

**Evaluating PI3K/AKT inhibitors' sensitivity in
prostate cancer cell lines harboring androgen receptor
gene rearrangements**

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BY

Radha Manohar Kapgate

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Advisor: Dr. Scott Dehm

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Dedication

This dissertation is dedicated to my parents. Thank you for the support and sacrifices for letting your daughter live the American dream.

Abstract

Prostate cancer, particularly its advanced form known as castration-resistant prostate cancer (CRPC), continues to pose significant clinical challenges. The persistence and progression of CRPC are often driven by the androgen receptor (AR) reactivation, even under conditions of androgen deprivation. This study focuses on the PI3K/AKT pathway, a critical oncogenic driver in prostate cancer is often activated in tumors with AR gene structural rearrangements (*AR*-GSRs) due to loss of *PTEN* function. This thesis evaluates the hypothesis that the PI3K/AKT pathway could serve as an effective therapeutic target in CRPC cell lines harboring *AR*-GSRs, given its role in bypassing the androgen signaling cascade and independently promoting tumor survival and proliferation. Using prostate cancer cell lines engineered to express specific *AR*-GSRs, this research employed crystal violet growth assays to determine the sensitivity of cells to PI3K/AKT inhibitors. The findings reveal that cell lines with *AR*-GSRs display a distinct response to PI3K/AKT pathway inhibition compared to those without these rearrangements. Specifically, cells lacking the ligand-binding domain of AR due to gene rearrangements showed increased sensitivity to PI3K/AKT inhibitors, suggesting a potential therapeutic advantage in targeting this pathway. This thesis provides insight into the complex interactions between AR signaling and the PI3K/AKT pathway. It suggests a targeted therapeutic strategy that may enhance treatment outcomes in CRPC patients with specific genomic alterations. These findings could guide the development of personalized medicine approaches in the treatment of prostate cancer, particularly for those resistant to conventional therapies.

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Chapter 1: Introduction

Overview of Prostate Cancer:

Prostate cancer (PC) accounts for more than 25% of newly diagnosed cancer cases and one in seven men in the United States is likely to develop this disease. PC is the second leading cause of cancer-related deaths of men in the US with 34,700 deaths in 2023¹. Normal prostate homeostasis is crucial for the development of male reproductive organs and secondary sexual characteristics and is regulated through the activation of the Androgen receptor (AR) by androgens. Similarly, PC cells heavily rely on activating the AR through androgens, utilizing this pathway to promote their progression and survival².

Due to the dependence of PC cells on AR, current treatments for locally advanced or metastatic PC focus on Androgen Deprivation therapy (ADT) that includes lowering circulating androgens and inhibiting AR activity through methods like orchiectomy or luteinizing hormone-releasing hormone (LHRH) agonists³. Additionally, first-generation antiandrogens like bicalutamide are employed to competitively hinder androgen binding to the AR, preventing its activation⁴. However, most patients eventually develop resistance to these treatments and the disease progresses to castration-resistant prostate cancer (CRPC). Patients with CRPC can be treated with second-generation AR-targeted treatments such as abiraterone acetate, a potent biosynthesis inhibitor, and enzalutamide, an AR antagonist with a higher affinity for AR. A major limitation of these second-generation AR-targeted therapies is that primary and secondary resistance is driven by the reactivation of AR signaling⁵.

Androgen Receptor structure and activity:

The androgen receptor is a ligand-responsive nuclear transcription factor classified within the steroid hormone receptor family. This receptor family also includes the estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). Leydig cells in the testes primarily synthesize androgens under luteinizing hormone (LH) regulation from the anterior pituitary gland, which is itself controlled by gonadotropin-releasing hormone (GnRH). Testosterone mainly circulates bound to serum sex hormone-binding globulin (SHBG) and albumin, with only the free-form entering prostate cells. Upon entering the cells, testosterone is converted into the more potent DHT through the action of the enzyme 5-alpha-reductase, which binds strongly to AR, triggering a series of events including heat-shock protein displacement from AR and its nuclear translocation. In the nucleus, AR dimers bind to androgen response elements (AREs) in target gene promoter and enhancer regions, such as those of prostate-specific antigen (PSA protein is encoded by the *KLK3* gene) and transmembrane protease serine 2 (*TMPRSS2*), recruiting co-regulatory proteins for transcription, leading to cell growth and survival^{6,7}.

Androgen Receptor gene structure and function:

The *AR* gene is located at the locus Xq11-Xq12 of the X chromosome. The modular organization of the *AR* gene reflects the modular organization of the 110 kDa AR protein⁶. *AR* exon 1 encodes an N-terminal domain, which functions as the AR transcriptional activation region. Despite its intrinsically disordered nature, it contains activation function 1 (AF1), which is constitutively active and has a key role in protein-protein interactions. A central AR DNA binding domain (DBD)

is encoded by *AR* exons 2 and 3. The AR DBD consists of two zinc fingers that are critical for dimerization and sequence-specific DNA binding. A nuclear localization signal (NLS) encoded by *AR* exon 4 is located C-terminal to the AR DBD, within a hinge region that regulates AR nuclear import. AR exons 5-8 encode a C terminal ligand binding domain (LBD)⁸. The LBD facilitates the interaction of AR with heat shock proteins and chaperones and engages with the AR N-terminal domain in response to the binding of androgen hormones. The activation function-2 (AF-2), found within the LBD, is essential for creating a co-regulator binding site and facilitating direct interactions between the N-terminal domain and the ligand-binding domain. The presence of a ligand activates the AF-2 which in turn activates the downstream target genes of the AR.^{7,8,6}

Androgen receptor gene structural rearrangements (AR-GSR)

In PC, AR signaling fundamentally mirrors that in normal cells but is repurposed to drive cancer cell proliferation and survival⁹. After an initial heavy response to AR-targeted treatments, CRPC eventually develops concurrently with *AR* transcriptional reactivation¹⁰. AR-Variants (AR-Vs) have become recognized as a critical factor in developing resistance to treatments that target the androgen/AR pathway over the past few years. Diverse AR-Vs have been reported and higher levels of *AR* splice variants have been detected in CRPC when compared to hormone-naïve (HN) PC¹¹. Like the full-length Androgen receptor (AR-FL), AR-Vs have NTD and DBD domains but do not have the LBD, which is essential for hormonal regulation in the full-length receptor. The AR-Vs display constitutive transcriptional activation, even without a ligand.

One of the mechanisms contributing to the expression of AR-Vs involves Androgen receptor gene structural rearrangements (*AR-GSRs*). *AR* is one of the most frequently rearranged genes in CRPC after TMPRSS2:ERG fusion and PTEN mutations¹². *AR-GSRs* refer to structural alterations within the *AR* gene, characterized by at least one breakpoint that rearranges the *AR* gene body. These alterations can include duplications, deletions, inversions, and translocations, occurring at specific points within the gene's structure^{13, 14, 15}. For instance, a 35 kb tandem duplication of *AR* exon 3 and several cryptic exons, including CE3, contributes to the formation of truncated AR variants in the androgen-independent 22Rv1 cell line (Fig.1c). Due to this duplication, there is an increased distance between exons 3 and 4, potentially enhancing the likelihood of exon 3 splicing to exon CE3 or other cryptic exons. The duplication has been demonstrated to increase the expression of AR-V7 and other AR variants, in addition to the full-length AR in 22Rv1. Another prostate cancer cell line R1-AD1, which expresses the *AR-FL*, was modified using genetic engineering to create a new isogenic cell line named R1-D567(Fig 1b). This new cell line includes a deletion of exons 5 through 7 of the *AR* gene. This gene rearrangement is responsible for the expression of *AR* variant ARv567es in R1-D567, resulting from the *AR* gene rearrangements in the parental R1-AD1 line¹⁶.

Clinical implications of AR-GSRs

Studies have shown that *AR-GSRs* are more prevalent in CRPC compared to primary PC, where they are rare or nonexistent. However, these rearrangements are restricted in mCRPC cases, with occurrence rates between 25% and 50% of the patients who have already been treated with ADT or *AR*-targeted therapy^{17, 13, 18}. Findings by Henzler et al. reveal that *AR-GSRs* are present in 10 of the 30 metastatic CRPC tumors studied, affecting 6 out of 15 patients, displaying at least one *AR*-

GSR event. In contrast, these rearrangements were absent in primary prostate cancer samples¹⁷. Complementing these findings, De Laere et al. documented *AR*-GSRs in 15 out of 30 CRPC patients using liquid biopsy techniques, highlighting the prevalence of such genetic alterations in more advanced stages of the disease¹³. The occurrence of *AR*-GSRs in CRPC compared to patients with primary cancer, particularly in patients who have already been treated with AR-targeted treatments suggests that these genetic alterations provide a survival advantage to tumor cells in the androgen-depleted environment of CRPC, contributing to treatment resistance and disease progression.

