AWARD NUMBER: W81XWH-16-1-0246

TITLE: Effects of Phthalates on Androgen Receptor Regulation Associated with Castration-Resistant Prostate Cancer Development

PRINCIPAL INVESTIGATOR: Sarah Neuman, Ph.D.

CONTRACTING ORGANIZATION:
University of Wisconsin System
MADISON, WI, 53715-1218

REPORT DATE: March 2020

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2020	Final	06/30/2016 to 12/31/2019

4. TITLE AND SUBTITLE
Effects of Phthalates on Androgen Receptor Regulation Associated with Castration-Resistant Prostate Cancer Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0246

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sarah Neuman
E-Mail: sdneuman@wisc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Wisconsin-Madison
University of Wisconsin System
21 N. Park St., STE 6401
Madison, WI 53715-1218

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
Androgen deprivation therapy is initially effective for most prostate cancer patients, but it recurs after several years and becomes resistant, defined as castration-resistant prostate cancer (CRPC). The molecular mechanisms of the transition to CRPC are poorly understood. The androgen receptor (AR) is known to play a key role in the prostate cancer progression. Numerous mechanisms were introduced to show how CRPC continues to grow through AR signaling. Current CRPC therapies are mostly aimed to either inhibit AR expression or block androgen biosynthesis based on those findings. However, there is considerable population of CRPC patients that are typically ignored, showing a loss of AR in the tumor. For this group of patients, current treatments are ineffective. However, little research is conducted to investigate the pathways that are associated with the AR negative CRPC development. Therefore, the overall goal is to elucidate molecular mechanisms involved in the CRPC development specifically in the AR negative cell population. Nowadays, high attention is given to the effects of environmental toxicants on biological systems because we are all exposed to these environmental factors for decades and many studies have shown association of environmental toxicant exposure with adverse health outcomes. Phthalates are widely used in plastics and many everyday products. However, very little is known of their impacts on PRCA progression. The proposed project will investigate the effects of phthalates on AR regulation at molecular levels and determine its role in the CRPC development. This project will use PRCA models both in vitro and in vivo. PRCA xenografts will be implanted into mice and assessed for PRCA-aggressiveness. Additionally, AR levels and their epigenetic alterations will be measured.

15. SUBJECT TERMS
None listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	15	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

Prostate cancer is one of the leading causes of death for men in the United States. Initially, prostate cancer patients are treated with androgen deprivation therapy. This treatment is usually successful at first; however, prostate cancer can recur years later as castration-resistant prostate cancer (CRPC). CRPC is much more aggressive and is frequently lethal. Despite the immense clinical significance of CRPC, little is known about the molecular mechanisms that cause prostate cancer to become castration-resistant. A subset of patients with CRPC lack expression of the androgen receptor (AR) in the tumor; for these patients, androgen deprivation therapy will not be effective. However, very little is known about how a tumor can become AR-negative. Environmental toxicants, such as phthalates, small molecules found in many plastics, often function as hormone mimetics and may contribute to the development of prostate cancer. These studies are focused on identifying a relationship between phthalate exposure, loss of AR expression, and the development of CRPC. We are investigating two potential mechanisms that may affect AR expression in this context: silencing of AR mRNA expression through DMNT1-mediated methylation, and translational control of AR protein mediated by the RNA helicase DDX3.

2. KEYWORDS:

phthalates, di-butyl phthalate (DBP), castration-resistant prostate cancer (CRPC), androgen receptor (AR), steroidogenesis, translational control

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Determine if phthalate treatment reduces AR expression in prostate cancer cell lines or in mice tissue. (Months 1-12, 100% completed)

Major Task 2: Determine the ability of phthalate treated prostate cancer cells to metastasize *in vivo* as the basis of castration-resistant prostate cancer (CRPC) development. (Months 10-24, 100% completed)

Major Task 3: Determine the relationship between AR mRNA expression and AR protein expression in the prostate. (Months 12-24, 100% completed)

What was accomplished under these goals?

Major Task 1: Determine if phthalate treatment reduces AR expression in prostate cancer cell lines or in mice tissue.

Subtask 1: Subcutaneously treat (8 weeks) adult male C57BL/6J mice with slow releasing implants containing phthalate or anti-androgen.

