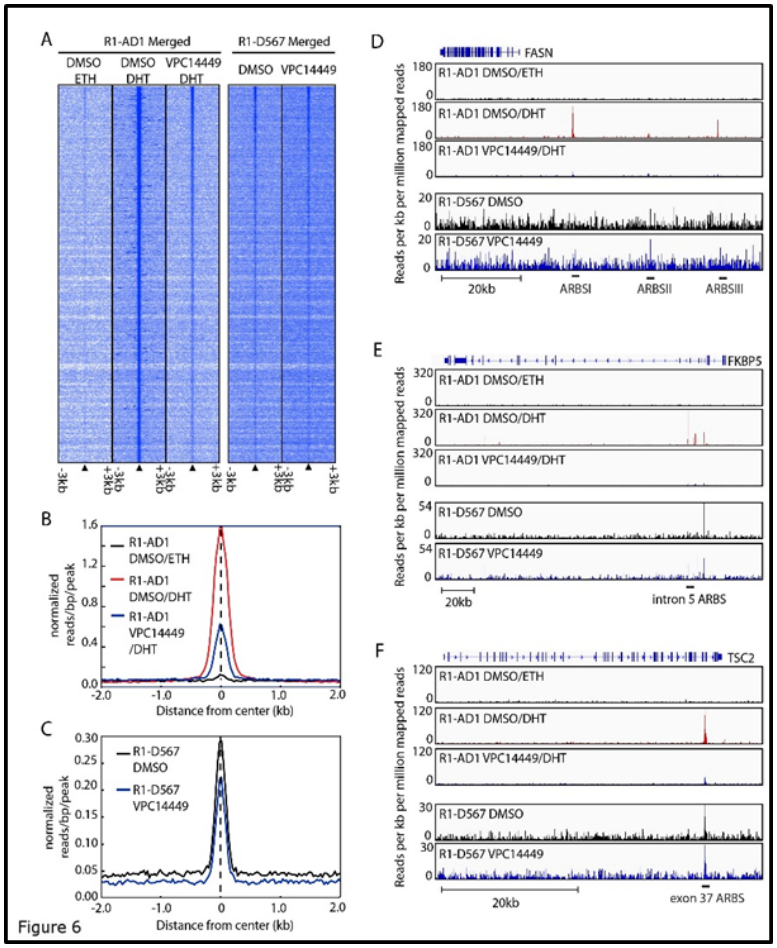


Figure 6

Fig. 6. (A) Heatmap of ChIP-seq signals ± 3kb around R1-AD1 AR peak midpoints from two biological replicate experiments for R1-AD1 cells (left panel). The same set of binding sites was also called from three biological replicate experiments for R1-D567 cells (right panel). Data are from cells treated with 50 uM VPC14449 or vehicle (DMSO) in the presence of 1 nM DHT or vehicle (ETH) as indicated. Average ChIP-seq signals shown in mapped reads per base pair per peak normalized per 106 reads from 2 datasets at binding sites identified in R1-AD1 cells (B) and 3 datasets in R1-D567 cells (C). (D) Gene track view of ChIP-seq data at FASN locus in R1-AD1 and R1-D567 cell with indicated treatments. Common AR/ARv567es binding site (ARBS) inside common ARBSs is indicated. (E) Gene track view of ChIP-seq data at FKBP5 locus. Putative downstream ARBS is indicated. (F) Gene track view of ChIP-seq data at TSC2 locus. Common ARBS in TSC2 exon 37 is indicated.

Key Outcomes for Hauptman Woodward Institute Site (Gewirth, PI)

Key Outcome 1: Direct binding measurements of the purified ARdbd with VPC-14449 using Isothermal Titration Calorimetry. Given the difficulty in co-crystallizing ARdbd and VPC-14449, we decided that it was imperative to confirm that an interaction between the two was possible using unbiased biophysical approaches. To this end, ITC experiments were conducted to measure the binding affinity between the purified ARdbd and VPC-14449 in order to help guide condition for crystallizations. Although ITC experiments were not explicitly proposed in the SOW, it is certainly an implied option in any structure determination project, and we felt that in this case it was a necessary step to better understand the protein-ligand interaction and to determine what concentration of VPC-14449 would be required for co-crystallization or soaking trials. ITC experiments indicate a K_D of approximately 64 μM binding affinity between VPC-14449 and the purified ARdbd in the absence of DNA. It is predicted the binding affinity would be significantly greater in the presence of DNA, but this experiment would be difficult to perform by an ITC approach, which traditionally measures an interaction between two (not three) purified components. A control ITC was also performed between the purified ARdbd and a DNA oligonucleotide (called ADR3) to confirm the protein was able to bind to its normal target (K_D=56 nM, shown in our published article).

