

AWARD NUMBER: W81XWH-21-1-0436

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

PRINCIPAL INVESTIGATOR: Irfan Asangani, PhD

CONTRACTING ORGANIZATION: University of Pennsylvania

REPORT DATE: JULY 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			<i>Form Approved</i> <i>OMB No. 0704-0188</i>		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) JULY 2023		2. REPORT TYPE Annual		3. DATES COVERED 1JUL2022 - 30JUN2023	
4. TITLE AND SUBTITLE PP2A Activation as a Therapy for AR-Addicted Refractory Castration-Resistant Prostate Cancer			5a. CONTRACT NUMBER W81XWH-21-1-0436		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Irfan Asangani			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania 3451 Walnut Street Franklin Building, 5th Floor Philadelphia, PA, 19104			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES:					
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					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
U	U	U	UU	11	USAMRDC

TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	10
5. Changes/Problems	11
6. Products	11
7. Participants & Other Collaborating Organizations	12
8. Special Reporting Requirements	12
9. Appendices	12

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.

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

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 hyperphosphorylation 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	
<p>Task 1: To evaluate the AR dependency in anti-androgen resistant prostate cancer cells</p> <ul style="list-style-type: none"> • 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 <p>Cell lines and models used: LNCaP, LNCaP-AR, VCaP and LAPC4 cells [ATTC] and their respective enzalutamide resistant derivatives developed by us [Asangani lab].</p> <p>Techniques: CRISPR mediated gene knockdown, phenotypic assays, drug treatment, immunoblotting, qRT-PCR, RNA-seq and ChIP-seq</p>	1-24	Asangani Lab

<p>Progress: 80% Completed. AR depletion leads to the death of EnzaR PCa cells.</p> <p>RNASeq following AR depletion by ARD-69 show loss of AR transcriptional signature.</p> <p>ChIP-seq for AR and MED1 following AR depletion show genome wide loss of AR and MED1. Some of these results have been published recently (Please see PMID: 37644036)</p>		
<p>Task 2: To identify the major sites of phosphorylation and novel interactions of AR-MED1-BRD4 complex in EnzaR prostate cancer cells</p> <ul style="list-style-type: none"> • 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. <p>Cell lines and models used: LNCaP, and LAPC4 and their respective enzalutamide resistant derivatives developed by us [Asangani lab].</p> <p>Techniques: Immunoprecipitation, Mass spectrometry, immunoblotting.</p> <p>Progress: 50% Completed. Immunoprecipitation followed by immunoblotting show increased interaction between AR, BRD4 and MED1 in EnzaR cells. Mass spectrometry has been completed pending analysis.</p>	6-18	Asangani Lab Garcia Lab
<p>Task 3: To evaluate the p-AR, p-MED1, p-BRD4 and Methyl-C as prognostic and predictive biomarkers for ADT.</p> <ul style="list-style-type: none"> • Immunohistochemistry with antibodies against phospho-S81 AR, p-S484/S488 BRD4, p-T1457 MED1, and Methyl-C on multiple progression, and outcome TMA <p>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.</p> <p>Techniques: Immunohistochemistry, pathology evaluation and analysis</p> <p>Progress: 70% 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.</p>	6-24	Asangani Lab Lal Lab
<p><i>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</i></p>	24	
<p>Specific aim2: Study the PP2A restoration on pAR-MED1-BRD4 complex and AR-signaling in anti-androgen refractory CRPC</p>		