As stated in the first annual progress report, to determine if phthalate exposure leads to reduced AR protein expression, adult male C57BL/6 mice (6-8 weeks) were subcutaneously treated with a slow-releasing compressed pellet containing either 25 mg of MBP or cholesterol control for 1 month (*n*=5 per treatment). After treatment, mice were euthanized and tissues including prostate lobes (dorsal and lateral, ventral, and anterior), bladder, testis, seminal vesicles, liver, and kidney were collected. Half of the set were formalin-fixation for IHC and the other half were snap-frozen for RT-qPCR analysis. In addition, serum (approximately 300 µL each) from individual mice were collected and stored at -80^{o}C for LC-MS/MS analysis to determine circulating MBP levels and multiple steroid hormone levels.

Subtask 2: Measure circulating phthalates levels in mice serum through LC/MS followed by further pharmacokinetics analysis.

LC-MS/MS analysis was used to measure MBP levels in the serum collected in Subtask 1. Unfortunately, we were unable to detect circulating MBP, likely due to a technical issue in preparation of the compressed pellet. It appeared that the pellet dissolved too quickly, and we were unable to achieve chronic exposure to MBP. In the future, we will need to devise a different method of delivery to achieve long-term MBP delivery/exposure. However, we did develop a new LC-MS/MS method to quantitatively measure 12 steroid hormones of interest (colored yellow in Figure 1) in mice serum and in cell culture media (referenced in the first annual progress report).

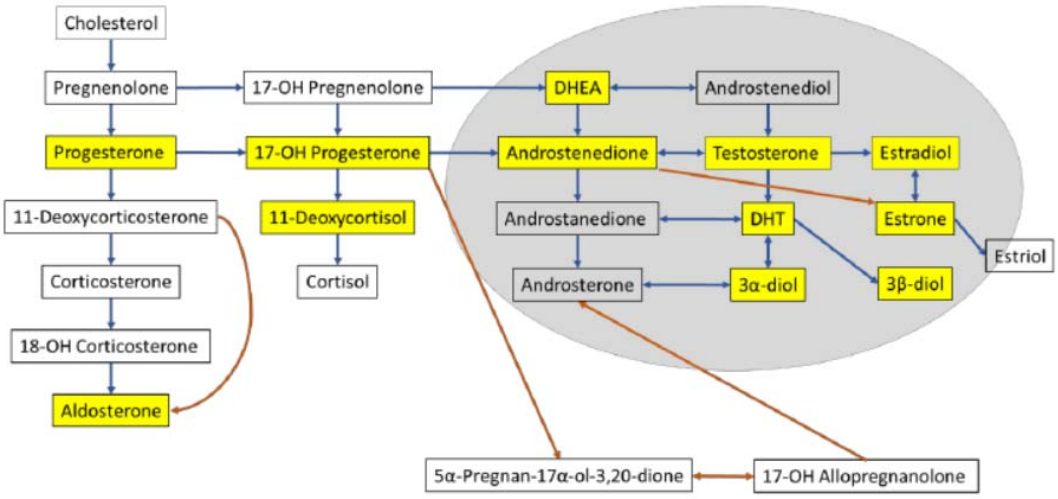

Figure 1. Steroidogenesis pathway indicating steroids of interest for LC-MS/MS analysis.

We used this LC-MS/MS panel to measure steroid hormone levels in prostate cancer cell lines (C4-2B) after 24 hour treatment with MEHP (the major metabolite of DEHP) or MEHP + testosterone (T) compared to DMSO vector control. As shown in Figure 2, MEHP treatment alone raised testosterone and 4-androstenedione levels and lowered estradiol levels. MEHP+T slightly raised testosterone levels and strongly raised estradiol levels, while 4-androstenedione was unaffected. This LC-MS/MS steroid panel will be a crucial research tool as we continue to analyze the effects of phthalates on prostate cancer development and progression.