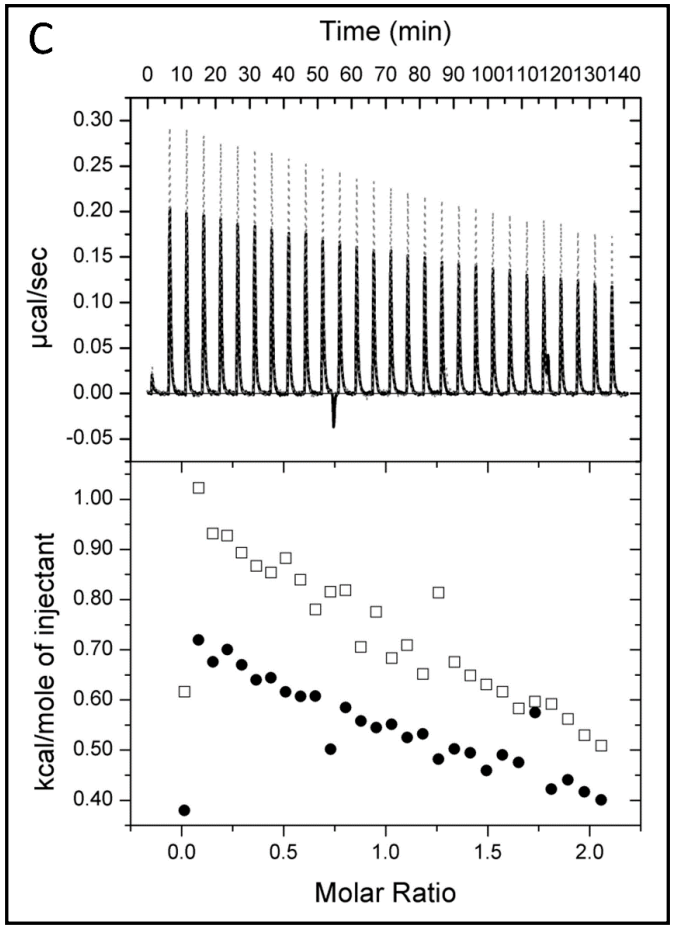


Figure KO1. Isothermal titration calorimetry of AR-DBD against VPC-14449 (dotted lines) is overlaid with the titration of the protein into buffer (heavy lines). (***Top***) The raw ITC data expressed as the change in thermal power with respect to time over the titration period. (***Bottom***) The lower panel shows the integrated heats. The AR-DBD C552A protein (1 mM) was in the syringe and the inhibitor VPC-14449 (100 μM) was in the cell.

Key Outcome 2.Protein crystallization of ARdbd in the presence of DNA and soak of VP14449. Crystals of ARdbd plus DNA were formed and shown to have the expected diffraction properties.

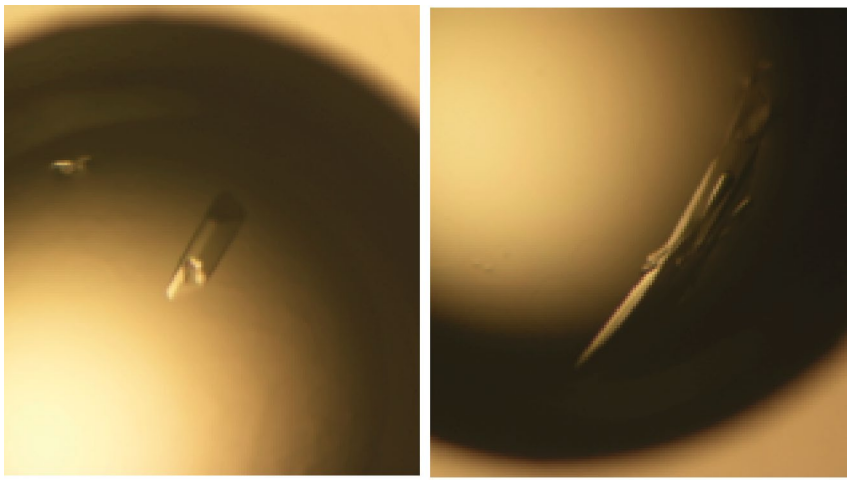


Figure KO2. Crystals of ARdbd + DNA. The crystals were subsequently soaked with 2 mM VP14449 compound. Evidence for the bound ligand was not seen in the electron density maps calculated from diffraction data from these soaked crystals.

The crystals were soaked with compound VP14449 at a concentration of 2 mM. X-ray diffraction data sets were collected from the soaked crystals and the structure was solved by molecular replacement. There was no evidence for the bound ligand in the crystal structure. The concentration of the ligand used in the soaking experiment was higher than the experimentally observed binding constrant (2 mM vs. 64 uM). The failure to observe the ligand in the crystal structure indicates that the ligand could not access the ARdbd binding surface. This indicates that the likely target for the inhibitor is the ARdbd prior to DNA complex formation.

Key outcome 3: Crystals of ARdbd.

The ARdbd protein was subjected to crystallization trials. Small crystals were obtained (red boxes). Evaluation by SONICC testing confirmed that the crystals were proteinacious. Attempts to reproduce the crystals yielded small crystals that exhibited oxidation sensitivity. Due to the small size and difficulty in handling these crystals, the diffraction properties of these crystals were not able to be evaluated.

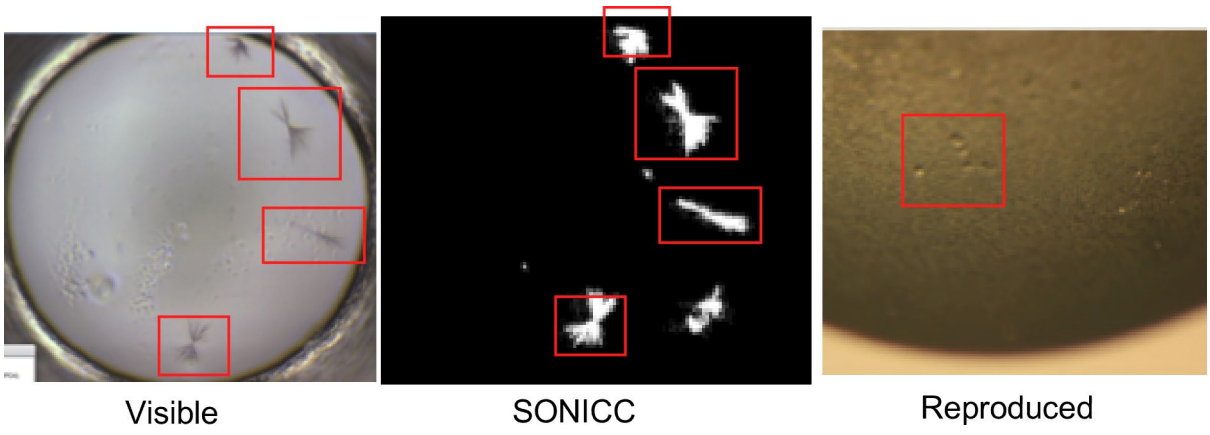


Figure KO3. Crystals of apo ARdbd. Visible and SONICC imaging photos are displayed in the first 2 panels. Crystal correspondences are indicated by red boxes.

Key outcome 4: Crystals of ARdbd cocrystallized with VP14449.

A broad screen of crystallization conditions was tested in order to obtain co-crystals of ARdbd in complex with VP14449. Several crystallization hits were observed (red boxes). The crystals were evaluated with SONICC imaging to distinguish protein crystals from salt. At least 4 conditions were observed to be SONICC positive. Attempts to reproduce these crystals were hampered by oxidation sensitivity of the ARdbd protein. However, these experiments indicate that co-crystallization of the dbd-ligand complex may be a feasible strategy for visualizing the VP ligand binding site and mode of binding. A steroid hormone receptor DNA binding domain has never before been crystallized in the absence of DNA.

