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TITLE: Malignant Changes in Bladder Cancer Associated with Defoliant Exposure

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CONTRACTING ORGANIZATION: Kansas University Medical Center

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

Kansas University Medical Center
3901 Rainbow Boulevard,
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Bladder cancer is a common solid tumor in the VA Health System. The Institute of Medicine acknowledged that defoliants such as Agent Orange and Agent Blue used in Vietnam Era conflicts can potentially increase risk of bladder cancer. The active agents in these compounds (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in Agent Orange and arsenicals/arsenic in Agent Blue) and are known to induce changes in gene expression pathways that can transform cells. We hypothesize that AO/AB exposure causes unique alterations in gene expression and methylation in the urothelium which contribute to malignant degeneration. The objective of this study is to define alterations that occur in the urothelium with exposure to AO/AB in both laboratory models and in Vietnam Era Veterans.

15. SUBJECT TERMS
NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. **INTRODUCTION:**

Bladder cancer is a common solid tumor and prevalent in the VA Health system. Recently, the Institute of Medicine has acknowledged the fact that rainbow defoliants such as Agent Orange and Agent Blue used in southeast Asian conflicts can increase the risk for bladder cancer. While some laboratory studies have suggested modest evidence in favor of these agents being directly carcinogenic, little work has been done on Vietnam Era Veterans to ascertain potential alterations in soldiers with exposure. We *hypothesize* that AO/AB exposure causes unique alterations in gene expression and methylation in the urothelium which contribute to malignant degeneration. The *objective* of this study is to define alterations that occur in the urothelium with exposure to AO/AB in both laboratory models and in Vietnam Era Veterans.

2. **KEYWORDS:**

TCDD, agent orange, agent blue, bladder cancer, RNA sequencing, arsenic, mouse model

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Specific Aim/Major Goal 1: Determine pathway activation/suppression and signaling in an annotated data set of veteran and active duty military personnel with emphasis on potential AO/AB exposure.

Major Task 1- Assess and compare global transcriptomic changes in BCa tissue from Vietnam Era Veterans with AO/AB exposure and non-exposed BCa patients. (**approximately 50% complete).**

Major Task 2: Assess epigenetic methylation changes in BCa specimens from Vietnam Era Veterans with potential TCDD exposure and compare this to non-exposed BCa patients and transformed cells. **(approximately 50% complete).**

Milestone 1**:** Define sequencing results from both data sets (**Expected completion during no cost extension.**)

Milestone 2**:** Compare sequencing analysis between human patients and human cell lines garnered in SA1 - (**Expected completion – months 9-24/no cost extension**).

Specific Aim/Major Goal 2 – Determine the impact of acute and long-term low doses of TCDD and/or cacodylic acid on benign urothelial cell lines

Major Task 1: Determine the impact of acute and long term low doses of TCDD and/or cacodylic acid on benign urothelial cell lines (UROtsa, SV-HUC-1) (**complete, ~month 15)**

Milestone 1**:** Acquisition of transformed malignant cells as defined by increased growth rates and alteration of growth on agar or low attachment plates. (**complete, ~month 15***)*

Milestone 2**:** Acquisition of transformed malignant cells as defined by xenograft formation. (**experiments are complete, but cells failed to transform***)*

Milestone 3**:** Sequencing and analysis of primary data including *in vitro* and *in vivo* experiments as well as sequencing data. (**Completed as capable, ~ month 15-16***)*

Major Task 2: Assess tumor development and global transcriptomic and epigenetic changes in a murine model of carcinogen induced BCa exposed to arsenic. (**complete, Month 12.).**

Milestone 1**:** Completion of BBN/CA induced tumor analysis including bladder size, stage, grade, cell death status and immunohistochemistry. (**Complete, month 16***)*

Milestone 2: Completion of RNA sequencing. We aim to make comparison between CA/nonCA treated samples in addition in to between mouse/human samples. (**Expected completion – months 15-24**)

What was accomplished under these goals?