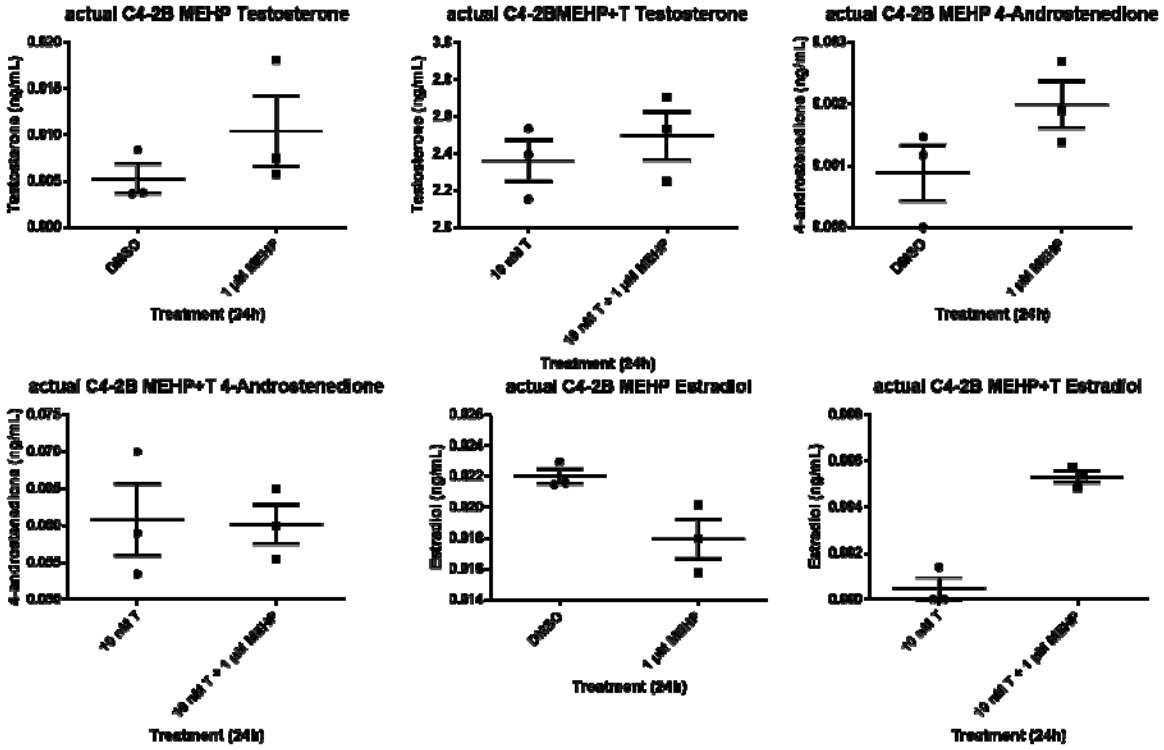

Figure 2. LC-MS/MS analysis of steroid hormone levels on prostate cancer cell lines treated with MEHP or MEHP+Testosterone.

Subtask 3: Immunohistochemistry (IHC) for AR expression and RT-qPCR for AR mRNA analysis in mice tissues obtained from Subtask 1.

As shown in the first annual report, we used RT-qPCR to measure AR mRNA expression levels relative to the reference genes YWHAZ and B2M in the ventral prostate, dorsal/lateral prostate, and anterior prostate (tissues collected as described in Subtask 1). No changes were observed in the ventral prostate or the dorsal/lateral prostate (Figure 3A, B), but a significant reduction in AR mRNA expression was detected in the anterior prostate (Figure 3C). To determine if MBP exposure induced changes in the weight of androgen-regulated tissues by lowering the AR expression, and presumably AR signaling, the masses of tissues including the prostate lobes, seminal vesicles (SV), and testes were measured. However, no changes were observed between the control group and the MBP-treated group in all measured tissues.

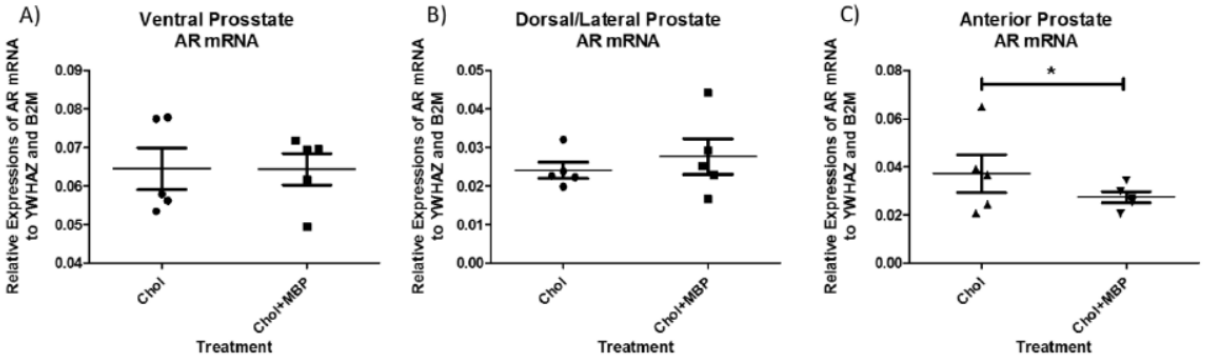

Figure 3. RT-qPCR analysis of AR mRNA expression levels in prostate compartments of mice treated with MBP. (A) AR mRNA expression in the ventral prostate (VP) is unchanged upon MBP treatment. (B). AR mRNA expression in the dorsal/lateral prostate (DLP) is unchanged in response to MBP treatment. (C) AR mRNA expression in the anterior prostate (AP) is significantly downregulated in response to MBP treatment. *p<0.05