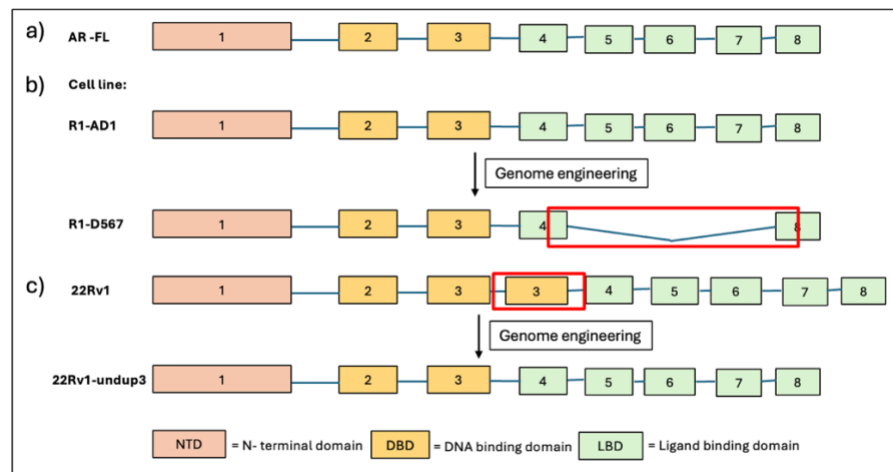


Figure 1: Schematic Representation of AR gene rearrangements in various mCRPC cell lines. a) Full-length AR protein b) R1-AD1 cell line expressing full-length AR and R1-D567, derived through genome engineering resulting in a deletion encompassing exons 5-7, leading to an exon 4-8 fusion. c) **22Rv1** contains a tandem duplication involving exon 3 and **22Rv1-undup3** an engineered version of 22Rv1 with the duplicated exon 3 region removed to restore a structure closer to AR-FL.

Functional significance of AR-GSRs

In CRPC, tumors harboring *AR*-GSRs frequently display elevated levels of both full-length AR and AR-Vs. *AR*-GSRs play a pivotal role in maintaining high AR activity under conditions of

androgen deprivation. Studies have shown that these rearrangements often lead to both the overexpression of full-length *AR* mRNA and the production of truncated, constitutively active AR-Vs capable of promoting AR signaling independent of androgen levels.^{19, 12} . Furthermore, in tumors with concurrent *AR* gene amplification and *AR*-GSRs, frequent expression of AR-V4, AR-V7, AR-V9, and additional novel AR-V species has been observed, potentially exhibiting additive effects on AR activity¹⁹.

However, the specific impact of *AR*-GSRs can vary with some resulting in the expression of AR-Vs. By bypassing the regulatory mechanisms that normally control *AR* activity, these variants confer a growth advantage to tumor cells in low-androgen environments and contribute to therapeutic resistance. The association between *AR*-GSRs and high AR activity is significant because it highlights how *AR*-GSRs can lead to sustained high AR signaling pathways, enabling CRPC cells to thrive even under androgen-deprived conditions.

Additionally, a previous study by the lab also provides significant insights into the relationship between *AR*-GSRs and PTEN loss in the context of CRPC. While *AR* gene rearrangements generally occur independently of changes in other key genes typically involved in prostate cancer progression, there is a notable exception with PTEN. For PTEN, the study suggested that instances of *AR* gene rearrangements are more frequently observed in the mCRPC tumor samples that had alterations in the *PTEN* gene¹².

AR and PI3K/AKT pathway in prostate cancer

The androgen receptor pathway and PI3K/AKT signaling pathways are two major oncogenic drivers in prostate cancer. In normal prostate homeostasis, the AR signaling pathway and the PI3K/AKT pathway regulate each other through reciprocal negative feedback inhibition. This regulatory interaction ensures that activation of one pathway can attenuate the other, maintaining cellular balance and preventing unchecked growth. Under normal physiological conditions, AR activity can inhibit PI3K/AKT signaling indirectly by modulating levels of the phosphatase PHLPP, which dephosphorylates and inactivates AKT. Conversely, activation of PI3K/AKT signaling, typically through growth factors like Insulin, Insulin-like Growth Factors (IGFs), Epidermal Growth Factor (EGF), etc. can suppress *AR*-mediated transcriptional activity, possibly through feedback effects on receptor tyrosine kinases that impact *AR* stability and localization.²⁰

In the context of PTEN loss, which is common in many prostate cancers, this reciprocal regulation is disrupted, leading to simultaneous activation of both pathways and promoting prostate tumor progression. PTEN normally acts to restrain PI3K/AKT signaling; thus, its loss leads to enhanced AKT activation. Enhanced PI3K/AKT signaling in the absence of PTEN suppresses AR activity initially, but therapeutic inhibition of PI3K or AR paradoxically activates the other pathway. For instance, PI3K inhibition in PTEN-deficient tumors can lead to increased AR activity by relieving negative feedback on upstream regulators such as HER kinases, which can enhance AR stability and function.²⁰ Similarly, AR inhibition can lead to increased AKT activation by reducing the levels of PHLPP, mediated by changes in the expression of *FKBP5*, a chaperone protein that stabilizes PHLPP. With less PHLPP available, AKT dephosphorylation is blocked, leading to

increased AKT activity²¹. This reciprocal feedback loop allows PC cells to adapt and survive when either pathway is inhibited alone but combined pharmacological blockade of both *AR* and PI3K/AKT signaling induces profound tumor regressions in preclinical models. Thus, in *PTEN*-deficient prostate cancers, targeting only one pathway is often insufficient due to compensatory activation of the other pathway.^{21,20}

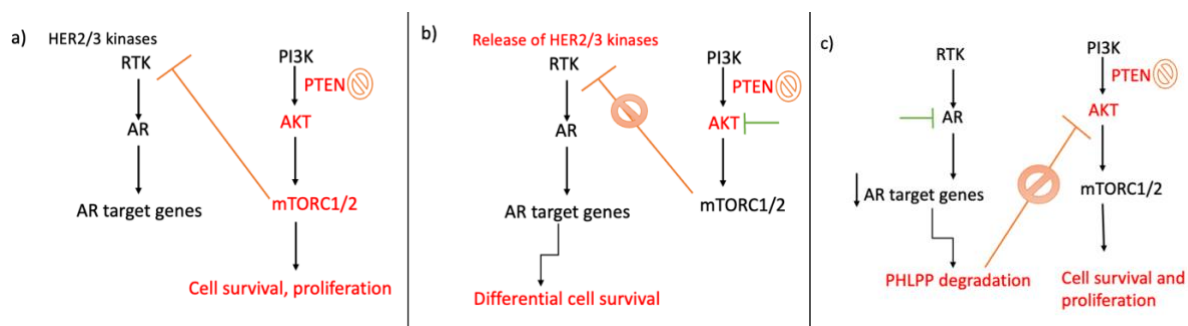


Figure 2: Crosstalk between AR and PI3K/AKT pathway prostate cancer with PTEN loss. a) In the case of cancer with PTEN loss, the activated AKT can suppress AR transcriptional activity by inhibiting the release of HER2/3 kinases at Receptor tyrosine kinase. b) Subsequently, inhibiting AKT in case of cancer with PTEN loss leads to the release of HER2/3 kinases that activate receptor tyrosine kinases (RTKs) and stimulate the AR that promotes the expression of AR target genes. This leads to the differential survival of the PC cells through the canonical AR pathway. c) AR inhibition by ADT can further activate the PI3K/AKT pathway as a compensatory survival mechanism via reduced levels of FKBP5, relieving PHLPP-mediated suppression of AKT.

AR-GSRs are found in the tumor sample with PTEN loss. Moreover, high *AR* expression is maintained despite the active PI3K/AKT pathway. This situation is particularly counterintuitive because activation of the PI3K/AKT pathway should generally suppress *AR* activity and vice versa. This observation suggests that *AR*-GSRs may uncouple *AR* from its typical inhibition by the PI3K/AKT pathway, thereby allowing both pathways to function highly and independently and

helping for the survival and proliferation of prostate cancer cells, particularly in the hormone-deprived environment of CRPC.

Moreover, a novel approach involves drug sensitivity imputation based on gene expression profiles. This method leverages data from the extensive Genomics of Drug Sensitivity in Cancer (GDSC) study, which assesses 367 drugs across 958 cell lines, combined with RNA-seq data from 99 CRPC biopsies analyzed by the SU2C-WC consortium.^{22,23,24} This approach has facilitated the identification of a panel of drugs that were predicted to have high potency specifically in CRPC tumors harboring *AR*-GSRs compared to CRPC lacking *AR*-GSRs. Of note, a subset of these drugs, constituting 21% of the identified compounds, target the PI3K/AKT/mTOR pathway directly. This discovery underscores the potential of targeting this pathway that is upregulated in the presence of *AR*-GSRs.