Our previous progress report yielded a few major points from initial year of research. First, we surprisingly found that cacodylic acid (CA), a methylated form of arsenic akin to what is in Agent Blue, did not promote tumor formation in our BBN model of BCa. This result was the opposite of our hypothesis. Second, we did not observe obvious promotion of tumorigenicity in our cell lines, another case of where we found the opposite of our hypothesis. The major goals of this year's work was to continue our cell culture experiments and determine why CA did not produce more tumor formation.

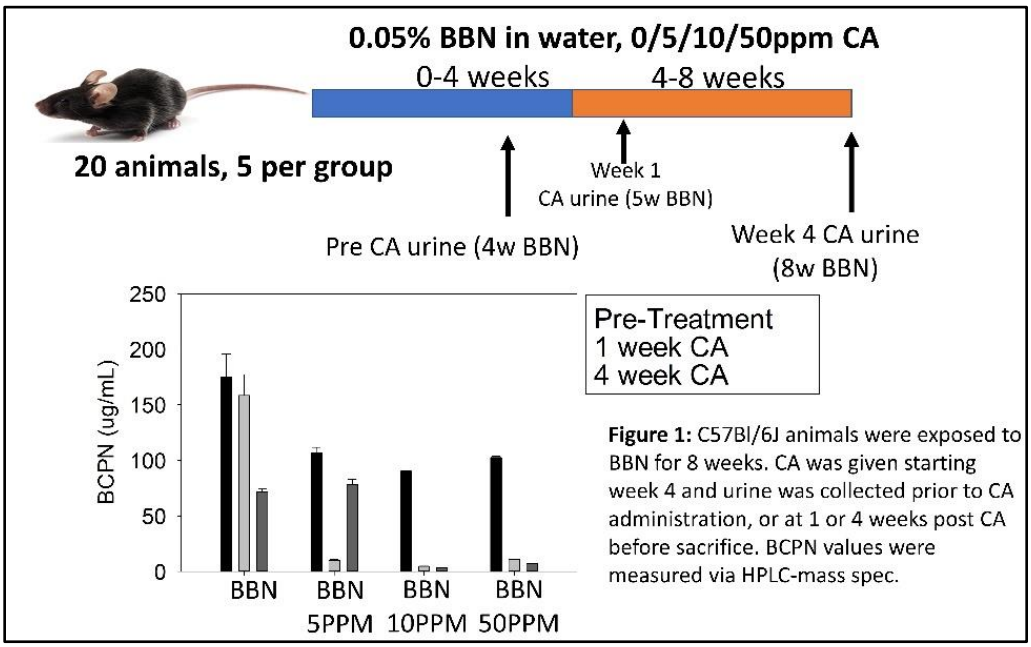

Figure 1: C57Bl/6J animals were exposed to BBN for 8 weeks. CA was given starting week 4 and urine was collected prior to CA administration, or at 1 or 4 weeks post CA before sacrifice. BCPN values were measured via HPLC-mass spec.

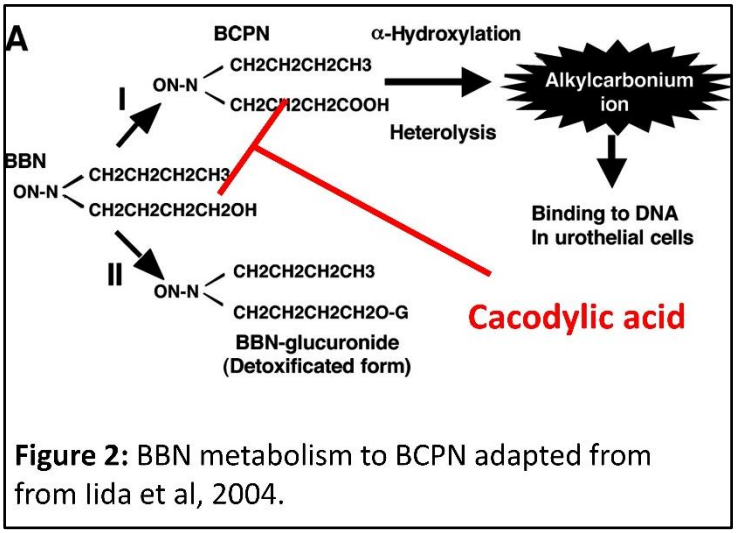

Figure 2: BBN metabolism to BCPN adapted from from Iida et al, 2004.