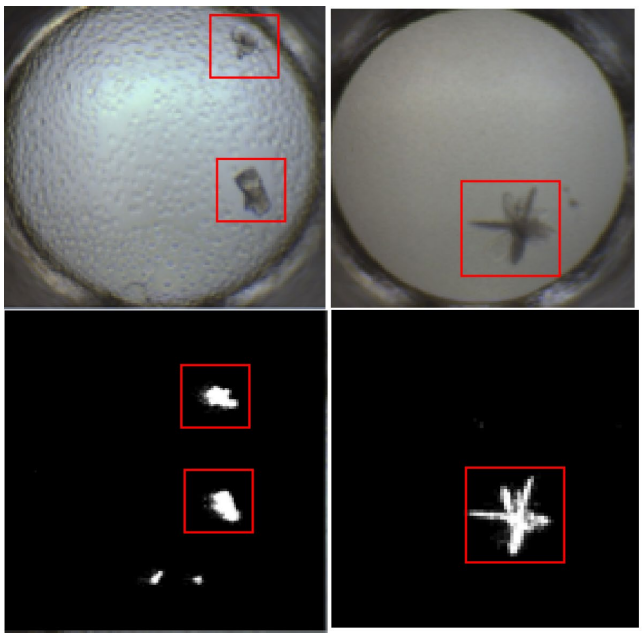


Figure KO4. Co-crystals of ARdbd in complex with VP14449. Correspondences between visible and SONICC images are in red boxes.

Key Outcomes for Vancouver Prostate Centre Site (Rennie, PI)

Key outcome 1: Using our in-silico model we identified and characterized 8 novel small molecule compounds inhibitor of the AR with an IC_{50} under 500 nM in transcription assays.

A new round of medicinal chemistry was performed using the original X-ray structure from Dr. Gewirth's laboratory (Shaffer et al, PNAS 2004) and structural docking information from compounds VPC-14228 and VPC-14449. Weak structural points in their stability were also taken into account. The virtual molecular derivatives were docked into the DBD binding site using Glide and eHits program. Compounds that received moderate to higher scores by Glide SP were selected and re-docked using the eHiTS docking protocol. Selected docked ligands were subjected to additional on-site scoring using the Ligand Explorer (LigX) program and the pKi prediction module of the Molecular Operating Environment (MOE). With this information, a cumulative scoring of four different predicted parameters (Glide score, eHiTS score, LigX score and pKi predicted by the MOE) were generated with each molecule; receiving a binary 1, 0 score for every "top 20% appearance". The final cumulative vote resulted in approximately 22 compounds that consistently demonstrated high predicted binding affinity toward the DBD site.

Internal Number	IC_{50}_eGFP (μM)	PSA IC_{50} (μM)
14449	0.10	0.17
14512	0.16	0.16
14513	0.12	0.14
14515	0.25	0.36
14518	0.19	0.08
14524	0.40	0.29
14530	0.19	0.18
14534	0.04	0.07

All the 22 compounds were then screened for their ability to inhibit AR transcriptional activity using a nondestructive, cell-based enhanced green fluorescent protein (eGFP) AR transcriptional assay. All compounds were subjected to concentration-dependent titration to establish their corresponding IC_{50} values. To ensure these values were true positive hits in the AR transcriptional eGFP assay, we validated their activities by a second transcription assay, based on light detection instead of fluorescence, by quantifying their effect on the production of the prostate specific antigen (PSA) in prostate cancer cell lines. As expected, hit compounds induced an equivalent decrease in PSA levels in LNCaP cells similar to the IC_{50} values found with the eGFP assay. The compounds that were found to display an IC_{50} below 1 μM. For comparison purposes, in this assay, gold standards Enzalutamide and Bicalutamide displayed IC_{50}'s of 100 nM and 600 nM, respectively. Importantly, seven new compounds displayed excellent activity under 500 nM.

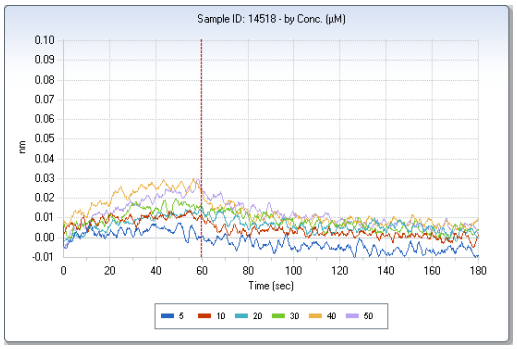
Key outcome 2: These new compounds effectively reduced the growth of prostate cell lines including an Enzalutamide-resistant cell line. They also showed no direct interaction with the AR by Biolayer Interferometry experiments.

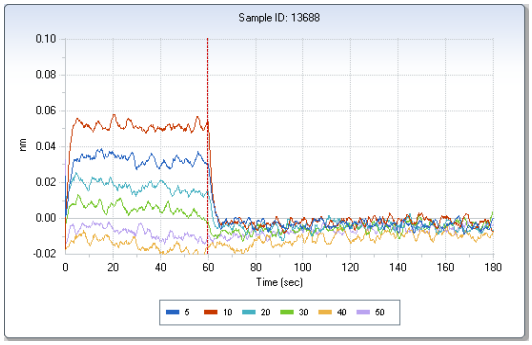
Internal Number	IC_{50} (μM)
14449	0.50
14512	0.60
14513	0.30
14515	0.75
14518	0.45

14524	0.75
14530	0.30
14534	0.15

To determine the translational potential of the most potent DBD inhibitors, we evaluated their ability to reduce growth of prostate cell lines stimulated by the androgen R1881. The cell viability was assessed after 3 days of incubation with the test compound in a concentration dependent manner. The selected compounds effectively inhibited the growth of LNCaP cells as scored by IC_{50}'s between 0.15 – 0.75 μM. These compounds did not show any growth inhibiton on AR-independent PC3 cell lines, confirming their AR-specificity. Moreover, these compound were also very effective in inhibiting the growth of MR49F cells resistant to Enzalutamide at IC_{50}'s similar to those observed in LNCaP cells.