This study focuses on evaluating the PI3K/AKT pathway as a potential therapeutic target in CRPC cell lines that harbor *AR*-GSRs. *AR*-GSRs are associated with both elevated *AR* activity and a prevalence in tumors exhibiting PTEN loss, which results in the hyperactivation of the PI3K/AKT pathway. This correlation is significant given that *AR*-GSRs not only lead to enhanced *AR* signaling—even in androgen-depleted environments—but also may uncouple the *AR* from its reciprocal negative feedback interactions with the PI3K/AKT pathway. This uncoupling suggests that while *AR*-targeted therapies remain standard for CRPC treatment, they might be insufficient due to potential compensatory mechanisms via the PI3K/AKT pathway. Therefore, we hypothesize that the PI3K/AKT pathway could represent a viable target in cell lines with *AR*-GSRs, proposing

that inhibition of this pathway may effectively reduce cancer cell viability and disrupt the cancer progression, particularly in cases resistant to conventional AR-directed therapies. To evaluate the therapeutic potential of targeting the PI3K/AKT pathway in CRPC cell lines with *AR*-GSRs, we used growth assays based on crystal violet staining to determine the half-maximal inhibitory concentrations (IC50) of the drugs directly targeting the PI3K/AKT pathway. For this study, we used PC cell line models that have defined *AR*-GSRs, along with PC cell lines lacking *AR*-GSRs as controls.

Currently, there are no established therapies that directly target *AR*-GSRs. The significance of this study is that it has the potential to identify inhibitors of PI3K/AKT signaling as therapeutics that could overcome the growth-promoting effects of *AR*-GSRs in CRPC. By testing these inhibitors, our research aims not only to enhance understanding of the role of *AR*-GSRs in therapy resistance but also to accelerate the development of targeted interventions. The findings from this study are expected to provide a foundation for future therapeutic innovations, potentially leading to personalized treatment strategies that improve outcomes for patients with advanced prostate cancer.

Chapter 2: Materials and Methods

Cell lines and culture conditions

22Rv1(CRL-2505) cells were acquired from the American Type Culture Collection (ATCC). R1-AD1 and R1-D567 cell lines have been characterized in¹⁶ and 22Rv1unduped-3 has been described in²⁵. All these cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) with 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin.

For the experiments evaluating the effects of androgen deprivation, cells were grown in the media containing 10% charcoal-stripped serum (CSS) and for experiments where the effect of serum deprivation was to be assessed, cells were grown in media without any serum.

All cells were maintained in a humidified atmosphere of 5 % CO₂ at the ambient temperature of 35- 37° Celsius. Mycoplasma testing occurred monthly, and cells were cultured maximum of three months.

Drugs

Capivasertib (HY-15431), Ipatasertib (HY-15186), GSK-690693(HY-10249), Dactolosib (HY-50673), Temsirolimus (HY-50910) and Torin-2(HY-13002) were purchased from MedChem Express for the drug treatment of cells. Stock solutions were made using DMSO.

Cell growth assays

R1-AD1 (15000 cells/well), R1-D567 (9000 cells/well), 22Rv1- undup3 (16000 cells/well), and 22Rv1 (9000 cells/well) cells were seeded on 48-well plates in RPMI 1640 with 10% FBS and P/S. In the experiments where the effect of androgen deprivation was to be studied on these cell lines, 10% CSS serum was used instead of FBS. Cells were allowed to adhere for 24 hours, and dilutions of drugs and DMSO (vehicle control) were added at an equivalent volume to the existing medium. After 3 days or 6 days of growth, cells were fixed with glutaraldehyde and stained with crystal violet²⁶. Dye was eluted from cells using Sorenson's buffer (0.89% (w/v) Tri-sodium citrate (w/v), 0.1N HCl, 40% (v/v) ethanol) and absorbance was measured at 560 nM using a Biotek plate reader.

Concentration ranges of the drug for drug treatment:

Capivasertib: R1-AD1 and R1-D567 were treated with a concentration range of 11uM- 0.0003 uM. The endpoint of the experiment was kept at 72 hours. 22Rv1- undup 3 and 22Rv1 were treated with the concentration range of 100 uM- 0.003 uM and the endpoints were kept for 72 hours or 144 hours. The drug dilution was 1:3.

Ipatasertib: R1-AD1 and R1-D567 were treated with the concentration range of 11uM- 0.0003 uM and 72 hours was kept as the endpoint. 22Rv1-undup 3 and 22Rv1 were treated with the concentration range of 33.33 uM- 0.001 uM and the endpoints were kept for 72 hours or 144 hours. The drug dilution was 1:3.

GSK 690693: R1-AD1 and R1-D567 were treated with the concentration range of 11 uM- 0.0003 uM and 72 hours was kept as the endpoint. 22Rv1 -undup 3 and 22Rv1 were treated with the concentration range of 33.33 uM- 0.001 uM and 10 uM- 0.003 uM for CSS conditioned study and the endpoints were kept for 72 hours or 144 hours. The drug dilution was 1:3.

Torin 2: : R1-AD1, R1-D567, 22Rv1- undup3 and 22Rv1 were treated with the concentration range of 10uM- 0.0001 nM. For R1-AD1 and R1-D567, 72 hours was kept as the endpoint. For 22Rv1-undup 3 and 22Rv1 endpoints were kept for 72 hours or 144 hours. The drug dilution was 1:10

Temsirolimus: R1-AD1, R1-D567, 22Rv1-undup3 and 22Rv1 were treated with the concentration range of 10uM- 0.0001 nM. For R1-AD1 and R1-D567, 72 hours was kept as the endpoint. For 22Rv1-undup 3 and 22Rv1 endpoints were kept for 72 hours or 144 hours. The drug dilution was 1:10

Antibodies

Antibodies were purchased from Santa Cruz for western blot detection of AR (N- 20, sc-185, 1:1000; SP107, 1:1000), α -tubulin (B-5-1-2, sc-23948, 1:5000), p70S6k (Cell Signaling, 2708,1:1000), and Phospho-p70S6k (T389) (Cell Signaling, 9205, 1:1000)

Western blot

Cells were washed with ice-cold 1X phosphate-buffered saline (PBS) buffer, and whole cell lysates obtained in RIPA complete buffer (1M Tris-HCl pH8.0, 5M NaCl, 10% (v/v) NP-40, 10% (w/v) sodium deoxycholate, 10% (w/v) SDS, 1X eComplete (MilliporeSigma), and 1X PhosStop (MilliporeSigma). After centrifuging at 13,000 x g for 5 minutes, the supernatant was collected. Equal masses of total protein were separated via electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene (PVDF) membranes (Immobilon-P, Millipore). Membranes were incubated in primary antibodies in blocking buffer (5% (w/v) milk tris-buffered saline (TBS) with 0.1% (v/v) Tween 20) at 4°C overnight and HRP-conjugated secondary antibodies were incubated for 2 hours at room temperature. Blots were incubated with Super Signal West Pico (Thermo Fisher, 34580) and/ or ProSignal Femto (Prometheus Protein Biology Products, 20-302B) and imaged using an iBright CL750 system (Thermo Fisher) for chemiluminescence detection.

Si-AR-FL Transient Transfection

For, siRNA transfections, 22Rv1 cells in a medium supplemented with 10% FBS were allowed to grow to 80% confluence was achieved and then mixed with 200 pmol targeted or nontargeting control siRNAs (Horizon Discovery). siRNAs for nontargeting negative control (D-001210-01) were obtained from Horizon discovery. siRNA sequences designed to target AR exon 7 were as follows: siAR-LBD: SENSE: 5'- UUUUACUAUGCUAGCUCAAAGG-3', ANTISENSE: 5'- AAUGAUACGAUCGAGUCCUU-3'. Mixtures of cells (22Rv1) and siRNAs were electroporated in a 4-mm gap-width cuvette (BTX) with a 10ms pulse at 350V by BTX Square

Wave Electroporator. After a 15-minute recovery time, cells were seeded in the appropriate 10% FBS medium for western blotting lysate collection to ensure that the transfection was successful and/or how long the transfection lasted, and a 6-day end point crystal violet assay.

Data Analysis and Statistics

For experiments that involve two groups, unpaired Student's t-tests were performed. For experiments that involved more than two groups, one-way or two-way ANOVA was utilized followed by Tukey's post-hoc test for comparison between different groups. Statistical tests were performed using GraphPad Prism software and Microsoft Excel. P-values less than 0.05 were considered statistically significant.

Chapter 3: Results

AR-GSR positive R1-D567 cells are more growth-sensitive to Pan-AKT drugs compared to AR-GSR negative R1-AD1 cells

R1-AD1 and R1-D567 cells are isogenic cell lines wherein R1-D567 cells were derived from R1-AD1 cells by targeted deletion of *AR* exons 5-7, which models *AR* deletions occurring in CRPC tissues that prevent splicing of *AR* exons 5-7¹⁶. The three AKT isoforms are activated via phosphorylation on S473 and T308 by the upstream protein kinases PDK1 and mTORC2²⁷. Capivasertib, ipatasertib, and GSK-690693 are pan-AKT drugs that bind and inhibit all the isoforms of AKT. These three drugs were all predicted by computational imputation to have a higher potency in cells harboring *AR*-GSRs compared to cells lacking *AR*-GSRs. To test this, we evaluated whether R1-AD1 and R1-D567 cells display differential growth sensitivities to these pan-AKT inhibitors using growth assays with a crystal violet staining endpoint. Crystal violet staining intensity was used to determine the half-maximal inhibitory concentration (IC₅₀)²⁸ for each drug in these cell lines.

