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TITLE: PP2A Activation as a Therapy for AR-Addicted Refractory Castration-Resistant Prostate Cancer

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14. ABSTRACT: The aims of this proposal are to elucidate the mechanism of restoration of AR-signaling in enzalutamide /darolutamide/apalutamide refractory metastatic castration-resistant prostate cancer with specific emphasis on understanding the mechanism of PP2A loss and evaluating its activation by SMAPs (small molecular activator of phosphatase) as a potential novel therapy. We hypothesize that AR-driven transcriptional addiction displayed by anti-androgen resistant cells is caused by hyperphosphorylation of core transcriptional complex consisting of AR, MED1, and BRD4 due to the persistent CDK7 kinase activity and concomitant loss of PP2A phosphatase activity. We will test this hypothesis by pursuing the following three specific aims, Aim 1: Investigate the PP2A loss associated AR addiction in second generation anti-androgen refractory CRPC. Study the PP2A restoration on pAR-MED1-BRD4 complex and AR-signaling in anti-androgen refractory CRPC. Aim 3: Evaluate the reactivation of PP2A by SMAPs as a therapeutic strategy in the enzalutamide refractory CRPC.

15. SUBJECT TERMS: Castration-resistant Prostate cancer, Androgen Receptor, Anti-Androgens, Small Molecule Activator of Phosphatase, DT-061, Protein Phosphatase 2A, Transcription, Mediator 1, Bromodomain containing 4

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1. INTRODUCTION:

Prostate cancer (PCa) is the second most common cancer in men worldwide, and the eighth leading cause of cancer-related deaths. In 2020 alone, prostate cancer is expected to be diagnosed in 180,890 men, and close to 26,000 will die of the metastatic castration-resistant prostate cancer (mCRPC) in the United States. Androgen receptor (AR) is a major driver alteration in mCRPC, and most prostate tumors continue to rely on AR signaling. The second generation anti-androgen therapies such as abiraterone and enzalutamide have become standard of care treatments for mCRPC. However, most patients eventually develop resistance to these therapies and, thus, alternate approaches to target androgen signaling and other survival signaling pathways are needed. In addition to enzalutamide, structurally similar anti-androgen drugs such as apalutamide and darolutamide have recently been approved by the FDA for non-metastatic CRPC (nmCRPC), which is also expected to lead to refractory disease. The mechanism of acquired resistance to enzalutamide is mostly driven by AR where overexpression, genomic amplification, and point mutations, overexpression of splice variants have shown to contribute to an AR addicted refractory metastatic disease. The restoration of AR-signaling observed in a vast majority of the refractory diseases presents challenges for long-term disease control and progression-free survival. Therefore, better understanding and targeting of AR transcriptional addiction should be considered. Using enzalutamide as a model second-generation anti-androgen drug, we have identified hyper-phosphorylation of MED1 and BRD4 – two important co-activators of AR, in enzalutamide resistant PCa cells. Additionally, AR itself was found to be hyperphosphorylated that was accompanied by reduction in the phosphatase PP2A levels. Furthermore, we have identified transcriptional reduction of LCMT1 methyltransferase as a potential mechanism for the loss of PP2A activity through decreased methylation in enzalutamide resistant PCa cells.

2. KEYWORDS:

Castration-resistant Prostate cancer, Androgen Receptor, Anti-Androgens, Small Molecule Activator of Phosphatase, DT-061, Protein Phosphatase 2A, Transcription, Mediator 1, Bromodomain containing 4

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1: Investigate the PP2A loss associated AR-addiction in second generation antiandrogen refractory CRPC	**Months**	
Task 1: To evaluate the AR dependency in anti-androgen resistant prostate cancer cells • Validation of AR restoration in EnzaR PCa cells - PSA • Effect of AR depletion and AR silencing (CRISPR kd) on the growth of EnzaR PCa cells – proliferation assay, colony formation assay, TUNNEL assay, immunoblotting for apoptosis markers, FACS to detect apoptotic cells • RNAseq following AR depletion by ARD-69 • ChIP-seq for AR, BRD4, and MED1 following AR depletion in EnzaR cells Cell lines and models used: LNCaP, LNCaP-AR, VCaP and LAPC4 cells [ATTC] and their respective enzalutamide resistant derivatives developed by us [Asangani lab]. Techniques: CRISPR mediated gene knockdown, phenotypic assays, drug treatment, immunoblotting, qRT-PCR, RNA-seq and ChIP-seq	1-24	Asangani Lab