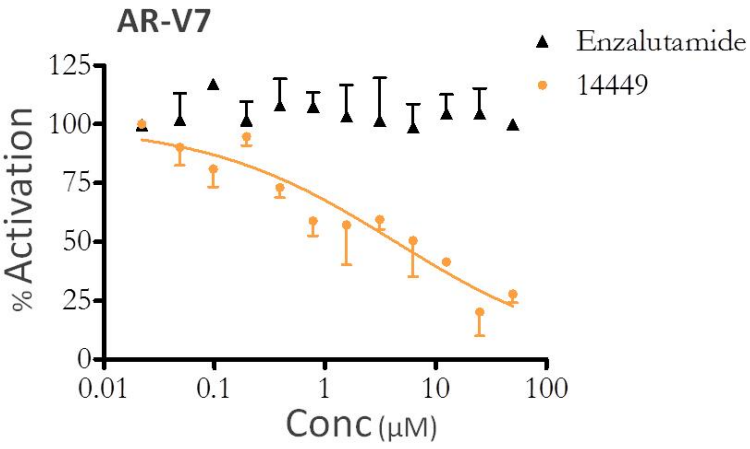
Biolayer interferometry (BLI) studies demonstrate if a direct reversible interaction between compounds and a purified AR ligand binding domain (LBD) occur in a dose dependent manner. As our compounds are predicted to bind to the DBD we should not be able to detect such an interaction. Our data confirmed that there is indeed no interaction of the lead compound VPC-14518 with the LBD of AR (left panel), compared to the LBD binding positive control VPC 13688 (right panel).





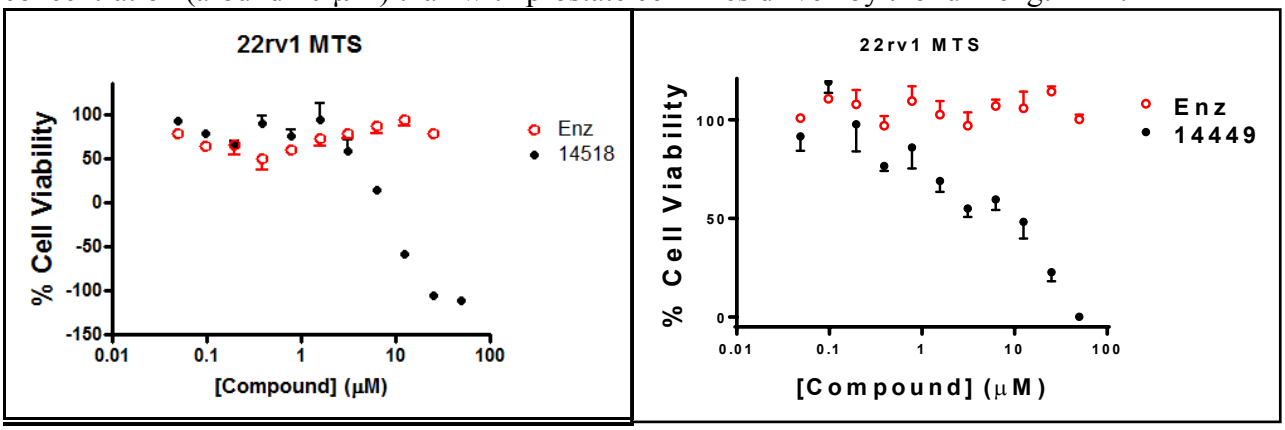
<u>Key outcome 3: These compounds effectively inhibited AR-V7 variants and reduced the growth of prostate cell lines driven by the AR variant V7.</u>

Because most AR splice variants retain the DBD domain they should be vulnerable to inhibition by DBD-inhibitor compounds. We evaluated transcriptional activity inhibition of variant AR-V7 using a luciferase reporter assay under the control of an AR promoter. The activity of transiently expressed AR-V7 was reduced with increasing concentrations of VPC-14449 without altering AR-V7 expression. Control experiments with Enzalutamide showed no effect on AR-V7 activity and were consistent with the absence of the LBD from this variant. Notably, the compounds did not achieve complete inhibition and were less effective against the transcriptional activity of AR-V7 (IC_{50} 4–8 μM) when compared with inhibition of the full-length receptor. All



compounds with IC_{50}'s less than 500 nM were tested in this system.

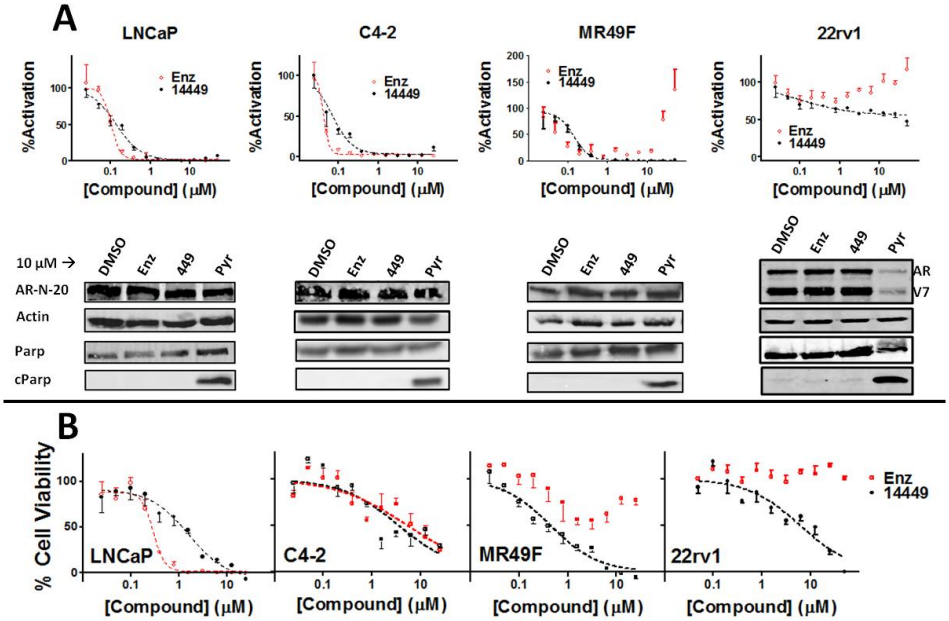
Furthermore, we investigated the capability of these compounds to inhibit the growth of the prostate cancer cell line 22rv—that is known to be driven by an AR variant (AR V7). Lead compounds VPC-14449 and VPC-14518 effectively inhibited the growth of this cell line, albeit at a higher concentration (around 10 μM) than with prostate cell lines driven by the full length AR.