IC₅₀ values for all tested Pan-AKT inhibitors were lower for R1-D567 cells than R1-AD1 cells, indicating that R1-D567 cells were more sensitive (Fig 3). In particular, the IC₅₀ of capivasertib in R1-AD1 cells was approximately 3 times greater than the IC₅₀ of in R1-D567 cells (Fig 3a,b). These data from an isogenic cell line model indicate that Pan-AKT inhibitors are more potent growth inhibitors in cells harboring an *AR*-GSR that deletes *AR* exons 5-7.

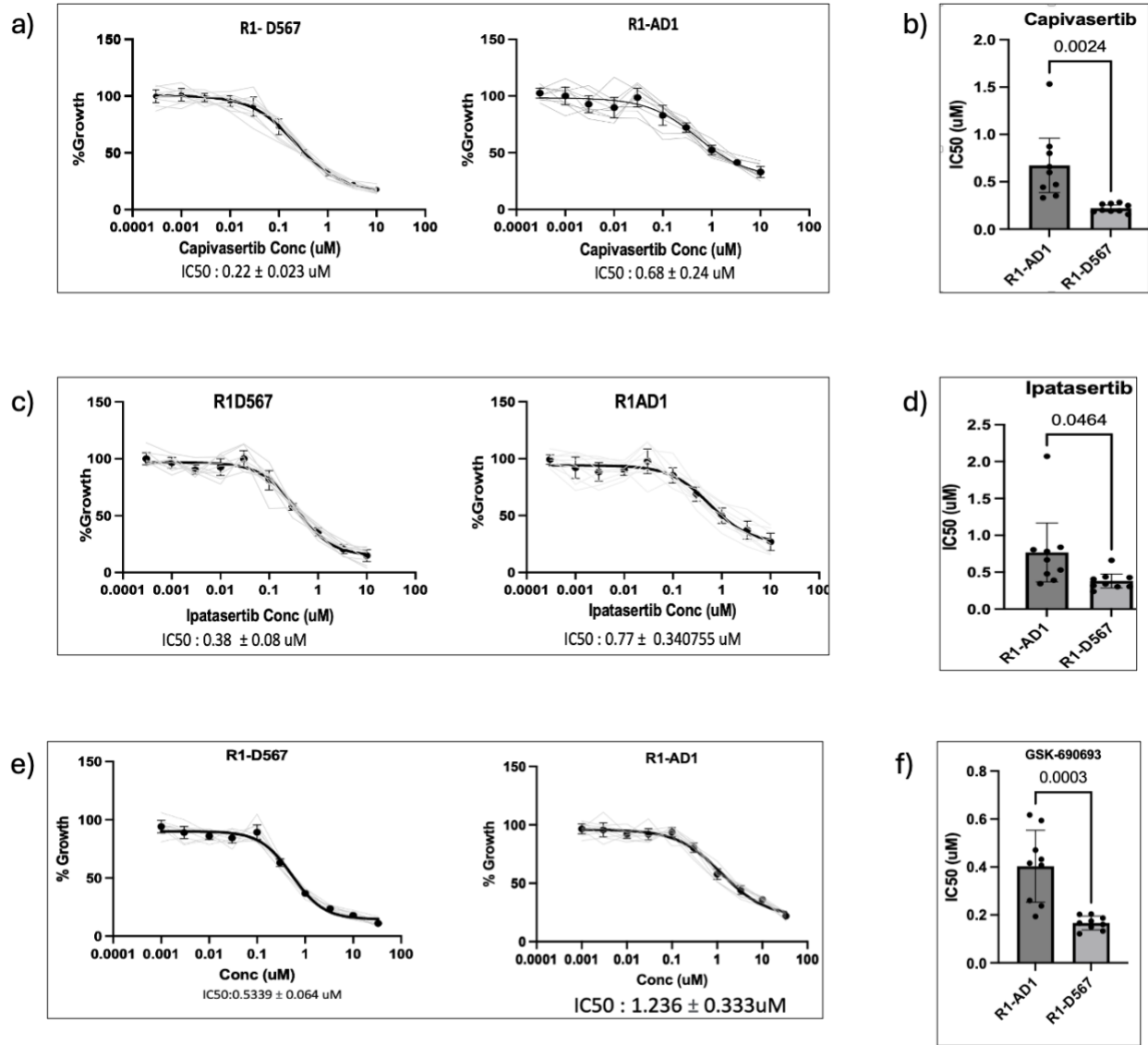


Figure 3: R1-D567 cells are more sensitive to pan-AKT drugs than R1-AD1 cells

Results from growth assays of R1-D567 and R1-AD1 cells treated with a) capivasertib, c) ipatasertib, and e) GSK-690693. (b), (d) and (f) represent statistical significance using an unpaired student T-test ($p > 0.05$). Data are mean \pm 95% CI from three independent experiments in biological triplicate ($n=9$).

AR-GSR positive R1-D567 cells and AR-GSR negative R1-AD1 cells display similar growth sensitivities to mTORC1 inhibitors

AKT phosphorylates and inhibits the tuberous sclerosis complex (TSC1/2), which in turn activates mTORC1²⁹. Two inhibitors of mTORC1, Temsirolimus, and Torin-2, were also predicted by computational imputation to have a higher potency in cells harboring *AR*-GSRs. However, when we determined the IC₅₀ of these drugs for inhibiting the growth of R1-AD1 and R1-D567 cells, we observed no difference (Fig 4 a,b,c,d). This suggests that the *AR*-GSR status does not determine sensitivity to mTORC1 inhibitors.

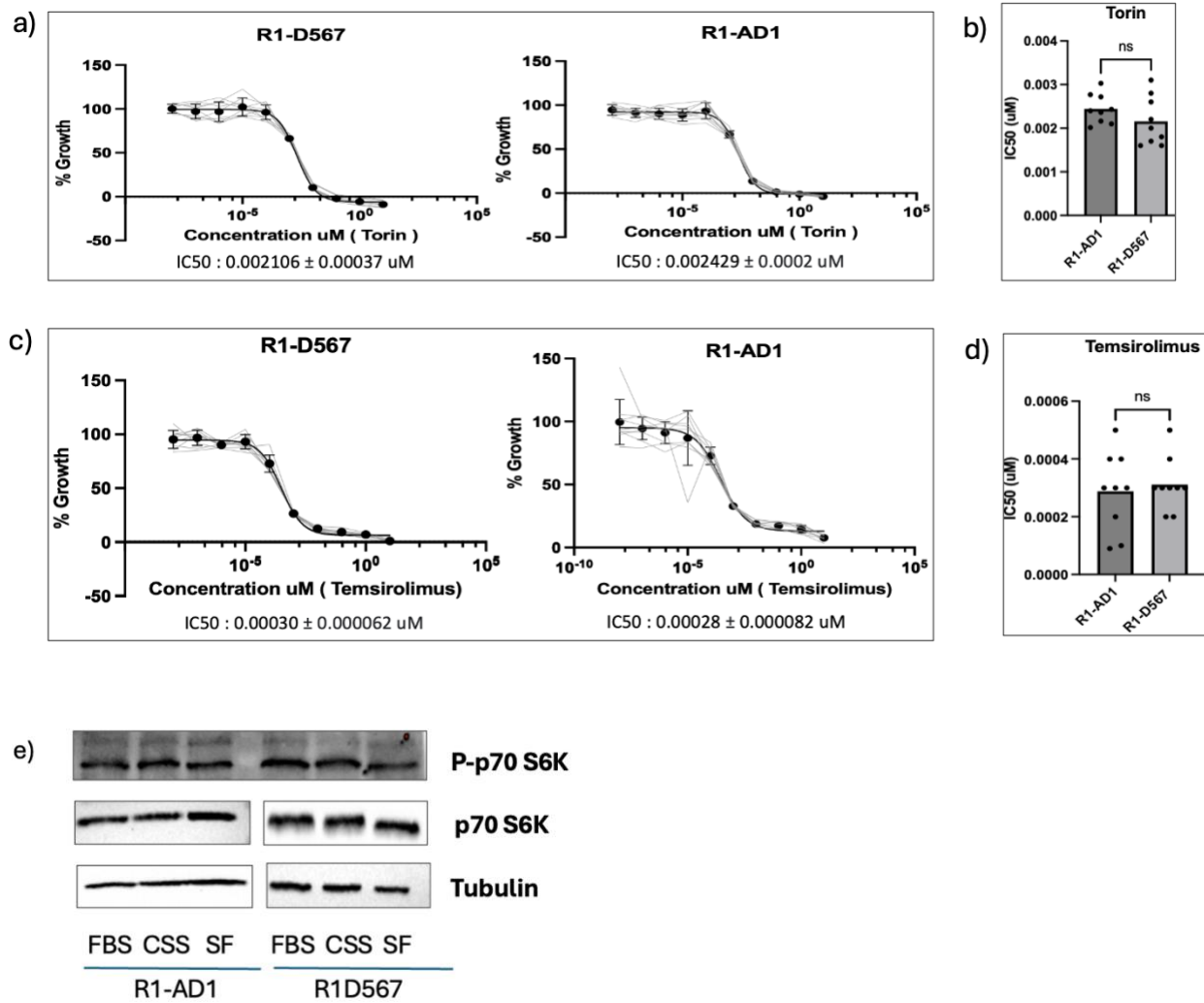


Figure 4: R1-D567 and R1-AD1 have no difference in the sensitivity towards mTORC1 inhibitors
 Viability assays of R1-D567 and R1-AD1 cells treated with Torin-2 (a,b) and Temsirolimus (c,d). Statistical significance was determined using an unpaired student T-test ($p > 0.05$). Data are mean ± 95% CI from three

independent experiments in biological triplicate (n=9). e) Western blotting for p70 S6K in R1-AD1 and R1-D567 in FBS, CSS, and Serum-free conditions to determine any difference in the phosphorylation of the downstream target of mTORC1.