Progress: 60% Completed. AR depletion leads to the death of EnzaR PCa cells. RNASeq following AR depletion by ARD-69 show loss of AR transcriptional signature. ChIP-seq for AR and MED1 following AR depletion show genome wide loss of AR and MED1.		
Task 2: To identify the major sites of phosphorylation and novel interactions of AR-MED1-BRD4 complex in EnzaR prostate cancer cells • Immunoprecipitation followed by mass spectrometry with nuclear lysates from parental and EnzaR derivatives of LNCaP and LAPC4 • Identify new interacting partners as well differentially phosphorylated amino acid residues on AR, BRD4, and MED1 in EnzaR cells • Immunoprecipitation followed by immunoblotting to validate the increased interaction between AR, BRD4 and MED1 in EnzaR cells. Cell lines and models used: LNCaP, and LAPC4 and their respective enzalutamide resistant derivatives developed by us [Asangani lab]. Techniques: Immunoprecipitation, Mass spectrometry, immunoblotting. Progress: 30% Completed. Immunoprecipitation followed by immunoblotting show increased interaction between AR, BRD4 and MED1 in EnzaR cells. Mass spectrometry has been completed pending analysis.	6-18	Asangani Lab Garcia Lab
Task 3: To evaluate the p-AR, p-MED1, p-BRD4 and Methyl-C as prognostic and predictive biomarkers for ADT. • Immunohistochemistry with antibodies against phospho-S81 AR, p-S484/S488 BRD4, p-T1457 MED1, and Methyl-C on multiple progression, and outcome TMA We have already received the TMA slides from Dr. Arul Chinnaiyan at U.Mich. Preliminary data for Methyl-C in outcome TMA is shown in figure 5 of the proposal. The p-MED1, p-BRD4 and p-AR antibodies have been tested positively for immunohistochemistry using cell blocks. Techniques: Immunohistochemistry, pathology evaluation and analysis Progress: 60% Completed. • Immunohistochemistry with antibodies against Methyl-C on multiple progression, and outcome TMA show association of decreased methyl-C with worst prognosis. We anticipate the IHC with the rest of the antibodies will be completed this year.	6-24	Asangani Lab Lal Lab
Milestone(s) Achieved: With this aim we will be able (1) confirm the critical role of AR and associated co-regulator MED1 and BRD4 in driving enzalutamide resistance in PCa; (2) confirm increased phosphorylation of AR, BRD4 and MED1 and loss of PP2A (Methyl-C), as well as identify new interactors in enzalutamide resistant state; and*;(3) evaluate these as novel prognostic and predictive biomarkers and finally*	24	
Specific aim2: Study the PP2A restoration on pAR-MED1-BRD4 complex and AR-signaling in anti-androgen refractory CRPC		