Key outcome 4: Half-life of these compounds in metabolic conditions were improved, as few of the very active compounds initially displayed a half-life under 20 minutes in microsome studies.

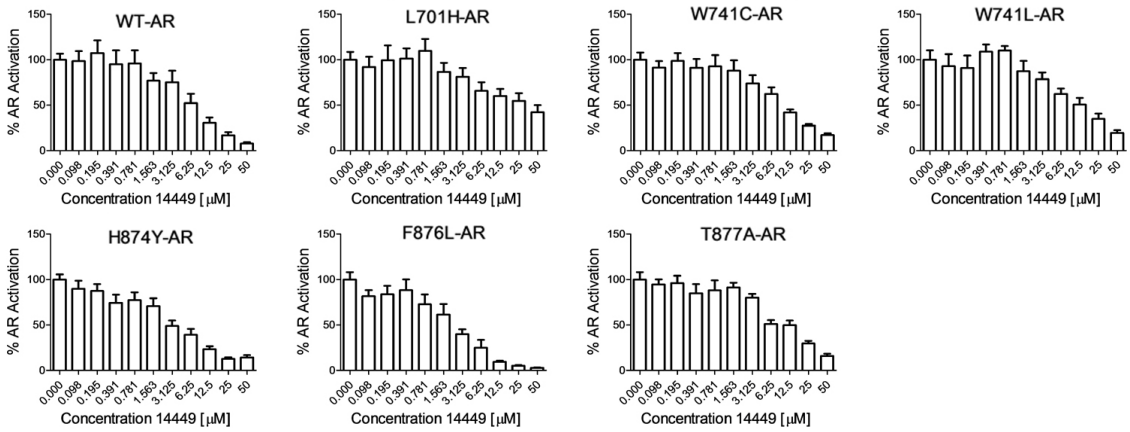
All compounds effective in *in vitro* assays were evaluated for their metabolic stability. All compounds tested were soluble up to 50 μM in media (data not shown). Preliminary microsomal stability data demonstrated our previous compounds displayed little metabolic stability. For example, compounds VPC-14228 and 14449 displayed 18 and 30 minutes of half-life in microsome experiments, respectively. However, our new lead compound VPC-14518 was found to have a half-life of 263 minutes. All the novel compounds were tested using this technology.

Key Outcome 5: VPC-14449 inhibited AR-transcriptional activity and cell viability in drug-resistant PCa cell lines. VPC-14449 was tested in transcriptional activation and cell growth assays using various cell lines that express mutated versions of the AR (e.g., LNCaP, C4-2, MR49F) or truncated variants (e.g., 22rv1). The compound was effective to varying degrees in every cell line tested, indicating the bypass of drug-resistance mechanisms.



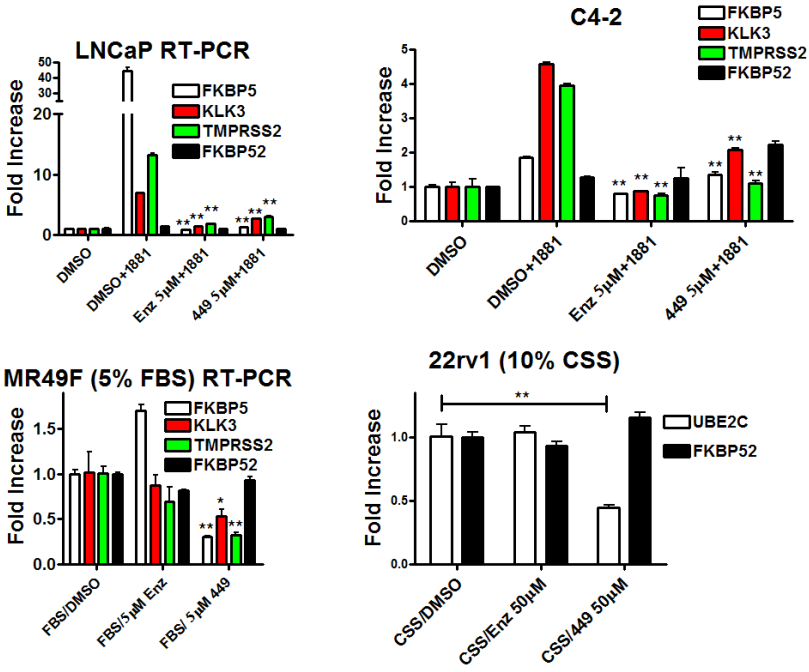
(Panel A) The indicated cell-lines were seeded (96 well plates, RPMI+CSS media) and co-transfected with ARR_3tk-Luc (50 ng/well) and Renilla-Luc (2 ng/well) plasmids (48 hours). With the exception of 22rv1 cells, 0.1 nM R1881 was used to stimulate the endogenously produced AR. Either enzalutamide (Enz) or VPC-14449 were administered (24 hours) at increasing compound concentration. Error bars represent the mean and SEM of 4 replicates for each concentration and are normalized to constitutive Renilla expression. 100% refers to normalized luminescence in the absence of compounds. Bottom panels in *A* show western blots of cell lysates after the indicated cell lines were treated for 24 hours with indicated compounds (10 μM) or DMSO control. *Pyr* refers to pyrvinium pamoate, a previously discovered AR-DBD inhibitor with toxic properties. Lysates were also probed with anti-PARP/cleaved PARP antibodies as a measure of apoptosis; AR-N20/ antibody for AR & ARV7; and actin antibodies. (Panel B) Cell viability was measured using an MTS assay with the indicated cell-lines, cultured in RPMI+CSS as in *A*, following 72 hours compound treatment with 1 nM R1881 (except 22rv1 = no R1881 treatment).

Key Outcome 6. VPC-14449 bypassed AR-LBD mutations involved in anti-androgen resistance. An AR-DBD specific inhibitor should bypass all mutations in the AR ligand binding domain (LBD) that are known to confer enzalutamide or bicalutamide resistance in tumor cells. After sequencing the androgen receptor in circulating free DNA from the blood of prostate cancer patients (Lallous et al., 2016, *Genome Biol.*) we selected six additional AR-LBD substitutions—that were ostensibly generated after anti-androgen treatment—and performed luciferase reporter assays in PC3 cells with transiently expressed AR encoding each individual point mutation. Consistent with the DBD as the site of action, the transcriptional activity of every tested AR-LBD mutant was inhibited by VPC-14449.