<p>Task 1: To investigate the role of LCMT1 in PP2A loss in EnzaR PCa cells.</p> <ul style="list-style-type: none"> • 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 mutagenesis) into eukaryotic expression vector for rescue studies • Transcriptome analysis for LCMT1 expression in enzalutamide refractory tumors <p>Cell lines and models used: LNCaP, and LAPC4 cells [ATTC] and their respective enzalutamide resistant derivatives developed by us [Asangani lab].</p> <p>Techniques: Molecular cloning, qRT-PCR, ChIP-qPCR, bisulfite sequencing, immunoblotting, transfection, cell proliferation and colony formation assays</p> <p>Progress: 75% Completed. LCMT1 knockout by CRISPR has been completed. Cloning of LCMT1 overexpression construct is completed. Cloning of PP2A-C wild-type and L309A mutant has been completed. We anticipate the rest of the experiments to be completed in the next 6 months.</p>	12-24	Asangani Lab Feng Lab
<p>Task 2: To evaluate the effect of PP2A-C knockdown in PCa cells using CRISPR-Cas9 system</p> <ul style="list-style-type: none"> • 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 <p>Cell lines and models used: LNCaP and LAPC4 [ATCC] and enzalutamide resistant LNCaP and LAPC4 [Asangani lab].</p> <p>Techniques: Cloning, transfection, qRT-PCR, immunoblotting, colony formation assays.</p> <p>Progress: 70% 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.</p>	12-30	Asangani Lab Narla Lab
<p>Task 3: Investigate the effect of PP2A activation by SMAP on AR, MED1 and BRD4 chromatin binding in anti-androgen resistant PCa cells</p> <ul style="list-style-type: none"> • Chromatin fractionation followed by immunoblotting • ChIP-seq for AR, BRD4, and MED1 following SMAP treatment in EnzaR cells <p>Cell lines and models used: EnzaR LNCaP and EnzaR LAPC4 cells [Asangani lab].</p> <p>Techniques: drug treatment, cell fractionation, immunoblotting, and ChIP-seq</p> <p>Progress: Completed. ChIP-seq for AR and MED1 following SMAP treatment show genome wide loss of AR and MED1. Similar results observed with chromatin fractionation followed by immunoblotting. Some of these results have been published recently (Please see PMID: 37644036)</p>	18-36	Asangani Lab
<p>Task 4: To evaluate the effect of SMAP on transcriptional landscape and growth in anti-androgen resistant PCa cells.</p>	18-36	Asangani Lab

<ul style="list-style-type: none"> • RNA-seq following SMAP treatment in EnzaR cells • Sensitivity to SMAP in enzalutamide, darolutamide and apalutamide resistant PCa cells <p>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].</p> <p>Techniques: drug treatment, immunoblotting, RNA-seq, qRT-PCR, proliferation and colony formation assays.</p> <p>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. Some of these results have been published recently (Please see PMID: 37644036)</p>		
<p><i>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.</i></p>	36	
<p>Specific aim3: Evaluate the reactivation of PP2A by SMAP as a therapeutic strategy in the refractory CRPC</p>		
<p>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</p> <ul style="list-style-type: none"> • 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 <p>Cell lines and models used: Enzalutamide resistant LNCaP and VCaP (Asangani Lab)</p> <p>Techniques: castration, tumor generation and surgical extraction, extraction of bone marrows and organs, immunohistochemistry, qRT-PCR, Alu-PCR to detect micromets</p> <p>Progress: 30% completed.</p>	12-36	Asangani Lab
<p>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</p> <ul style="list-style-type: none"> • 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 	12-36	Asangani Lab Navone Lab

<p>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)</p> <p>Techniques: castration, tumor generation and surgical extraction, extraction of bone marrows and organs, immunohistochemistry, qRT-PCR, Alu-PCR to detect micromets</p> <p>Progress: Not done. We anticipate starting these experiments soon.</p>		
<p><i>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.</i></p>	36	

What was accomplished under these goals?

Significant majority of the goals were achieved that resulted in the high impact publication titled “Loss of LCMT1 and biased protein phosphatase 2A heterotrimerization drive prostate cancer progression and therapy resistance” in the journal Nature Communications (2023 Aug 29; PMID: 37644036). For complete description of the data and conclusion, please refer to the published manuscript.

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

I was invited as speaker to present the PP2A data at AACR Special Conference on Advances in Prostate Cancer Research, Denver, Colorado, March 2023.

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 CRPC growth in vitro and in vivo. Thus, targeting AR signaling by PP2A activation is a novel approach in treating refractory CRPC.

We believe the publication of some of these data in high impact journal Nature Communication will have a significant impact on the relevant area of prostate cancer research.

What was the impact on other disciplines?

Our published results will help explain the tumor suppressor role of PP2A in diverse cancer types as the mechanism regulating the activity of this critical protein phosphatase appears to be conserved across cancer types.

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?

None

What other organizations were involved as partners?

None

8. Special Reporting Requirements

Nothing to report

9. Appendices

Loss of LCMT1 and biased protein phosphatase 2A heterotrimerization drive prostate cancer progression and therapy resistance.

Rasool RU, O'Connor CM, Das CK, Alhusayan M, Verma BK, Islam S, Frohner IE, Deng Q, Mitchell-Velasquez E, Sangodkar J, Ahmed A, Linauer S, Mudrak I, Rainey J, Zawacki KP, Suhan TK, Callahan CG, Rebernick R, Natesan R, Siddiqui J, Sauter G, Thomas D, Wang S, Taylor DJ, Simon R, Cieslik M, Chinnaiyan AM, Busino L, Ogris E, Narla G, Asangani IA. Nat Commun. 2023 Aug 29;14(1):5253. doi: 10.1038/s41467-023-40760-6. PMID: 37644036 Free PMC article.