Task 1: To investigate the role of LCMT1 in PP2A loss in EnzaR PCa cells. • Cloning two independent sgRNA targeting LCMT1 into single lentivirus CRISPR-Cas9 plasmid. • Generation of LCMT1 knockdown using CRISPR/Cas9 system • Cloning of LCMT1 into eukaryotic expression vector for overexpression studies • Cloning of PP2A-C wild type and L309A mutant (through site-directed mutagensit) into eukaryotic expression vector for rescue studies • Transcriptome analysis for LCMT1 expression in enzalutamide refractory tumors Cell lines and models used: LNCaP, and LAPC4 cells [ATTC] and their respective enzalutamide resistant derivatives developed by us [Asangani lab]. Techniques: Molecular cloning, qRT-PCR, ChIP-qPCR, bisulfite sequencing, immunoblotting, transfection, cell proliferation and colony formation assays Progress: 60% Completed. LCMT1 knockout by CRISPR has been completed. Cloning of LCMT1 overexpression construct is completed. We anticipate the rest of the experiments to eb completed in the next 8-10 months.	12-24	Asangani Lab Feng Lab
Task 2: To evaluate the effect of PP2A-C knockdown in PCa cells using CRISPR-Cas9 system • Cloning two independent sgRNA targeting PP2A-C into single lentivirus CRISPR-Cas9 plasmid. • SMAP and anti-androgen (enzalutamide/darolutamide/apalutamide) sensitivity assays • Immunoblotting for PP2A, p-MED1, p-AR and p-MED1 Cell lines and models used: LNCaP and LAPC4 [ATCC] and enzalutamide resistant LNCaP and LAPC4 [Asangani lab]. Techniques: Cloning, transfection, qRT-PCR, immunoblotting, colony formation assays. Progress: 60% Completed. Enzalutamide resistant PCa cells demonstrate exquisite sensitivity to SMAP. SMAP treatment leads to reduced p-MED1 and p-AR. CRISPR knockout of PP2A-C is in progress.	12-30	Asangani Lab Narla Lab
Task 3: Investigate the effect of PP2A activation by SMAP on AR, MED1 and BRD4 chromatin binding in anti-androgen resistant PCa cells • Chromatin fractionation followed by immunoblotting • ChIP-seq for AR, BRD4, and MED1 following SMAP treatment in EnzaR cells Cell lines and models used: EnzaR LNCaP and EnzaR LAPC4 cells [Asangani lab]. Techniques: drug treatment, cell fractionation, immunoblotting, and ChIP-seq Progress: Completed. ChIP-seq for AR and MED1 following SMAP treatment show genome wide loss of AR and MED1. Similar results are observed with chromatin fractionation followed by immunoblotting.	18-36	Asangani Lab
Task 4: To evaluate the effect of SMAP on transcriptional landscape and growth in anti-androgen resistant PCa cells. • RNA-seq following SMAP treatment in EnzaR cells	18-36	Asangani Lab

• Sensitivity to SMAP in enzalutamide, darolutamide and apalutamide resistant PCa cells Cell lines and models used: EnzaR LNCaP, LAPC4, LNCaP-AR and VCaP; DaroR LNCaP, LAPC4, LNCaP-AR and VCaP; ApaR LNCaP, LAPC4, LNCaP-AR and VCaP [Asangani lab]. Techniques: drug treatment, immunoblotting, RNA-seq, qRT-PCR, proliferation and colony formation assays. Progress: Completed. RNASeq in SMAP treated EnzaR cells show reduction in AR target gene expression. SMAP treatment in enzalutamide, darolutamide and apalutamide resistant PCa cells leads to apoptotic cell death.		
Milestone(s) Achieved: Upon completion of Aim 2, we expect to: (1) confirm the role of LCMT1 in PP2A loss in EnzaR cells, (2) reactivation of PP2A by SMAPs and its negative effect on the growth, AR-cistrome and transcriptome through dephosphorylation of AR-MED1-BRD4 core transcriptional complex in second generation anti-androgen resistant PCa cells.	36	
Specific aim3: **Evaluate the reactivation of PP2A by SMAP as a therapeutic strategy in the refractory CRPC**		
Task 1: Determine the effect of DT-061 on enzalutamide refractory prostate tumors in vivo. To assess the effect of DT-061 on Enzalutamide resistant LNCaP-AR and VCaP tumors in mice • Castration of SCID mice • CRPC LNCaP-AR and VCaP enzalutamide refractory tumor generation • Treatment group 1) control; 2) Enzalutamide; 3) DT-061; 4) Enzalutamide plus DT-061 • [12 mice per group x 4 groups x 2 cell lines = 96 mice total] • Measure tumor growth • Post treatment analysis of tumors for on-target activity and apoptosis • Effect of drug treatment on micrometastasis **Cell lines and models used**: Enzalutamide resistant LNCaP and VCaP (Asangani Lab) Techniques: castration, tumor generation and surgical extraction, extraction of bone marrows and organs, immunohistochemistry, qRT-PCR, Alu-PCR to detect micromets Progress: We anticipate performing these experiments shortly upon ACURO approval.	12-36	Asangani Lab
Task 2: Determine the effect of DT-061 in enzalutamide refractory PDX tumors in vivo. To assess the effect of DT-061 on Enzalutamide resistant AR positive PDX • Naïve and enzalutamide resistant PDX in castrated mice • Treatment group 1) control; 2) Enzalutamide; 3) DT-061; 4) Enzalutamide plus DT-061; • [12 mice per group x 4 groups x 2 PDX model = 96 mice total] • Measure tumor growth • Post treatment analysis of tumors for on-target activity and apoptosis • Effect of drug treatment on micrometastasis **Cell lines and models used**: Naïve and acquired enzalutamide resistant AR positive PDX (PDX will be provided by Dr. Navone – please see the support letter)	12-36	Asangani Lab Navone Lab