PC3 cells were co-transfected with a plasmid encoding the wild-type (WT) or mutant receptor and ARR_3tk-Luc reporter (50 ng/well each) and stimulation with 0.1 nM R1881. L701H, W741C/L, H874Y, F876L and T877A are all mutations that occur in the AR-LBD. 100% refers to the luminescence recorded in the absence of compounds.

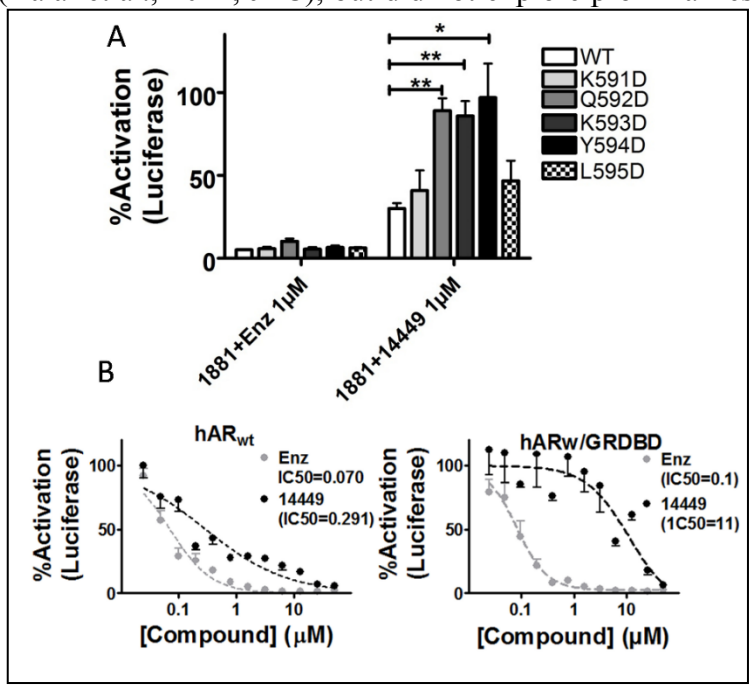
Key Outcome 7. Down-regulation of full length and variant target-genes by VPC-14449 treatment. The cell lines used in Key Outcome 5 were treated with VPC-14449 to evaluate AR target-genes suppression. The results demonstrated that VPC-14449 suppressed three archetypical downstream targets of the AR, including FKBP5, TMPRSS2 and KLK3 (aka PSA). In 22rv1 cells, high concentration of VPC-14449 suppressed UBE2C—an identified mitosis gene target of ARV7 (Hu et al., 2012, *Cancer Res.*).



PCa cells were cultured with 1 nM R881 and 5 μM compounds where indicated for 24 hours. LNCaP/C4-2 cells (RPMI+CSS) were cultured with or without R1881 and the indicated compounds, whereas MR49F cells were cultured in RPMI + 5% FBS (containing natural androgens) and compounds. 22rv1 cells (RPMI+10% CSS) were treated with 50 μM compound (no R1881). RT-PCR was performed to determine relative amounts of the indicated genes and FKBP52 negative control *=p-value ≤ 0.05, **=p-value ≤ 0.01 comparing expression b/w DMSO+R1881 and compound+1881 (LNCaP, C4-2), b/w FBS/DMSO and FBS/compounds (MR49F) and b/w CSS/DMSO and CSS/compounds (22rv1) treatment conditions.

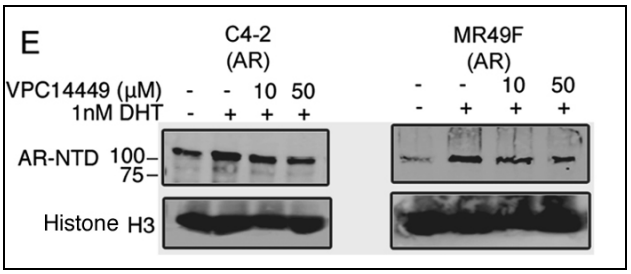
Key Outcome 8: Mutagenesis of the ARDBD delineated the binding site for VPC-14449. We originally proposed that the VPC-14449-DBD interaction depends on Q592 and Y594 side-chains

(Dalal et al., 2014, JBC), but did not explore proximal residues predicted to also facilitate binding. Thus, to identify other key residues, we mutated K591-L595 (KQKYL) of the AR and performed reporter assays in PC-3 cells. Three point mutants - Q592D, K593D and Y594D - weakened inhibition of the full-length AR (1 μM VPC-14449), revealing the central lysine as another critical side-chain for ligand recruitment (see figure below). In addition, simultaneous mutations of the binding site were introduced with a chimera construct in which the AR-DBD was replaced with the glucocorticoid receptor DBD, where the KQKYL sequence is replaced by QHNYL. Reporter assays with over-expressed AR showed that the chimera remained R1881-inducible and sensitive to Enzalutamide inhibition, but resisted VPC-14449 inhibition compared to the wild type AR.



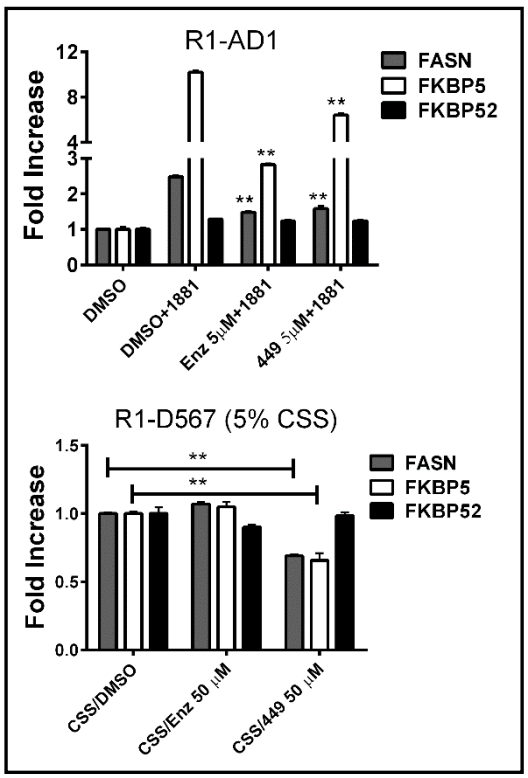
(A) PC-3 cells were transfected with the wild type/mutant AR and ARR_3tk-luc (48 hr) as described in Dalal et al., 2017, Mol Canc. Ther. Compound treatment and R1881 stimulation (24 hr) is followed by cell lysis and luciferase measurement. Error bars = mean ± SEM of 6 replicates per point. *=p-value < 0.05; **=p-value < 0.01. **(B)** PC-3 cells were co-transfected with wild-type or ARw/GRDBD receptor plasmids and ARR_3tk-Luc as in *A*. 100% = luminescence recorded without compounds (DMSO).