To determine why CA might reduce cancer formation in BBN treated animals, we examined the literature and observed prior publications indicating CA might function as a nuclear factor erythroid 2-related factor 2 (Nrf2) agonist, and that Nrf2 agonism may reduce BBN induced carcinogenesis through an effect on BBN metabolism (Iida et al., 2004). If this was true, CA might affect the metabolism of BBN in such a way that the model was no longer functioning appropriately as BBN metabolism itself was being affected. Reduced levels of the active metabolite (BCPN) would explain our findings. To assess this, we collaborated with the KU-Lawrence campus to generate an assay for evaluating the presence of BCPN, a metabolite of BBN in mouse urine. C57Bl/6J animals were given BBN for 4 weeks and urine was collected. CA (5ppm, 10ppm, 50ppm as in prior work in this grant) or regular water also containing 0.05% BBN was given for an additional 4 weeks. Urine was collected at week 1 and week 4 from whole cages of animals via a metabolic caging system. We found dramatic reductions in urine concentrations of BPCN after only 1 week of CA treatment (Figure 1). This reduction in BCPN levels was sustained out to 4 weeks in the case of both 10ppm and 50ppm doses, but not the 5ppm dose. We previously observed

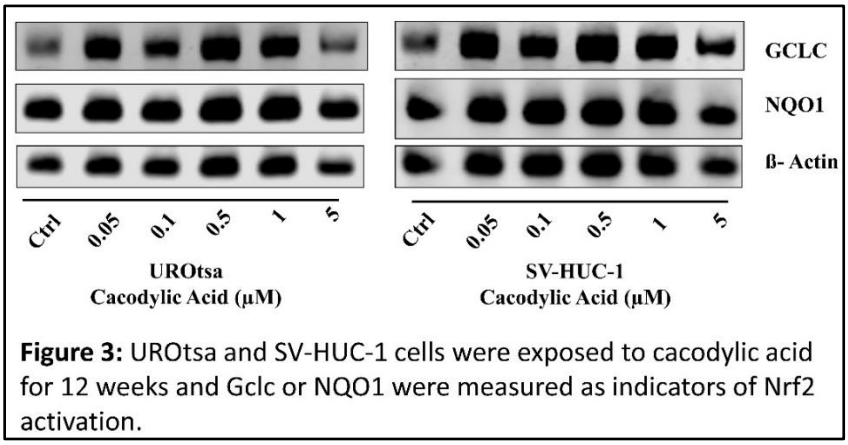

Figure 3: UROtsa and SV-HUC-1 cells were exposed to cacodylic acid for 12 weeks and Gclc or NQO1 were measured as indicators of Nrf2 activation.