AR-GSR positive R1-D567 cells and AR-GSR negative R1-AD1 cells display similar activation of p70 S6K

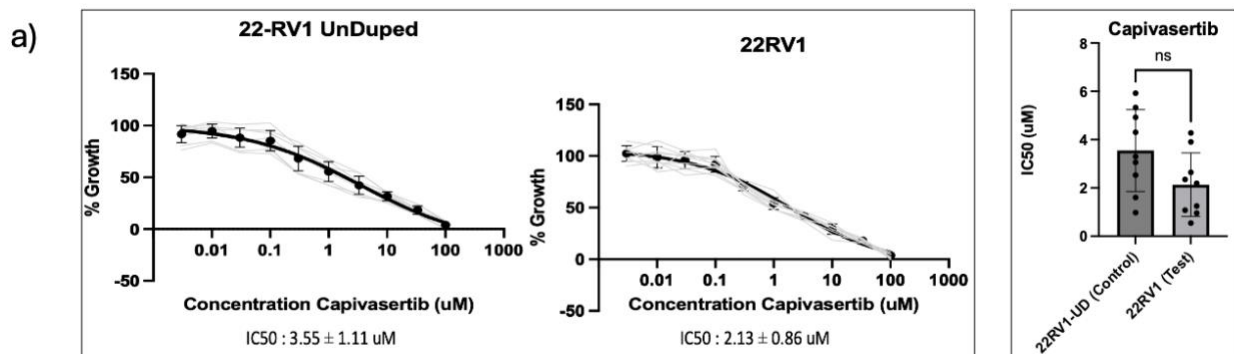
To further elucidate the molecular mechanisms underpinning these observations, western blot analyses were conducted to evaluate p70 S6K phosphorylation. p70 S6K is a 75 kDa protein that can be phosphorylated on Thr 389 by mTORC1. The phosphorylation status of p70 S6K can provide insight into the PI3K/AKT/mTORC1 pathway activity beyond cellular proliferation rates. We examined p70 S6K phosphorylation in cells cultured in a medium containing FBS, steroid-depleted CSS, and serum-free media to test the influence of growth factors, cytokines, and hormones. The Western blot results were similar to those of the crystal violet assays, showing no discernible difference in p70 S6K phosphorylation between the R1-AD1 and R1-D567 cell lines across all media conditions (Fig 4e).

AR-GSR positive 22Rv1 cells and AR-GSR negative 22Rv1- undup3 cells display similar growth sensitivities to AKT and mTORC1 inhibitors

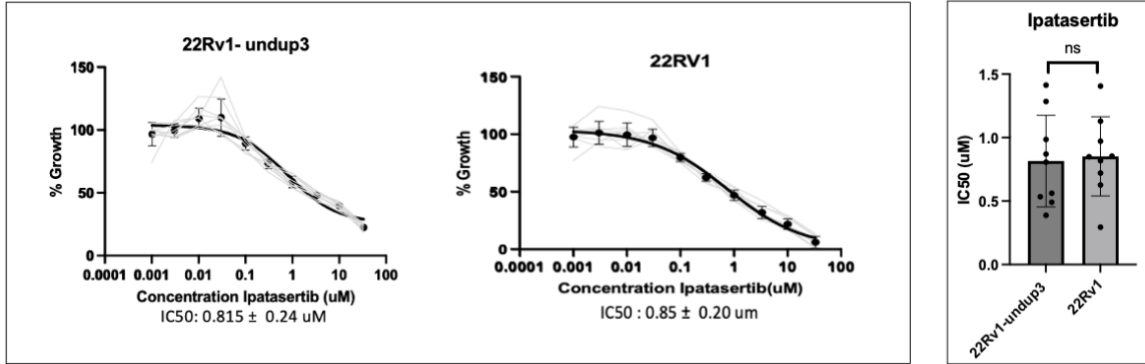
To deepen our understanding of drug sensitivity as a function of the type of *AR-GSR*, we quantified the IC₅₀ values of the selected drugs within 22Rv1 cells and the isogenic derivative, 22Rv1-undup3 using crystal violet growth assays. The 22Rv1 cell line features a 35kDa tandem duplication of exon 3 in the *AR* gene. In contrast, 22Rv1-undup3 was engineered to lack this

duplication, serving effectively as a control to assess the impact of this specific genomic alteration on drug response.

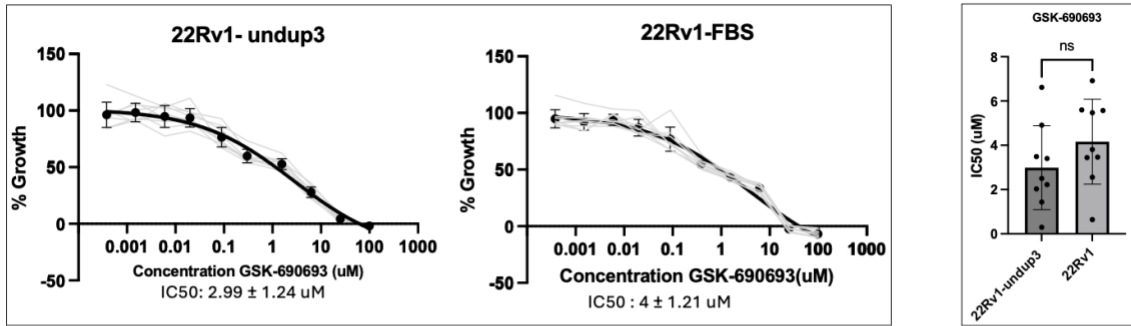
Again, inhibitors directly targeting the PI3K/AKT signaling cascade, capivasertib, ipatasertib, GSK-690693, temsirolimus, and torin-2 were tested on these cell lines. Through statistical evaluation using unpaired T-tests, we observed that the IC₅₀ values for these drugs did not differ significantly between these two cell lines, demonstrating a uniform sensitivity regardless of the *AR*-GSR status (5a,b,c,d). This uniformity underscores that the presence or absence of the *AR* exon 3 duplication does not influence the effectiveness of these treatments under hormone-rich conditions simulated by FBS media. However, an exception was noted in the response to Temsirolimus (Fig 5e) where differences in sensitivity were evident, and 22Rv1 was more sensitive to 22Rv1-undup3, suggesting that the cellular mechanisms influenced by Temsirolimus may interact differently with the hormonal environment or genetic alterations.



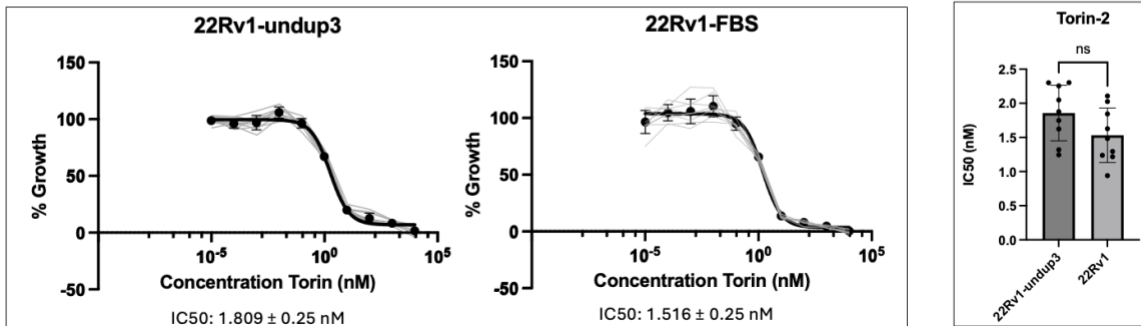
b)



c)



d)



e)