Key Outcome 9: VPC-14449 effects on AR-chromatin interactions in MR49F and C4-2 drug-resistant cell lines. We performed chromatin fractionation in an expanded panel of AR-positive cells treated with VPC-14449. In C4-2 (androgen insensitive) or MR49F (Enzalutamide resistant) cell lines, VPC-14449 at higher concentrations (>10 μM) was required to partially inhibit binding of AR [nuclear translocation stimulated by dihydrotestosterone (DHT)] to chromatin in the isolated chromatin-bound fraction. Successful chromatin fractionation was indicated by western blot of histone H3, a protein tightly associated with DNA.



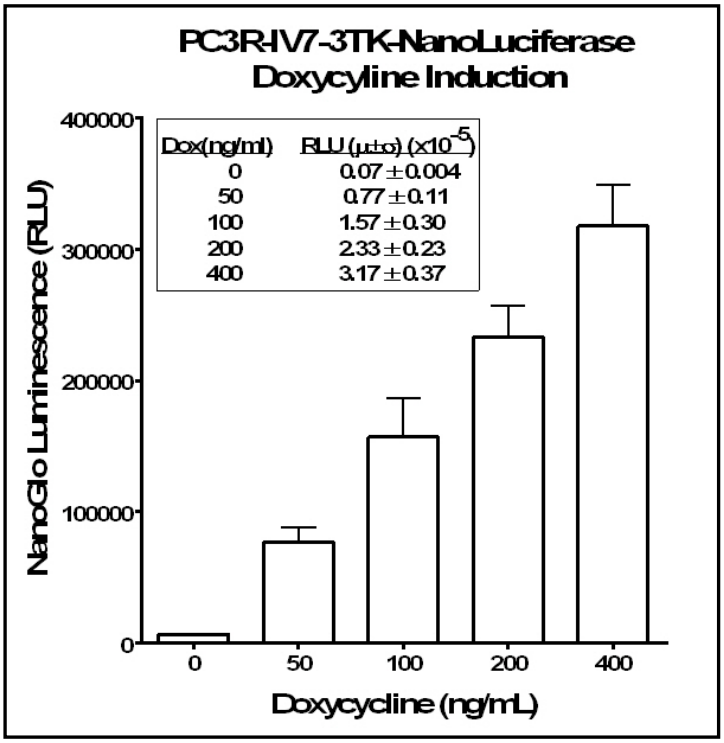
Western blot to detect the full-length AR in chromatin fractions from C4-2 (androgen insensitive) and MR49F (Enzalutamide resistant) cells treated with VPC-14449 and DHT. Each cell-line was treated with VPC-14449 for 24 hours followed by DHT mediated AR stimulation for 4 hours before lysis and isolation of the chromatin-bound fraction for western analysis. Antibodies against the AR-N-terminus (AR-NTD) and Histone H3 were employed.

Key outcome 10: VPC-14449 inhibition of AR-specific genes in R1-AD1 and R1-d567 cells. R1-AD1 cells, which were derived from heterogeneous CWR-R1 cell line and express full-length AR (developed in the Dehm Lab), displayed VPC14449-mediated inhibition of *FKPB5* and *FASN*, two genes under AR regulation in this cell line (top panel). Similar results were observed with R1-D567 cells that express only the truncated ARv567es variant, with *FKBP5* and *FASN* expression moderately suppressed by VPC-14449 (bottom panel). The GR-regulated gene, *FKPB52*, served as a negative control and was unaffected by treatments with androgens or AR inhibitors. For R1-AD1 cells, the AR was stimulated with synthetic androgen R1881.



In all RT-PCR experiments**,** extracted mRNAs were analyzed for relative amounts of AR or AR variant target-genes in each cell line. **(**top panel**)** R1-AD1 (RPMI+CSS) were cultured with or without 1 nM R1881 and 5 μM of the indicated compounds (24 h). R1-D567 cells (bottom panel) were maintained in RPMI + 5% CSS and were not stimulated with androgens—since ARv567es does not require androgen simulation for translocation to the nucleus and subsequent transcriptional activity. *=p-value ≤ 0.05, **=p-value ≤ 0.01 comparing expression between DMSO+R1881 and compound+1881 (R1-AD1) and between CSS/DMSO and CSS/compounds (R1-D567). Error bars = the mean ± SD with 3 replicates per bar. Experiments were repeated in at least two independent biological replicates.

Key outcome 11: Development of a doxycycline inducible AR-V7 cell line for high throughput screening. It has been very challenging to simultaneously hit the "sweet spots" of drug development—high potency, DBD-target specificity, and metabolic stability (which in turn leads to clinically relevant bioavailability). Most of the potent compounds tended to bind to both the DBD and LBD regions. Therefore, Roche and the VPC joint operations team (JOT) decided to develop screening assays that eliminated the AR-LBD region—which in turn encoded the ARV7 variant protein—from the current screening assays. Compounds that bind the ARV7 DBD would also bind the full-length AR DBD protein; however, this ARV7 screen would eliminate any compounds that bind solely to the LBD.



We generated stable cell populations encoding ARV7 cDNAs expressed from doxycycline inducible promoters. We were able to exquisitely control the timing (relative to compound treatment) and the amount of ARV7 being produced—which in turn enabled a robust and reproducible response.

Key outcome 12: Validating High Throughput ARV7 screen and complementary profiling assays to screen Roche's proprietary 1,600,000-compound library. It became apparent during our research agreement with Roche, that after evaluating 937 compounds we exhausted the medical chemical modifications to the original scaffold (i.e., the 14449-chemotype family) to identify a potent, DBD-target specific, and metabolically stable candidate inhibitor for clinical development. Roche and the VPC jointly decided to initiate the "back-up" path of our research agreement – the screening of Roche's proprietary 1,600,000-compound chemical library to identify candidates with novel DBD-binding scaffolds.