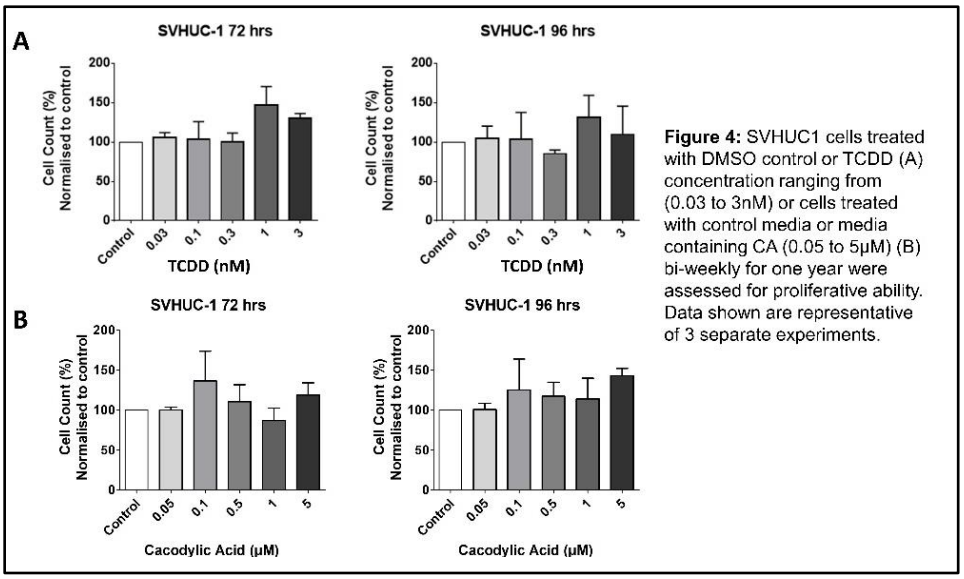

Figure 4: SVHUC1 cells treated with DMSO control or TCDD (A) concentration ranging from (0.03 to 3nM) or cells treated with control media or media containing CA (0.05 to 5µM) (B) bi-weekly for one year were assessed for proliferative ability. Data shown are representative of 3 separate experiments.

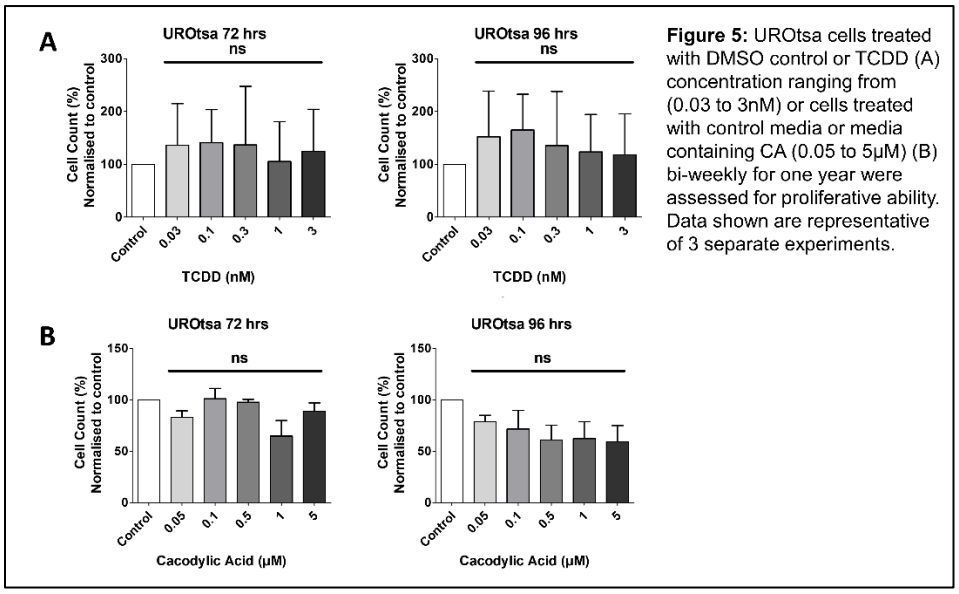

Figure 5: UROtsa cells treated with DMSO control or TCDD (A) concentration ranging from (0.03 to 3nM) or cells treated with control media or media containing CA (0.05 to 5µM) (B) bi-weekly for one year were assessed for proliferative ability. Data shown are representative of 3 separate experiments.

differences in tumor formation in size in the 10ppm and 50ppm groups consistent with our observed changes in BBN metabolism (reduced levels of BCPN). It can be inferred that the massive reduction in BCPN levels would result in dramatic reductions in BPCN exposure, and thus reduced exposure to carcinogen and reduced carcinogenicity (see model in Figure 2). We attempted to assess the ability of CA to activate Nrf2 in both acute and chronically treated BCa cell lines by checking protein levels of Nrf2 target genes. We did not see consistent alterations but did observe alterations in Glutamate—cysteine ligase catalytic subunit (Gclc) a known target of Nrf2 (Figure 3). As BBN is also metabolized in the liver, it may be that CA has more potent effects on liver metabolism of BBN and thus the lack of BCPN conversion is predominantly mediated by alterations in liver metabolism. This is an important finding and details the mechanism of why CA yielded a surprising result. Moreover, this result indicates the lack of increased carcinogenicity observed with CA is likely more related to the model in question than the compound and re-emphasizes the need for human data.