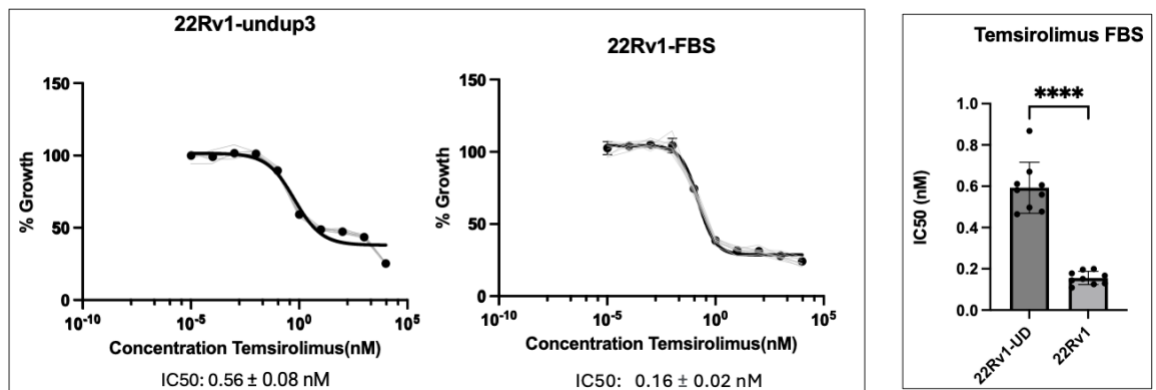
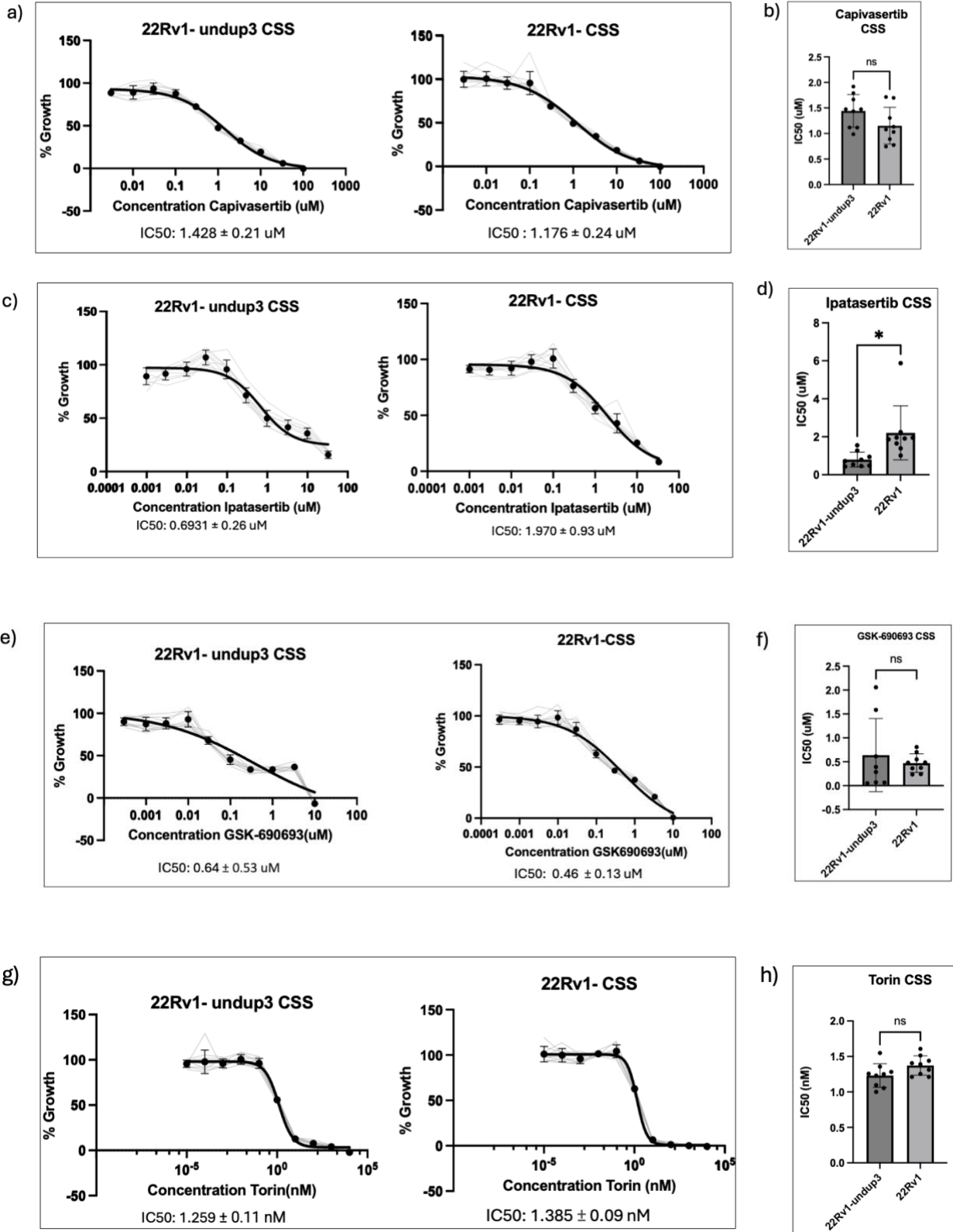


Figure 5: No Differential Sensitivity of 22Rv1 and 22Rv1-undup3 cell lines to PI3K/AKT/mTORC1 Inhibitors. Panels (a) through (d) depict the IC50 curves for Capiwasertib, Ipatasertib, GSK-690693, and Torin-2, respectively, showing no significant differences in drug response between 22Rv1 and 22Rv1-Unduplicated cell lines. Panel (e) demonstrates the unique response to Temsirolimus, with 22Rv1 cells exhibiting greater sensitivity compared to 22Rv1-undup3 ($p > 0.05$) (n=9)

Continued similar drug sensitivity of 22Rv1 and 22Rv1-Undup3 cell lines in CSS Conditions

Following our initial experiments in FBS, which showed no notable differences in IC50s among the control and test cell lines for most drugs, we decided to further our investigations under CSS conditions. This was crucial to determine if hormonal influences in FBS could be confounding our results. A 6-day growth endpoint was selected for the experiments because the 22Rv1-undup3 cell line was found to proliferate slowly in hormone-depleted conditions.

The experiments revealed that both 22Rv1 and 22Rv1-undup3 cell lines exhibited similar dose-response curves and IC50 values for capivasertib, ipatasertib, and Torin (Fig 6a,b,d). In contrast, we observed that temsirolimus was more potent in the 22Rv1 cell line relative to the 22Rv1-undup3 cell line even in CSS media (Fig 6e).



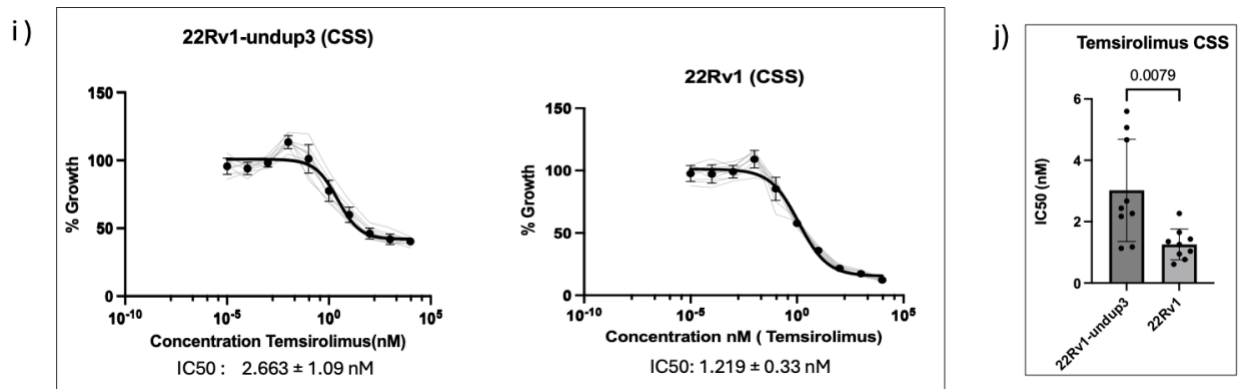


Figure 6: Drug Sensitivity of 22Rv1 and 22Rv1-undup3 Cell Lines Under CSS Conditions

Panels (a), (b), and (d) display the IC₅₀ curves for Capiasertib, Ipatasertib, and Torin, respectively, demonstrating similar sensitivity profiles in both 22Rv1 and 22Rv1-undup3 cell lines under steroid- and hormone-free conditions, underscoring the intrinsic effectiveness of these inhibitors independent of hormonal modulation. (e) highlights a distinctive sensitivity to Tamsulosin in 22Rv1 compared to 22Rv1-undup3. Potency between the 2 cell lines was compared using a student t-test ($p > -0.05$) ($n = 9$)

AR-FL Knockdown does not Influence Capiasertib's sensitivity in 22Rv1 Cells

In our comparative analysis of drug responses within mCRPC cell lines, Capiasertib demonstrated particularly strong efficacy in the R1-D567 cell line compared to R1-AD1, 22Rv1, and 22Rv1-undup3. This led us to recognize that, unlike the R1-D567 cell line, all other tested cell lines retain the full-length androgen receptor (AR-FL) (Fig 1). This observation prompted us to hypothesize that the presence of AR-FL might be contributing to lower capivasertib sensitivity. To further explore the hypothesis that the heightened drug sensitivity observed in the R1-D567 cell line was attributable to the absence of AR-FL, we chose to conduct targeted experiments on the 22Rv1 cell line, which displays the expression of AR-FL and AR splice variants. We used siRNA targeted to *AR* exon 7, which selectively knocks down AR-FL but not AR splice variants. As a control, we used non-targeted siRNA. Cells transfected with siRNAs were treated with capivasertib, and growth was assessed by crystal violet staining after 6 days. Western blot for AR

confirmed selective knockdown of AR-FL without any effect on the levels of AR-V proteins (Fig 7a). The crystal violet growth assay revealed that both curves (siControl and siAR) nearly overlap, indicating that the knockdown of AR-FL did not significantly alter the cell sensitivity to capivasertib (Fig 7b). This suggests that AR-FL might not be a key factor in capivasertib resistance, or other compensatory mechanisms might be active. The unpaired student t-test confirmed the results (Fig 7c).