During the Year-4 Extension, we focused on (a) developing and validating the inducible ARV7 cellular assay as a high throughput screen (HTS) for the chemical library and (b) developing additional stringent biochemical/cellular assays to assess compound specificity. The latter assays included the ARV7 gene signature (V7GS), nuclear hormone receptor (NHR), bio-layer interferometry (BLI), surface plasmon resonance (SPR), and fluorescence resonance energy transfer (FRET)—to identify compounds that bind to both the DBD and the LBD regions. We also established an assay that identified compounds that bound specifically to the NanoLuciferase report enzyme, which in turn could inhibit reporter activity—thereby eliminating a class of "false positives."

c) Opportunities for training and professional development

Nothing to Report

d) Results disseminated to communities of interest

Nothing to Report

e) Plans for the next reporting period to accomplish the goals?

Nothing to Report (final report)

4. **IMPACT:**

a) Impact on the development of the principal discipline(s) of the project

Nothing to Report

b) Impact on other disciplines

Nothing to Report

c) Impact on technology transfer

Nothing to Report

d) Impact on society beyond science and technology

Nothing to Report

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

1. Tam, K., Dalal, K., Hsing, M., Cheng, C.W., Chiang, Y.T., Sharma, A., Peacock, J.W., Wang, W., **Cherkasov, A., Rennie, P.S.,** Gleave, M.E., and Ong, C.J. (2017) Androgen Receptor transcriptionally regulates Sema 3C in a GATA2-dependent manner. *Oncotarget.* 8(6):9617-9633.

2. Butler, M., Roshan-Moniri, M., Hsing, M., Lau, D., Kim, A., Yen, P., Mroczek, M., Nouri, M., Lien, S., Axerio-Cilies, P., Dalal, K., Yau, C., Ghaidi, F., Guo, Y., Yamazaki, T., Lawn, S., Gleave, M., Gregory-Evans, C., McIntosh, L., Cox, M.E., **Rennie, P.,** and **Cherkasov, A.** (2017) Discovery and characterization of small molecules targeting the DNA-binding ETS domain of ERG in prostate cancer. *Oncotarget.* 8(26):42438-42454.

3. Dalal, K., Munuganti, R., Morin, H., Lallous, N., **Rennie, P.S., and Cherkasov, A.** (2016) Drug-discovery Pipeline for Novel Inhibitors of the Androgen Receptor. *Meth. Mol. Biol. 1443:31-54.*

4. Dalal, K., Che, M., Que, N., Sharma, A**.,** Yang, R., Lallous, N., Borgmann, H., Ban, F., Li., H, Roshan-Moniri, M., Tam, K.J., Tse, R., Osistanbullu, D., LeBlanc, E., Gleave, M.E., **Gewirth, D., Dehm, S., Cherkasov, A.,** and **Rennie, P.S.** (2017) Bypassing drug-resistance

mechanisms of prostate cancer with small-molecules that target androgen receptor chromatin interactions. *Mol. Canc. Ther.* 16(10):2281-2291.

5. Ban, F., Dalal, K., Li, H., LeBlanc, E., **Rennie, P.S.,** and **Cherkasov, A.** (2017) Best Practices of Computer-Aided Drug Discovery: Lessons Learned from the Development of a Preclinical Candidate for Prostate Cancer with a New Mechanism of Action. *J. Chem. Inf. Model.* 57(5):1018-1028.

6. Ban, F. LeBlanc, E., Morin, H., **Rennie, P.S.**, and **Cherkasov, A.** (2018) Chemoinformatics driven development of novel therapies for drug resistant prostate cancer. *Mol. Inform.* E180043 doi: 10.1002/minf.22800043.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report

- **Other Products**

  AR ChIP-seq data of prostate cancer cell lines treated with VPC14449 are deposited in NCBI Gene Expression Omnibus (GEO) under accession GSE96084.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a) Individuals who have worked on the project:

University of Minnesota Site (Dehm, PI)

Name	Dr. Scott Dehm
Project Role	PI
Researcher Identifier	orcid.org/0000-0002-7827-5579
Person months worked	1.2
Contribution to project	Dr. Dehm provided project oversight, supervised research and data analysis carried out by Drs. Che and Yang, secured regulatory approvals, managed grant budget and reporting, and coordinated with co-PIs Rennie and Gewirth.
Funding support	

Name	Dr. Meixia Che

Project Role	Research Associate
Researcher Identifier	
Person months worked	9.6
Contribution to project	Dr. Che collected and analyzed data under supervision of Dr. Dehm
Funding support	

Name	Dr. Rendong Yang
Project Role	Research Associate (Bioinformatics)
Researcher Identifier	
Person months worked	3
Contribution to project	Dr. Yang provided bioinformatics support and planned ChIP-seq experiments
Funding support	

Hauptman Woodward Institute Site (Gewirth, PI)

Name	Dr. Daniel Gewirth
Project Role	PI
Researcher Identifier	Orcid.org/0000-0003-2702-3731
Person months worked	2
Contribution to project	Dr. Gewirth carried out oversight of the project, provided guidance and consultation to Dr. Que, and assisted with the collection of diffraction data.
Funding support	

Name	Dr. Nanette Que
Project Role	Staff Scientist
Researcher Identifier	Orcid.org/0000-0002-6955-7988
Person months worked	6
Contribution to project	Dr. Que carried out the protein purification and crystallization trials, collected X-ray diffraction data, and carried out the ITC binding experiments.
Funding support	

Vancouver Prostate Centre Site (Rennie, PI)

Name	Dr. Paul S. Rennie
Project Role	PI
Researcher Identifier	
Person months worked	2.4
Contribution to project	Dr. Rennie provided project oversight, and supervised research carried out by Dr. Dalal for the characterization of derivatives using biochemical assays.
Funding support	

Name	Dr. Artem Cherkasov
Project Role	Collaborator
Researcher Identifier	
Person months worked	2.4
Contribution to project	Dr Cherkasov oversaw all aspects of computational drug design, molecular modeling and bioinformatics.
Funding support	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

This report is for a COLLABORATIVE AWARD, and was prepared jointly by the three study PIs. The tasks are clearly marked with the responsible PI and research site.

9. APPENDICES

None