This observation must be taken into account when interpreting our sequencing data in mice. While we have made many novel observations, some of these may be attributable to changes in metabolism. Once we are able to complete our human tissue analysis under the NCE we can them make comparisons to human data as possible.

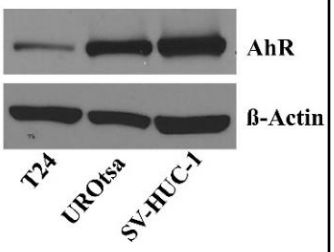

Figure 6:. Total cell extracts of various cell lines were analyzed by western blotting with antibody against AhR (Cell Signaling 83200S) or ß-Actin (cell Signaling 4970S).

The benign cell lines used in our study did not obviously undergo malignant transformation in the presence of either TCDD or CA (Figure 4, Figure 5). Cellular proliferation (Figure 4, Figure 5) and migration (not shown) were used as primary indicators of transformation. It is possible other aspects of cancer biology were affected however, the lack of changes observed favors the hypothesis these cells have been minimally transformed. Prior studies using other methylated arsenic compounds have shown demonstrable increases in metrics such as proliferation and migration (Bredfeldt TAAP 2006). The cells were reevaluated to ensure they were expressing the TCDD receptor AhR and substantial expression in both cell lines in addition to positive control cells T24, which have previously been shown to express AhR, was noted (Figure 6). As such, we believe that in spite of their pro-carcinogenic nature in other studies, neither TCDD nor CA obviously enhanced carcinogenicity in these experiments. In the case of CA, this may be because the organic form of arsenic (methylated arsenic, such as cacodylic acid) is generally less toxic to cells than

inorganic arsenic or monomethylated arsenic. It may be that the carcinogenic aspects of arsenic are somewhat dependent on cell death and/or ROS that are not generated by CA at the doses we used. In the case of TCDD, it remains to be determined why we did not see a more obvious promotion of carcinogenicity. Prior studies have shown acute treatment with TCDD enhanced invasiveness in T24 cells; however, these cells are already transformed cancer cells whereas we had hypothesized TCDD would transform benign cells.

Nonetheless, we sequenced a portion of our cells treated for 6 months with TCDD or CA to see if there was molecular evidence of transformation. We found >100 genes differentially regulated across all cell lines (not shown). To reduce the number of potentially relevant genes we filtered for genes that were regulated in the same direction (up/down) between the different cell lines. We found a number of genes (Table 1) differentially expressed across the dose response. Amongst these genes, we searched for gene pairs that were differentially expressed either 1) in both cell lines by the same chemical or 2) by both chemicals in the same cell line. Sorting by which genes were differentially expressed in both cell lines yielded a much lower number of hits. We will use both the refined list and our more general data containing all differentially regulated genes to compare to human data when this is available. We believe, depending on what is found in our human data, it may be possible to correlate changes in gene expression with changes in exosomal sequencing. The general lack of major changes partially supports the idea that these cells were minimally transformed.

Downregulated CA	Upregulated CA	Downregulated TCDD	Upregulated TCDD
NUAK1	none	RPS12	ACSL1
PRKCE		NPTXR	TIPARP
		SLPI	
		ZHX2	
		CEP57L1	
		KPNA5	

Table 1: RNA-sequencing was used to evaluate the transcriptome in SV-HUC-1 or UROtsa cells exposed to CA or TCDD for 6 months. Genes that were commonly either upregulated or downregulated in both cell lines were identified.