We also used immunohistochemistry (IHC) to assess AR protein expression levels in the epithelial and stromal compartments of the dorsal/lateral prostate (DLP). The IHC was conducted on formalin-fixed, paraffin-embedded mouse prostate tissues collected in Subtask 1. The primary antibody used for this analysis was rabbit anti-AR (1:250, Santa Cruz, CA). As shown in the first annual progress report, a reduction in AR protein levels was detected in the DLP of mice treated with MBP (Figure 4).

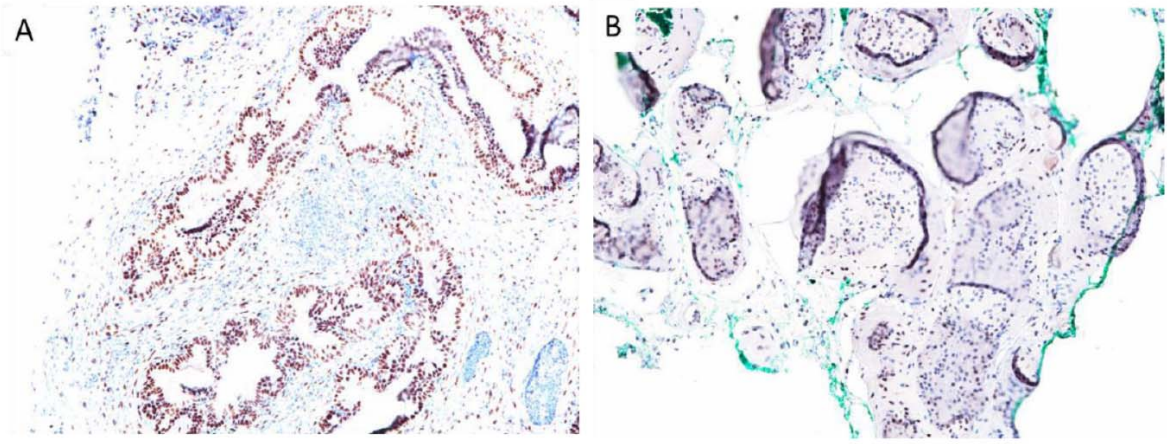

Figure 4. IHC analysis of AR protein expression in the dorsal/lateral prostate (DLP) of control and MBP-treated mice. (A) AR protein expression (shown in brown) in control mice treated with a cholesterol pellet. Tissues are counterstained to mark nuclei. (B) AR protein expression (brown) in MBP-treated mice. Note that there is a slight reduction in AR protein expression upon MBP treatment.

Subtask 4: Treatment of human prostate benign/cancer cell lines with phthalates followed by RT-qPCR for AR mRNA analysis and Western blot for AR protein analysis.

As described in the first annual progress report, we tested the effect of MBP treatment on AR expression levels in various human prostate cell lines, including the prostate cancer lines LNCaP and CWR22Rv1 and the non-tumorigenic prostate cell line BPH-1. 3 x 10^5 cells/well were seeded in a 6-well plate with 5% fetal bovine serum (FBS) medium. The next day, media was switched into 5% charcoal-stripped serum (CSS) medium and was incubated for 12-16 hours. After starvation, cells were treated with either dimethyl sulfoxide (DMSO) control or various doses of MBP (100 pM, 1 μM, or 10 μM) for 24 hours. After treatment, cells were collected and RNA were isolated with TRIzol Reagent (Life Technologies, Carlsbad, CA) followed by cDNA synthesis with Bio-Rad iScript kit. We then used qPCR to assess the expression levels of AR mRNA. Unfortunately, most of these treatments had no significant effect on AR mRNA expression levels, although we did observe a modest, but not significant, reduction in AR mRNA expression levels in CW22Rv1 prostate cancer cells after treatment with 1 μM MBP (Figure 5). We also did not observe any significant differences in AR protein expression via western blot.