Techniques: castration, tumor generation and surgical extraction, extraction of bone marrows and organs, immunohistochemistry, qRT-PCR, Alu-PCR to detect micromets Progress: Not done. We anticipate starting these experiments soon.		
Milestone(s) Achieved: : Upon completion of Aim 3, we expect to fully assess the anti-tumor activity of DT-061 in enzalutamide resistant cells and patient-derived xenograft CRPC models.	36	

What was accomplished under these goals?

Aim1: Investigate the PP2A loss associated AR-addiction in second generation antiandrogen refractory CRPC. We established four different enzalutamide resistant CRPC cell lines that show continued addiction to AR signaling. Treatment with AR degrader (ARD-69) in these cells led to loss of AR and associated coactivators such as MED1, BRD4 and p300 from the chromatin (Figure 1). Furthermore, AR degradation using ARD-69 led to cell death in enzalutamide, apalutamide and darolutamide resistant prostate cancer cells (Figure 2). Loss of methyl-C was found to be associated with worst relapse free survival, metastasis and anti-androgen resistance (Figure 3)

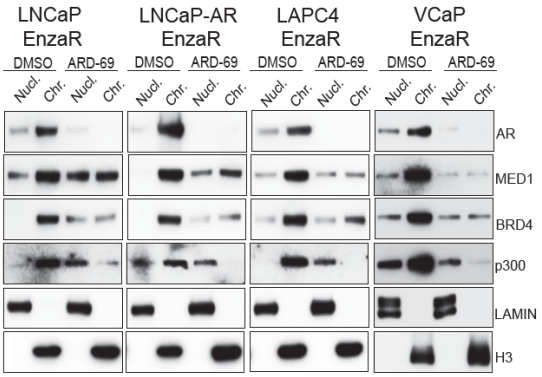


Figure 1: Immunoblot analysis demonstrating the loss of chromatin-bound AR, MED1, and BRD4 upon ARD-69 (AR PROTAC) treatment. Chromatin and soluble nuclear fractions extracted from LNCaP-, LNCaP-AR-, LAPC4-EnzaR, and VCaP-EnzaR cells treated with 100 nM ARD-69 for 12 h were probed for the indicated proteins. LAMIN and H3 served as controls for nuclear and chromatin fractions, respectively.