Data was compared to that acquired in our murine model but found no major notable correlation (not shown). Given that we have attributed our effects in the murine model to altered metabolism of BBN, it was not surprising that the differences in gene regulation between mice exposed to BBN and cells exposed to either TCDD or CA did not have similarity. Moreover, we expected a more carcinogenic phenotype in both scenarios. While the lack of BBN metabolism explains the findings in the mouse model, we are still attempting to understand exactly what mediated the lack of carcinogenicity present in the cell culture models.

The epigenetic data in our murine studies provides some novel and interesting data. We noted a number of different changes in pathways potentially relevant to bladder cancer biology. Somewhat surprisingly, we noticed a surprising number of genes that had both a change in methylation at very proximal spots on the gene. Many of these were similarly regulated i.e. we noticed an increase in methylation in one element, and a corresponding and similar decrease in an adjacent element. We extensively validated our data and this unique change was still present. We are currently investigating this phenomenon.

In addition, a number of pathways were observed to have some sort of difference in methylation status. Given the difference in cancer formation, we compared which genes had similar differential regulation in our dose response animal groups. Using both Reactome Pathways and the KEGG database we used this differential analysis and identified pathways relevant to these changes. Pathways with increased methylation in at least two of the sets of animals treated with cacodylic acid included those involved in tight junction formation, Hippo signaling, and a pathway associated with measles that contained changes in a number of Stat pathway genes. Pathways with reduced methylation include multiple pathways associated with the transcription factor Runx3, as well as calcium signaling, and pre-mRNA processing. Changes observed in *RUNX3* were of particular note as *RUNX3* methylation has been suggested as an “early” event in human BCa. Methylation of *RUNX3* is minimally present in normal human urothelium; however, is modestly present in mice according to our own data and TCGA. Reduced methylation of *RUNX3* has previously been suggested to be a mechanism of protection of nicotinamide, a class III histone deacetylatase, in murine of models of BCa (Kim et al., 2011). TCGA analysis using the

Pathway	Bioinformatic Database	Relevant Genes
Pathways with increased methylation in CA treated bladders		
Adherens junction - Mus musculus	KEGG	Actn1; Tcf7l2; 4930544G11Rik; Lef1; Ptprm; Fgfr1
Hippo signaling pathway - Mus musculus	KEGG	Bmp7; Axin2; Gdf6; Tcf7l2; Ppp2r2b; Nf2; Lef1; Tead4
Tight junction - Mus musculus	KEGG	Prkab1; Micall2; Jun; Prkaa1; Ppp2r2b; Magi1; Mpdz
Measles - Mus musculus (mouse)	KEGG	Irf7; Stat2; Jun; Stat3; Apaf1; Cd3g
Pathways with decreased methylation in CA treated bladders		
Ca2+ pathway	Reactome	Gnb5; Lef1; Camk2a; Tcf7l2
Triglyceride metabolism	Reactome	Agmo; Gpam; Lpin2
SLBP independent Processing of Histone Pre-mRNAs	Reactome	Snrpd3; Lsm11
SLBP Dependent Processing of Replication-Dependent Histone Pre-mRNAs	Reactome	Snrpd3; Lsm11
RUNX3 regulates CDKN1A transcription	Reactome	Runx3; Smad3
RUNX3 regulates WNT signaling	Reactome	Runx3; Tcf7l2

Table 2: Reduced representation bisulfite sequencing (RRBS) was used to evaluate methylation in 0.05% BBN treated C57Bl/6J animals exposed to CA (5ppm, 10ppm, 50ppm) or BBN alone for a 4 week period out of 16 weeks. Pathways that were differentially methylated in the same direction (hyper-/hypo-) in at least two of the CA treated animal sets are listed here.