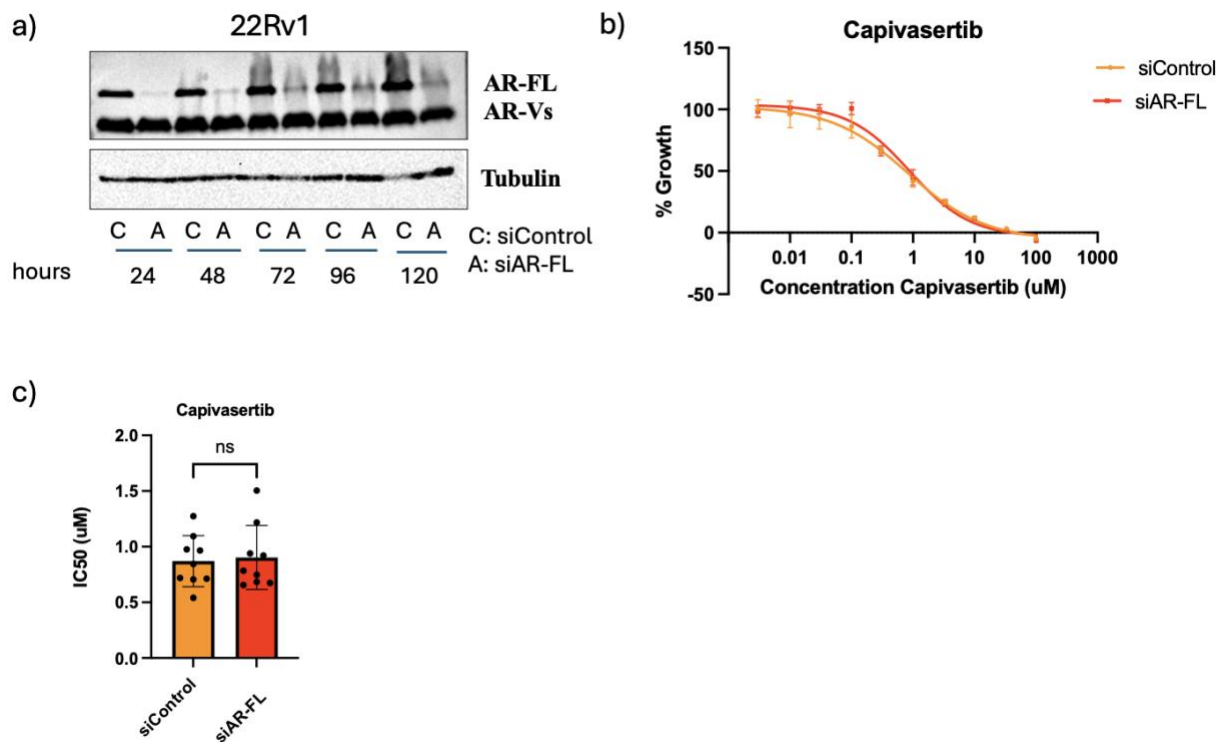


Figure 7: Lack of influence of AR-FL reduction on Capivasertib's response in 22Rv1 Cells

a) Western blot showing successful knockdown of AR-FL in 22Rv1 across 24, 48, 72, 96 and 120 hours post electroporation. b) Dose-response curve for capivasertib in 22Rv1 cells, c) Unpaired t-test comparing the IC50 values of capivasertib in 22Rv1 cells transfected with siRNA targeting AR-FL (siAR) versus non-targeting control siRNA (siControl). Each bar represents the average IC50 value from multiple biological replicates, with individual data points shown as dots. Error bars represent the standard deviation (n=9) (p>0.01).

Chapter 4: Discussion and Future Directions

The results of our study advance our understanding of the relationships between the presence of *AR*-GSRs in CRPC genomes and the sensitivity of prostate cancer cell lines to PI3K/AKT pathway inhibitors. Despite advances in AR-targeted therapies, resistance remains a significant clinical hurdle. This study aimed to explore alternative therapeutic targets by focusing on the PI3K/AKT signaling pathway, which interacts intricately with AR signaling.

Diverse *AR*-GSRs are crucial in understanding the complexity of CRPC. The study by Henzler et al. (2016) emphasizes that AR-GSRs can appear as deletions, duplications, inversions, and translocations, leading to varied functional impacts on AR signaling and drug sensitivity. Our findings align with this, demonstrating that the heterogeneity and diversity of *AR*-GSRs significantly influence CRPC behavior and treatment responses. For instance, in our study, the R1-D567 cell line, characterized by deletions of *AR* exons 5-7, exhibited increased sensitivity to pan-AKT inhibitors compared to its isogenic cell line R1-AD1 which does not have this deletion of *AR* exons suggesting that alterations in the AR structure particularly deletion of exon 5,6 and 7 of the AR, may predispose cells to a heightened reliance on the PI3K/AKT pathway for survival signals, thereby increasing their susceptibility to its inhibition. In contrast, the 22Rv1 cell line, with a tandem duplication of *AR* exon 3, and its isogenic counterpart 22Rv1- undup3 displayed uniform sensitivity to these AKT and mTORC1 inhibitors in both hormone-replete and hormone-depleted conditions. This uniformity across hormonal conditions supports the conclusion that the duplication in exon 3 of the *AR* gene in 22Rv1 does not influence the responsiveness of these cell lines to AKT and mTORC1 inhibitors. These findings suggest that not all *AR*-GSRs confer the

same survival advantages, underscoring the need for a more nuanced understanding of the molecular mechanisms underpinning these effects.

Moreover, contrary to the significant variance in sensitivity to pan-AKT inhibitors, our results do not support a significant influence of *AR*-GSRs on the sensitivity to mTORC1 inhibitors, as indicated by similar growth inhibitory effects in both these cell lines. This finding suggests that the activity of mTORC1, a direct downstream target of *AKT*, is independent of *AR*-GSRs, or that its role in CRPC progression could be overshadowed by other compensatory survival pathways like MAPK/ERK, JAK/STAT, and others that play significant roles in maintaining CRPC cell survival under therapeutic pressure³⁰. Moreover, uniform phosphorylation of *p70 S6K*, the downstream target of mTORC1 in both the R1-AD1 and R1-D567 in different media conditions further supports these results and the conclusion that *AR*-GSR status does not significantly impact the basal activity of the mTORC1 pathway or its downstream signaling, regardless of the hormonal or growth factor environment.

The enhanced efficacy of Capivasertib in the R1-D567 cell line, which lacks AR-FL, in contrast to other cell lines like R1-AD1, 22Rv1, and 22Rv1-undup 3 that express AR-FL, led to a compelling hypothesis that AR-FL may play a role in conferring resistance to PI3K/AKT pathway inhibitors. However, statistical analyses showed no significant difference in IC₅₀ between the siControl and siAR-FL in 22Rv1 cells, indicating that the knockdown of AR-FL does not significantly alter the sensitivity of 22Rv1 cells to capivasertib. This helps us conclude that AR-FL is not a major determinant of AKT inhibition response. Also, the deletion of exon 5-7 in R1-

D567 might be the reason for enhancing the reliance on the PI3K/AKT pathway and not the presence or absence of AR-FL but this needs further validation.

Limitations and future directions

While this study provides valuable insights into the differential sensitivity of prostate cancer cell lines with specific *AR*-GSRs to PI3K/AKT pathway inhibitors, it does not delve deeply into the mechanistic underpinnings of how these rearrangements alter cellular signaling and drug response pathways. The study mainly focuses on the pharmacological impacts of these genetic alterations without extensively exploring the molecular changes within the cells that could explain why certain *AR*-GSRs confer heightened sensitivity or resistance to treatments like capivasertib. For instance, a detailed examination of changes in gene expression, protein-protein interactions, or post-translational modifications resulting from *AR*-GSRs could be performed in future studies to understand these mechanisms.

Incorporating advanced methodologies such as CRISPR-Cas9 for knocking out distinct **AKT** isoforms could provide insights into drug sensitivity. Since these *AKT1-3* genes encode the isoforms of AKT, knocking out one or more of these genes in R1-AD1 and R1-D567, would directly reduce the levels of active AKT in the cells. Capivasertib targets AKT, so the absence of AKT would result in less target for the drug to inhibit, potentially leading to decreased drug efficacy and reduced sensitivity. Moreover, knocking out **PTEN** in R1-AD1, R1-D567, 22Rv1, and 22Rv1-undup3 should have a more pronounced effect, increasing their sensitivity to the drug.