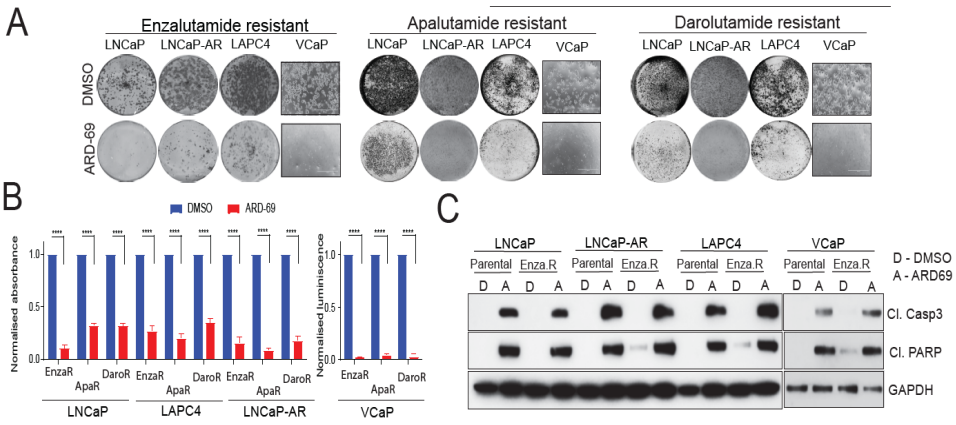


Figure 2: A) AR degradation affects the colony formation ability of enzalutamide, apalutamide, and darolutamide refractory PCa cells. Indicated cell lines were cultured either in the presence of vehicle control or 100 nM ARD-69 for 12-14 days, followed by crystal violet staining (n=3). In the case of VCaP - representative bright field images are shown. B) The quantification of the colony formation assay shown in A. C) Apoptotic induction upon AR degradation. The indicated parental cell lines or EnzaR derivatives were treated with either 100 nM ARD-69 or DMSO for 48 h. Lysates prepared from the cells were subjected to immunoblotting for cleaved caspase 3 and cleaved PARP. GAPDH was used as a loading control. Statistical significance is represented as ****p < 0.0001.

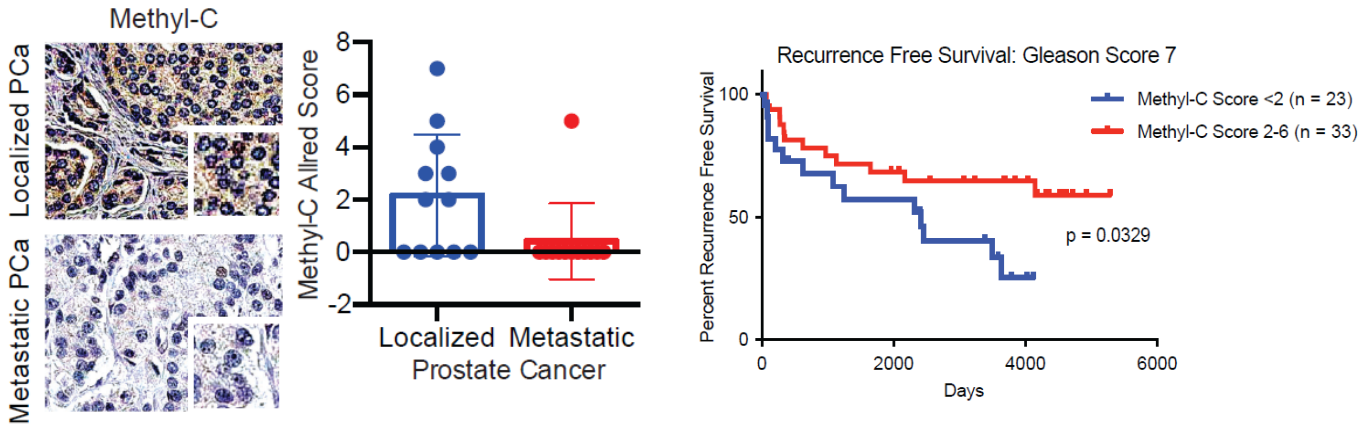


Figure 3: Representative immunohistochemistry images showing expression of methyl-C in localized and metastatic prostate cancer tissues. Box plots show the quantification of methyl-C score. Kaplan-Meier plot showing the association of methyl-C score with Gleason 7 prostate cancer.

Aim2: Study the PP2A restoration on pAR-MED1-BRD4 complex and AR-signaling in anti-androgen refractory CRPC. We have found that treatment of enzalutamide-resistant prostate cancer cells with DT-061 (SMAP) results in formation of PP2A holoenzyme and concomitant decrease in p-AR and p-MED1 from the chromatin. Similarly, ChIP-seq for AR and MED1 in enzalutamide-resistant LNCaP cells treated with DT-061 demonstrate genome wide loss of AR and MED1 resulting in the loss of AR mediated transcription (RNAseq) (Figure 4). These data suggests that restoration of PP2A in enzalutamide-resistant cells could be used to inactivate AR-MED1 transcriptional complex.