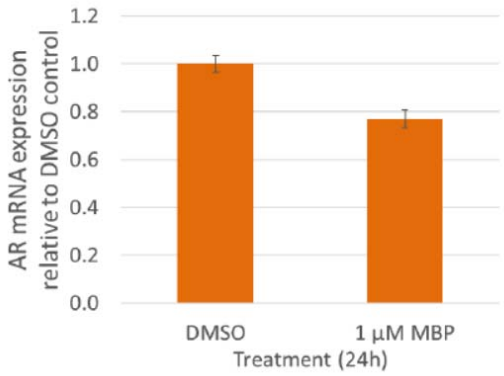

Figure 5. Analysis of AR mRNA expression in CW22Rv1 prostate cancer cell lines upon treatment with MBP. A modest, but not significant, reduction in AR mRNA levels is observed after 24 h treatment with 1μM MBP.

Major Task 2: Determine the ability of phthalate-treated prostate cancer cells to metastasize in vivo as the basis of castration-resistant prostate cancer (CRPC) development.

Subtask 1: Implant prostate cancer cell xenografts in adult male athymic mice with either with or without phthalate treatment and monitor for 8 weeks.

As outlined in the first annual progress report, we utilized prostate cancer cell xenografts to determine if MBP treatment enhanced the ability of cancer cells to metastasize into lymph nodes. We implanted xenografts containing 250,000 LNCaP prostate cancer cells (either untreated or MBP-treated, as described in Major Task 1, Subtask 4) under the kidney capsule of 5 immunocompromised male nude mice and monitored them for 8 weeks. The LNCaP cell line was chosen because previous research suggests that this cell line is capable of metastasis in mouse xenograft models [1].

Subtask 2: IHC and RT-qPCR analysis of AR expression on kidney grafts, prostates, and lymph nodes followed by statistical analysis.

Tissues were collected 8 weeks after xenografting and analyzed for proliferation and AR mRNA expression. Unfortunately, many of the xenografts in both control and MBP-treated conditions did not take. This left few tissues for analysis. On the tissues we could collect, we did not see any significant differences in lymph node proliferation (assessed by Ki67 staining) between control and MBP-treated conditions. We also did not observe any significant differences in AR expression levels in the xenograft or in the mouse prostate. Given that MBP treatment does not appear to have a significant effect on AR expression levels in human prostate cancer cell lines (see Major Task 1, Subtask 4), these results are not surprising. Future studies will be required to identify additional cell lines that are more responsive to MBP treatment to fully assess the ability of phthalates to promote metastasis *in vivo*.

Major Task 3: Determine the relationship between AR mRNA expression and AR protein expression in the prostate.

Subtask 1: Treatment of cells (see Major Task 1, Subtask 4) with phthalates followed by RT-qPCR for DNMT1 and DDX3 mRNA expression levels and western blot for DNMT1 and DDX3 protein expression levels.

Samples were collected as described in Major Task 1, Subtask 4. We then used RT-qPCR to measure mRNA expression levels and western blots to measure protein expression levels of both DMNT1 and DDX3, two potential regulators of AR expression in CRPC. Unfortunately, MBP treatment did not have a significant effect on mRNA or protein expression levels of either DMNT1 or DDX3.

Subtask 2: Measurement of the methylation pattern on the AR promotor region in phthalate-treated cells through the methylated DNA immunoprecipitation (MeDIP) assay and analysis of the effect of DDX3 expression levels on AR mRNA and protein expression.

As shown in the second annual progress report, we obtained protein extracts from prostate tissue (dorsolateral prostate and anterior prostate lobes) of mice treated with a cholesterol pellet and mice treated with an MBP-containing pellet. We then performed an immunoprecipitation using the MeDIP assay, as previously published [2,3]. qPCR was then used to assess for the levels of methylation at the AR promoter. This analysis does not show a significant increase in methylation at the AR promoter in MBP-treated cells compared to cholesterol controls, suggesting that phthalate treatment does not have a significant effect on DNA methylation at the AR promoter (Figure 6).