PTEN is a tumor suppressor that negatively regulates the PI3K/AKT pathway by

dephosphorylating PIP3 back to PIP2, thereby inhibiting AKT activation. The loss of *PTEN* results in increased levels of PIP3, leading to constitutive activation of AKT. In the absence of *PTEN*, cells become more dependent on the PI3K/AKT pathway for survival. Thus, inhibiting AKT with capivasertib in *PTEN*-deficient cells should have a more significant impact, increasing their sensitivity to the drug. Additionally, RNA sequencing for comprehensive expression profiles could significantly enhance our understanding of these mechanisms. Using RNA-seq for R1-AD1, R1-D567, 22Rv1, and 22Rv1-undup3 cell lines can help us identify differentially expressed genes and pathways associated with *AR*-GSRs.

Additionally, it would be beneficial to expand this study to include a broader range of cell line models harboring *AR*-GSRs like R1-I567 that have the inversion of exons 5,6 and 7 of *AR*¹⁶. Also, exploring the potential of combining different classes of PI3K/AKT inhibitors with *AR* antagonists would tell us more in detail about the reciprocal negative feedback in terms of *AR*-GSRs. *In vivo*, studies using CRPC xenograft models like LuCaP35 and LuCaP105 that carry *AR*-GSRs could validate and extend our understanding of the therapeutic potential of targeting these pathways³¹. These efforts will be crucial in overcoming the adaptive capabilities of CRPC and improving patient outcomes, potentially transforming the therapeutic landscape for this challenging disease.

In conclusion, this study demonstrates that mCRPC cell lines harboring specific *AR*-GSRs exhibit distinct sensitivities to PI3K/AKT pathway inhibitors. The findings highlight the complexity of *AR* signaling interactions, underscoring the potential of targeting these pathways more effectively in therapeutic settings, particularly for castration-resistant prostate cancer. Importantly, the

differential responses among cell lines with various *AR*-GSRs, such as R1-D567 and 22Rv1, illuminate the critical roles these genetic variations play in drug resistance and sensitivity. Our findings that specific *AR*-GSRs, particularly deletions of exons 5-7, lead to increased sensitivity to PI3K/AKT pathway inhibitors provide novel insights into the molecular mechanisms driving CRPC. This suggests a new therapeutic avenue targeting the PI3K/AKT pathway in CRPC patients with these genetic alterations. This work not only advances our understanding of the molecular underpinnings of prostate cancer resistance mechanisms but also opens avenues for the development of more refined, personalized therapeutic strategies that consider specific genomic alterations.

Bibliography

1. Siegel, R. L., Miller, K. D., Wagle, N. S. & Jemal, A. Cancer statistics, 2023. *CA. Cancer J. Clin.* **73**, 17–48 (2023).
2. Huggins, C. & Hodges, C. V. Studies on Prostatic Cancer: I. The Effect of Castration, Of Estrogen and of Androgen Injection on Serum Phosphatases in Metastatic Carcinoma of the Prostate. *CA. Cancer J. Clin.* **22**, 232–240 (1972).
3. He, Y. *et al.* Targeting signaling pathways in prostate cancer: mechanisms and clinical trials. *Signal Transduct. Target. Ther.* **7**, 198 (2022).
4. Bales, G. T. & Chodak, G. W. A controlled trial of bicalutamide versus castration in patients with advanced prostate cancer. *Urology* **47**, 38–43 (1996).
5. Crona, D., Milowsky, M. & Whang, Y. Androgen receptor targeting drugs in castration-resistant prostate cancer and mechanisms of resistance. *Clin. Pharmacol. Ther.* **98**, 582–589 (2015).
6. Tan, M. E., Li, J., Xu, H. E., Melcher, K. & Yong, E. Androgen receptor: structure, role in prostate cancer and drug discovery. *Acta Pharmacol. Sin.* **36**, 3–23 (2015).
7. Davey, R. A. & Grossmann, M. Androgen Receptor Structure, Function and Biology: From Bench to Bedside.
8. Gelmann, E. P. Molecular Biology of the Androgen Receptor. *J. Clin. Oncol.* **20**, 3001–3015 (2002).
9. Chen, Y. *et al.* ETS factors reprogram the androgen receptor cistrome and prime prostate tumorigenesis in response to PTEN loss. *Nat. Med.* **19**, 1023–1029 (2013).

10. Ryan, C. J. & Tindall, D. J. Androgen Receptor Rediscovered: The New Biology and Targeting the Androgen Receptor Therapeutically. *J. Clin. Oncol.* **29**, 3651–3658 (2011).
11. Hörnberg, E. *et al.* Expression of Androgen Receptor Splice Variants in Prostate Cancer Bone Metastases is Associated with Castration-Resistance and Short Survival. *PLoS ONE* **6**, e19059 (2011).
12. Li, Y. *et al.* Diverse *AR* Gene Rearrangements Mediate Resistance to Androgen Receptor Inhibitors in Metastatic Prostate Cancer. *Clin. Cancer Res.* **26**, 1965–1976 (2020).
13. Ho, Y. & Dehm, S. M. Androgen Receptor Rearrangement and Splicing Variants in Resistance to Endocrine Therapies in Prostate Cancer. *Endocrinology* **158**, 1533–1542 (2017).
14. Zivanovic, A. *et al.* Co-evolution of *AR* gene copy number and structural complexity in endocrine therapy resistant prostate cancer. *NAR Cancer* **5**, zcad045 (2023).
15. Brand, L. J. & Dehm, S. M. Androgen Receptor Gene Rearrangements: New Perspectives on Prostate Cancer Progression. (2014).
16. Nyquist, M. D. *et al.* TALEN-engineered *AR* gene rearrangements reveal endocrine uncoupling of androgen receptor in prostate cancer. *Proc. Natl. Acad. Sci.* **110**, 17492–17497 (2013).
17. Henzler, C. *et al.* Truncation and constitutive activation of the androgen receptor by diverse genomic rearrangements in prostate cancer. *Nat. Commun.* **7**, 13668 (2016).
18. Kallio, H. M. L. *et al.* Constitutively active androgen receptor splice variants AR-V3, AR-V7 and AR-V9 are co-expressed in castration-resistant prostate cancer metastases. *Br. J. Cancer* **119**, 347–356 (2018).

19. Zhao, S. G. *et al.* The DNA methylation landscape of advanced prostate cancer. *Nat. Genet.* **52**, 778–789 (2020).
20. Carver, B. S. *et al.* Reciprocal Feedback Regulation of PI3K and Androgen Receptor Signaling in PTEN-Deficient Prostate Cancer. *Cancer Cell* **19**, 575–586 (2011).
21. Mulholland, D. J. *et al.* Cell Autonomous Role of PTEN in Regulating Castration-Resistant Prostate Cancer Growth. *Cancer Cell* **19**, 792–804 (2011).
22. Geeleher, P., Cox, N. J. & Huang, R. S. Clinical drug response can be predicted using baseline gene expression levels and in vitro drug sensitivity in cell lines. *Genome Biol.* **15**, R47 (2014).
23. Iorio, F. *et al.* A Landscape of Pharmacogenomic Interactions in Cancer. *Cell* **166**, 740–754 (2016).
24. Che, M. *et al.* Opposing transcriptional programs of KLF5 and AR emerge during therapy for advanced prostate cancer. *Nat. Commun.* **12**, 6377 (2021).
25. Van Etten, J. L. *et al.* Targeting a Single Alternative Polyadenylation Site Coordinately Blocks Expression of Androgen Receptor mRNA Splice Variants in Prostate Cancer. *Cancer Res.* **77**, 5228–5235 (2017).
26. Li, Y. *et al.* Intragenic Rearrangement and Altered RNA Splicing of the Androgen Receptor in a Cell-Based Model of Prostate Cancer Progression. *Cancer Res.* **71**, 2108–2117 (2011).
27. Hart, J. R. & Vogt, P. K. Phosphorylation of AKT: a Mutational Analysis. *Oncotarget* **2**, 467–476 (2011).

28. Aykul, S. & Martinez-Hackert, E. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. *Anal. Biochem.* **508**, 97–103 (2016).
29. Cai, S.-L. *et al.* Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. *J. Cell Biol.* **173**, 279–289 (2006).
30. Chandrasekar, T., Yang, J. C., Gao, A. C. & Evans, C. P. Mechanisms of resistance in castration-resistant prostate cancer. *Transl. Androl. Urol.* **4**, (2015).
31. Nguyen, H. M. *et al.* LuCaP Prostate Cancer Patient-Derived Xenografts Reflect the Molecular Heterogeneity of Advanced Disease and Serve as Models for Evaluating Cancer Therapeutics: Prostate Cancer PDXs: LuCaP Series. *The Prostate* **77**, 654–671 (2017).