UALCAN database confirms that *RUNX3* is hypermethylated in human BCa tumors, even as early as low grade disease. It is possible that the effect on metabolism observed by CA either A) directly reduced tumor formation and thus prevented *RUNX3* methylation by yet to be determined mechanisms or B) functions through *RUNX3* methylation in part if methylation of *RUNX3* is a major driver of tumor formation. Further study on interactions between *RUNX3* and bladder cancer are warranted. These data generally support the idea that *RUNX3* mutation is a critical early event in BCa tumorigenesis. The use of *RUNX3* methylation/expression as a marker of early tumor formation/early mutagenic change may be a valuable means for identifying tumor formation. Future plans include identification of methylated *RUNX3* DNA in plasma samples from human patients as a potential indicator of early disease formation.

We met the majority of the goals of the project, and will finish the project through a no-cost extension of the grant. The only outstanding goal is the comparison between human and murine data. Because of the surprising results in our mouse model, our human data will largely be a comparison to itself, but should still provide some highly novel and valuable results.

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

The grant has offered training for both our post-doctoral fellow Dr. Ganesh Rajendran and our Research Assistant Professor Dr. Ben Woolbright. Dr. Rajendran has had and will continue to have the opportunity to discuss sequencing methodology with our collaborators. As we continue to develop this aspect of the project, it is our anticipation he will continue to engage with them.

Dr. Woolbright was able to collaborate with chemists at the University of Kansas-Lawrence to both learn about and assist in developing an assay for detecting BBN metabolites. This collaboration allowed us to determine the mechanism associated with the observed protection and has been incredibly informative in this project. This finding and developed assay will be made widely available to the field through publication and should be highly beneficial to the field. This assay allows for detection of metabolism of BBN. It is likely that many results are erroneous based on effects on BBN metabolism and not the purported effect. Because of this, we believe that we will be able to provide this as a fee for service to the field for any knockout mouse models.

How were the results disseminated to communities of interest?

Abstracts have been presented at AACR[1] and we expect to submit another abstract for consideration this year. We are currently preparing the first publication on our animal data. We believe that this project should yield 2 publications, one on our murine data, and an additional publication on the human data. We will disseminate these through standard journals and are currently targeting upper level Toxicology journals such as Toxicological Sciences for these papers.

[1] Rajendran G, Abbott E, Thompson J, Patel S, Barchowsky A, Dennis K, Woolbright BL, Taylor JA III. Effect of Agent Orange and Agent Blue derived carcinogens on bladder cancer cell lines and a murine bladder cancer model. Cancer Res. 82_12. Supplement 224-4

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
These data should have an immediate impact on cancer research. Our finding that CA interferes with BBN metabolism is important. We were able to demonstrate a direct effect on the metabolism of the compound using HPLC. This direct effect should be applicable to many compounds and is a dramatically underexplored and potentially critical aspect of the BBN model that was previously unknown. While our data are not the only data on this aspect, we believe ours is the first demonstration of a clear and defined effect based on co-treatment between BBN and another compound that does not fully coincide with the treatment time frames.

We believe the most impactful research is still yet to come as we are still waiting on some samples for our project. The human data will be compared to our murine data and to other databases to establish a signature for exposure and its effects if possible.

What was the impact on other disciplines?
The field of Toxicology is likely to be impacted by these results, particularly with regards to arsenic exposure. Our new data suggests that cacodylic acid likely interferes with BBN by blocking its metabolism. Toxicokinetics and xenobiotic metabolism are critical aspects of Toxicology, and thus these data are likely to be highly impactful to other scientists studying organic arsenic species

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
These data may impact regulatory practice as they suggest that TCDD/CA might not be directly carcinogenic to the bladder. We want to emphasize that we do not necessarily believe this is the case, and these experiments represent only initial laboratory study and should not be broadly used to alter regulatory measures by themselves. Further and broader investigation is needed.

5. CHANGES/PROBLEMS:
Changes in approach and reasons for change
No significant changes occurred; We do anticipate the necessity to explore our alternate plans sequencing data in human patients. We have made additional potential alternative routes (whole exome sequencing, etc..). Depending on the quality of the material we get. This was an expected potential issue and we are prepared to deal with this through exploration of alternate outcomes.