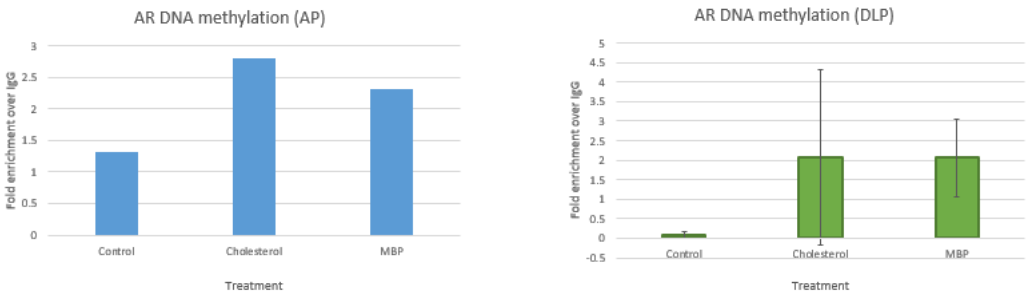

Figure 6. MeDIP assay to measure methylation at the AR promoter in response to phthalate treatment. An immunoprecipitation for methylated DNA was performed, and qPCR was used to measure methylation at the AR promoter. Control represents IgG control, cholesterol is a vehicle control, and MBP is the treatment sample. No significant enrichment of DNA methylation was detected in MBP-treated cells compared to cholesterol controls in either the anterior prostate (AP) or dorsal/lateral prostate (DLP).

We also analyzed the effects of DDX3 expression on AR mRNA and AR protein expression in CRPC tissues and cell lines. Pharmacological inhibition of DDX3 using the small molecule inhibitor RK33 significantly reduces expression levels of DDX3, and also significantly increases expression of AR (Figure 7). This data suggests that DDX3 an inverse relationship between DDX3 expression levels and AR expression levels in DDX3. This data also suggests that RK33 treatment might be a new way to alter AR expression levels and restore androgen sensitivity in CRPC.

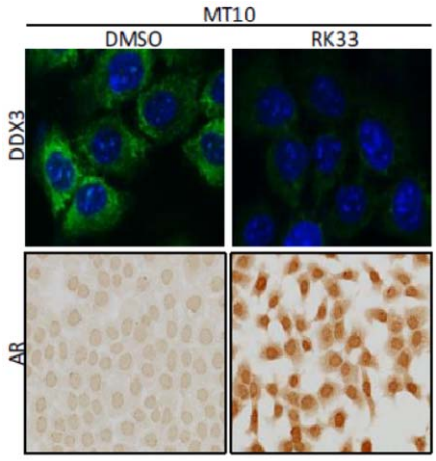

Figure 7. RK33, a small molecule inhibitor of DDX3, decreases DDX3 expression and increases AR expression in CRPC. Immunocytochemistry for DDX3 (green, top panels) and AR (brown, lower panels) was performed on MT10 CRPC cells. Note decrease of DDX3 and resolution of stress granules, along with increased AR expression with addition of RK33.

Subtask 3: Determine the co-localization pattern of DNMT1/DDX3 and AR in CRPC mice tissue through IHC.

Because we did not observe any effects on methylation of the AR promoter, as described in Figure 6, the remainder of our analysis focused strongly on DDX3. As described in the second annual report, we used a xenograft model to test the localization of DDX3 and AR in CRPC tissues in vivo. C42 xenografts (CRPC model) grown *in vivo* for two months showed two distinct populations of cells, consistent with the heterogeneity found in human CRPC (Figure 8). One population had high expression levels of DDX3 and low expression levels of AR, while a second population had low expression levels of DDX3 and high expression levels of AR. This data supports the concept that AR protein expression is inversely related to DDX3 protein expression.

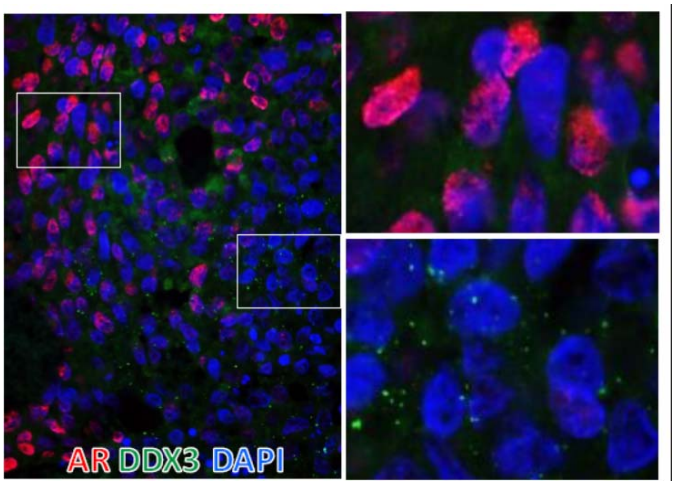

Figure 8. AR protein expression is inversely related to DDX3 protein expression. Using dual IHC, we co-localized AR (red) and DDX3 (green) protein in FFPE CRPC model C42B xenograft grown *in vivo* for 2 months. We observed two distinct populations of cells: population 1 consists of AR-negative and DDX3 puncta-positive cells (bottom right portion of micrograph), the second population was AR positive cells which were observed with little and diffuse DDX3 expression patterns (i.e. no stress granules; upper left portion of micrograph).