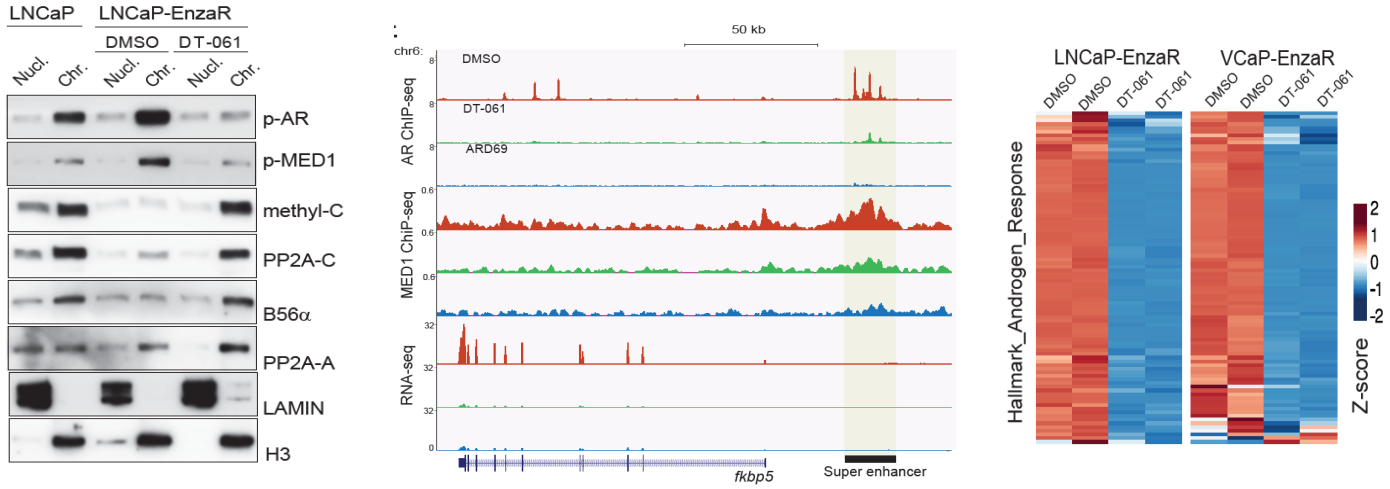
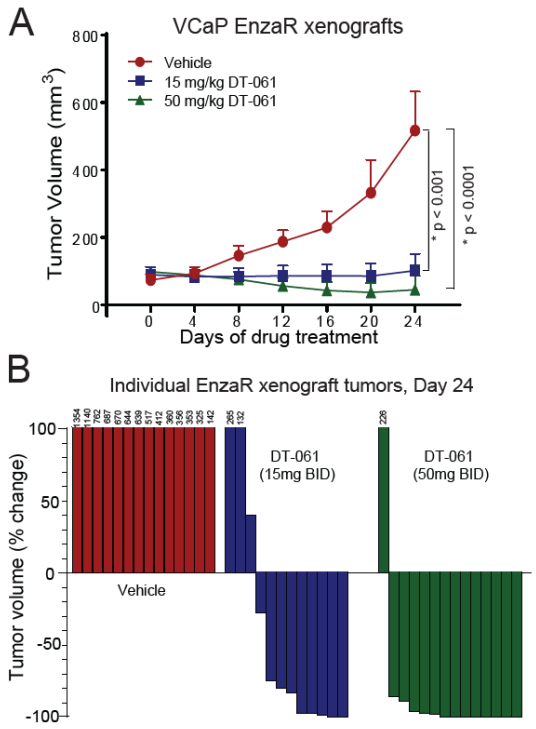