Actual or anticipated problems or delays and actions or plans to resolve them
We have experienced delay in acquisition of tissue from the JPC. This is likely related to labor intensive issues with sample identification and processing. We have been in constant communication with our colleagues and

have worked on facilitating the material transfer as feasible from the JPC. They understand the timeliness of the project and have been working on their end to meet study goals.

Changes that had a significant impact on expenditures
Because we did not find that our cell lines underwent malignant transformation in critical cancer outcomes that would specifically impact xenograft experiments we did pursue use of this model. The reduction in costs associated with these experiments will offset by the anticipated increase in our costs for sequencing human tissue. This is due to the need for alternate sequencing strategies which are more costly than planned RNA sequencing. The overall change should be largely neutral and thus we believe we will complete all goals of the project still.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No significant changes present.

Significant changes in use or care of vertebrate animals

We added additional experiments that were approved by both our institution and ACURO.

Significant changes in use of biohazards and/or select agents

None.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations
Rajendran G, Abbott E, Thompson J, Patel S, Barchowsky A, Dennis K, Woolbright BL, Taylor JA III. Effect of Agent Orange and Agent Blue derived carcinogens on bladder cancer cell lines and a murine bladder cancer model. Cancer Res. 82_12. Supplement 224-4

Journal publications.
We are anticipating two manuscripts targeting Toxicology journals within the next year. Preparation for the first manuscript is underway

Books or other non-periodical, one-time publications.

NA

Other publications, conference papers and presentations.

AACR 2021: Effect of Agent Orange and Agent Blue derived carcinogens on bladder cancer cell lines and a murine bladder cancer model. Ganeshkumar Rajendran, Erika Abbott, Jeffrey Thompson, Shachi Patel, Aaron Barchowsky, Katie Dennis, Benjamin L. Woolbright, and John A Taylor III

- **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

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:
Example:

Name: *John Taylor*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): 0000-0002-1780-3788
Nearest person month worked: *2*

Contribution to Project: *Dr. Taylor is the PI and is involved with all steps including data analysis, animal handling and more.*
Funding Support: *DoD*

Name: *Jeff Thompson*
Project Role: *Co-I*
Researcher Identifier (e.g. ORCID ID): 0000-0002-0876-2582
Nearest person month worked: *1*

Contribution to Project: *Dr. Thompson has focused on bioinformatic analysis of RNA-seq data.*
Funding Support: *DoD*

Name: *Ben Woolbright*
Project Role: *Co-I*
Researcher Identifier (e.g. ORCID ID): 0000-0003-3219-958X
Nearest person month worked: *2*

Contribution to Project: *Dr. Woolbright has helped setup experiments, assisted in writing reports and data analysis and monitored animal protocols and animal euthanasia.*
Funding Support: *DoD*

Name: *Katie Dennis*
Project Role: *Co-I*
Researcher Identifier (e.g. ORCID ID): 0000-0003-0994-8933
Nearest person month worked: *1*

Contribution to Project: *Dr. Dennis has served as our Board-Certified Pathologist and has staged all animals..*
Funding Support: *DoD*

Name: *Ganesh Rajendran*
Project Role: *Post-Doc*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*

Contribution to Project: *Dr. Rajendran has performed all cell culture, assisted in data analysis, and helped with animal culture.*
Funding Support: *DoD*

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?

- *Other.*

Name: *Michael Hagemean*
Project Role: *Collaborator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*

Contribution to Project: *Dr. Hageman's core facility served as a fee for service based center to generate and assess levels of BBN metabolites for this study.*
Funding Support: *Fees were supported by DoD grant through reductions in costs in other places*

Name: *Aaron Barchowsky*
Project Role: *Co-I*
Researcher Identifier (e.g. ORCID ID): 0000-0003-1268-8159
Nearest person month worked: *1*

Contribution to Project: *Dr. Barchoswky has assisted in analysis of data.*
Funding Support: *DoD*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
NA

9. APPENDICES:

NA