Subtask 4: Determine the co-localization pattern of DNMT1/DDX3 and AR in the human prostate cancer tissue microarray (TMA).

We analyzed DDX3 protein, AR mRNA, and AR protein expression levels in a tissue microarray (TMA) obtained from the Prostate Cancer Biorepository Network (PCBN). We used the LuCaP patient-derived xenograft (PDX) TMA (PCBN 89A-D) which contains 41 PDXs with 9 cores per xenograft. These PDXs include CRPC subtypes AR+ (n=5), AR low/- (DNPC, ARLPC) (n=3), and NEPC (n=4). DDX3 was detected with DAB and AR C-terminal was detected with Immpact Red, and counterstained with hematoxylin. Single stained slides were used to create a spectral library for the chromogens, and analysis was done using Vectra automatic image acquisition and InForm 1.4 software as previously described [4]. RNAscope was used to detect AR mRNA. This protocol was performed according to ACD Bio recommendations. Briefly, tissues were rehydrated with xylenes and ethanol, and antigen retrieval was performed in the decloaker with 1X ACD target retrieval buffer. 100uL of AR RNA probe was incubated at 40^{o}C for 2 hours. Sequential additions of AMP 1-6 buffers were added according to ACD protocol, followed by DAB for 5 min. Nuclei were counterstained with hematoxylin. Our results demonstrate that patient samples with high expression levels of AR protein have low expression levels of DDX3 protein; AR mRNA is also present in both cases (Figure 9). NEPC does not express AR mRNA or protein and has low expression levels of DDX3 (Figure 9).

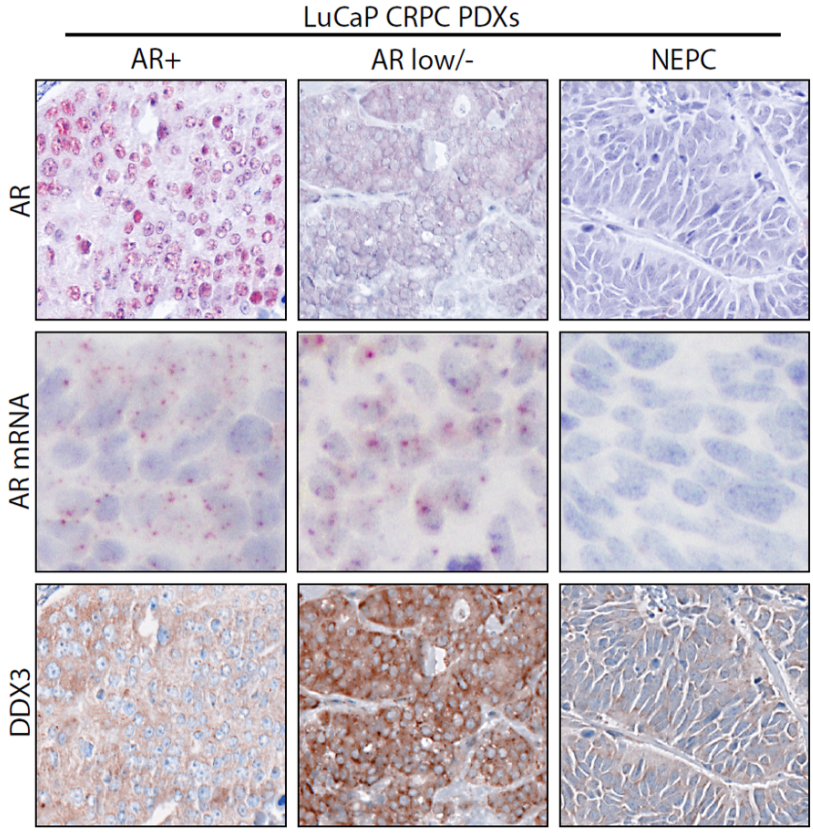

Figure 9. Analysis of AR protein, DDX3 protein, and AR mRNA expression in human prostate cancer tissue microarray (TMA) samples. Representative images of AR protein (red, first row), AR mRNA (brown, second row), and DDX3 protein (brown, third row) in PDXs from three subtypes of CRPC: AR+, AR low/-, and NEPC. Nuclei were counterstained with hematoxylin (blue). Note the inverse relationship between expression levels of AR protein and DDX3 protein.