Figure 4: Left, DT-061 stabilizes the PP2A holoenzyme complex on the chromatin resulting in the loss of chromatin-bound p-AR and p-MED1. Chromatin and nuclear fractions from parental LNCaP and LNCaP-EnzaR cells treated with either DMSO or 10 uM DT-061 for 12 h were used to probe the indicated proteins. LAMIN and total H3 served as controls for soluble nuclear and chromatin fractions, respectively. Middle, DT-061 decreases genome-wide AR and MED1 binding. Genome browser tracks of AR and MED1 binding at FKBP5 locus in the indicated condition for LNCaP-EnzaR cells. The SEs associated with this region is indicated with a black bar. The tracks at the bottom show the transcript levels of the corresponding loci. Right, RNA-seq heat maps displaying negative enrichment of msigDB Hallmark Androgen response signature in LNCaP EnzaR and VCaP EnzaR treated with vehicle or 10 uM DT-061 for 24 h.

Aim 3: Evaluate the reactivation of PP2A by SMAP as a therapeutic strategy in the enzalutamide refractory CRPC.

Following is a pilot experiment that was performed to test the efficacy of DT-061 in vivo, and this data has helped us identify the right dose of the compound (50mg/kg BID) that will be used in experiments proposed in this Aim.



To evaluate the efficacy of DT-061 as a potent inhibitor of AR-mediated transcription through AR-MED1 dephosphorylation, we sought to study its efficacy in blocking EnzaR CRPC growth in an in vivo mouse xenograft model. We utilized the EnzaR VCaP model, as it harbors the TMPRSS2–ERG gene fusion and AR amplification, both of which are frequent molecular aberrations observed in patients with CRPC. EnzaR VCaP tumor–bearing mice were established in 6-8 week old male mice. Upon tumors reaching ~100 mm3, mice were randomized into three groups and treated orally with vehicle (n = 12) or DT-061 (15 mg/kg/twice daily; n = 12) or DT-061 (50 mg/kg/twice daily; n = 14) for four weeks (Fig. 7A). Treatment with DT-061 led to a dramatic tumor regression compared with vehicle control (Fig. 7A and B). These data clearly demonstrate the in vivo efficacy of DT-061 in enzalutamide resistant prostate cancer xenograft models in non-castrated animals.

Figure 5: A) DT-061 blocks enzalutamide refractory prostate cancer growth in vivo. Mice bearing VCaP-EnzaR xenografts received vehicle or 15mg/kg or 50mg/kg DT-061 twice daily (b.i.d) for 3 weeks. Tumor volume measured twice per week using calipers Data are mean ± s.e.m., and p values were computed using two-tailed t-test. B) The percent change in volume for each tumor after 24 days of treatment is shown as a waterfall plot (y-axis).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We plan to pursue the remaining experiments outlined in our original submission. Moving forward, we will incorporate new things that we have learnt in the past year, such as the appropriate choice of cell lines and derivatives of SMAPs, into our experimental strategies.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Results from this reporting period show continued reliance on AR activity by second generation anti-androgen refractory prostate cancer cells. Importantly, small molecule activator of phosphatase (SMAP) can reactivate PP2A and block AR signaling to prevent AR-driven CPRC growth in vitro and in vivo. Thus, targeting AR signaling by PP2A activation is a novel approach in treating refractory CRPC.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Changes in approach and reasons for change

There has been no change in experimental approaches.

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, vertebrate animals, biohazards, and/or select agents

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

Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?
Name: Reyaz Rasool, PhD
Project role: Postdoctoral Fellow
Researcher identifier: 34980488 (UPenn ID)
Nearest person month work: 4

Name: Brijesh Kumar Verma, PhD
Project role: Postdoctoral Fellow
Researcher identifier: 8081312001 (UPenn ID)
Nearest person month work: 6

Name: Qu Deng, PhD
Project role: Postdoctoral Fellow
Researcher identifier: 86886212 (UPenn ID)
Nearest person month work: 2

Contribution to project: Drs. Rasool, Verma and Deng planned, performed, and analyzed experiments outlined in this progress report.
Funding support: This grant (W81XWH-21-1-0436)

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

Not applicable as this is the first reporting period.

What other organizations were involved as partners?

None

8. Special Reporting Requirements

Nothing to report

9. Appendices