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

Training Activity:

- Developed and learned lab techniques required for the research project described above, including LC-MS/MS, RT-qPCR, western blot, IHC, *in situ* hybridization, RNAscope, and immunoprecipitation (IP).
- Developed grant writing and manuscript writing skills by working one-on-one with Dr. William Ricke.
- Mentored three undergraduates to guide them with their own projects and develop my mentoring skills.
- Taught two lab modules for undergraduate Pharmacology-Toxicology (PharmTox) majors.

Professional Development:

- Attended weekly group lab meetings, relevant Cancer Biology seminars, monthly Prostate Center of Research Excellence seminar series, and monthly UW O'Brien Center seminar series.

- Mentored three undergraduates to guide them with their own projects and develop my mentoring skills.

- Taught two lab modules for undergraduate Pharmacology-Toxicology (PharmTox) majors.

- Journal Article Review (FASEB Journal, Development, Genesis)

- Invited Lectures and Talks
  PharmTox 558: Cell-based assays: an introduction to qPCR for gene expression analysis
  Invited speaker for the School of Science, Technology, and Education Colloquium at Lakeland University, my undergraduate alma mater

How were the results disseminated to communities of interest?

Volunteered to run a station about my research at two University of Wisconsin-Madison science outreach events: Science Expeditions and the Wisconsin Science Festival.

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

Nothing to report

4. IMPACT

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

Environmental toxicants often function as steroid hormone mimetics and thus may play important roles in the development of hormone-related cancers, including aggressive castration-resistant prostate cancer. The mechanism by which prostate cancer becomes castration-resistant has long been thought to be understood, and most studies point to a gain-of-function of AR as the molecular mechanism by which prostate cancer becomes resistant to androgen deprivation therapies. However, this work highlights an entirely new mechanism by which prostate cancer can become castration-resistant: through translational inhibition of AR expression. This finding represents an important new advance in prostate cancer research and may spur future studies that more carefully analyze the penetrance and importance of loss of AR expression in prostate cancer progression.

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?

Prostate cancer is the second leading cause of death for men in the United States. Early stages of cancer can be treated with high success rates; however, these cancers can eventually recur and become castration-resistant prostate cancer, which is very difficult to treat and often results in death. Exposure to environmental toxins, including phthalalates, may enhance the risk of developing CRPC. Therefore, the development of new therapeutics for CRPC is absolutely essential. This work has identified a new mechanism by which prostate cancers become castration-resistant, opening the door for development of novel therapeutic strategies to treat this deadly disease.

5. CHANGES/PROBLEMS

Nothing to report

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications:
Nothing to report

Books or other non-periodical, one-time publications:
Nothing to report

Other publications, conference papers, and presentations:

1. Neuman, S.D., Bashirullah, A. (2018). Reconsidering the Passive Diffusion Model of Steroid Hormone Cellular Entry. Developmental Cell 47(3): 261-262.
2. Neuman, S.D., Vellky, J.E., Ihry, R.J., Bashirullah, A., Ricke, W.A. Novel models to dissect the role of DDX3-dependent translational control in the prostate. Collaborating for the Advancement of Interdisciplinary Research in Benign Urology (CAIRIBU) Conference, Ellicott City, MD. December 2018. (poster presentation)
3. Neuman, S.D., Vellky, J.E., Ihry, R.J., Bashirullah, A., Ricke, W.A. Translational control in steroid hormone signaling. University of Wisconsin-Madison O'Brien Center Symposium. Madison, WI. April 2019. (oral and poster presentation)

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Sarah Neuman
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0001-5731-6426
Nearest person month worked:	12
Contribution to Project:	Dr. Neuman performed the experiments and analysis described in this report.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

N/A; Nothing to report

9. APPENDICES

References

1) Lewis, S.R., et al., *Steroidogenic factor 1 promotes aggressive growth of castration-resistant prostate cancer cells by stimulating steroid synthesis and cell proliferation.* Endocrinology, 2014. 155(2): p. 358-69.
2) Keil, K.P., et al., Androgen receptor DNA methylation regulates the timing and androgen sensitivity of mouse prostate ductal development. Dev Biol, 2014. 396(2): p. 237-45.
3) Kinoshita, H., et al., *Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer.* Cancer Res, 2000. 60(13): p. 3623-30.
4) Sehgal P.D., et al., *Tissue-specific quantification and localization of androgen and estrogen receptors in prostate cancer*. Human pathology, 2019. 89: p. 99-108.