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PRINCIPAL INVESTIGATOR: John A. Taylor, III, MD, MS

CONTRACTING ORGANIZATION: Kansas, University of, Medical Center Research Institute, Inc.
Kansas City, KS 66160

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John A Taylor, III, MD, MS Benjamin L Woolbright, PhD

E-Mail:jtaylor27@kumc.edu

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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.

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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. (**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. **(not completed – samples acquired but tissue deemed to be insufficient for this practice).**

Milestone 1**:** Define sequencing results from both data sets (**Complete.**)

Milestone 2**:** Compare sequencing analysis between human patients and human cell lines garnered in SA1 - (**Completed**).

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. (**Complete***)*

What was accomplished under these goals?

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.

The overall goal of this project was to assess potential changes in cancer associated signaling using transcriptomic and epigenomic signaling. We hypothesized that we would be able to define differences in samples from Veterans with active service in Vietnam and hypothetical exposure to Agent Orange as compared to samples from those that presumably would not have been exposed to Agent Orange.

Samples were acquired from The Joint Pathology Center in association with our collaborators Dr. Michael Lewin-Smith and Dr. Isabel Sesterhenn. All tissue was provided in a de-identified format. Taylor lab staff was blinded to the status of each tissue/patient status until after analyses were finished.

Tissue was macro-dissected to exclude areas of obvious non-tumor and then RNA was isolated from these tissues using the Qiagen RNEasy FFPE kit. DNA was isolated using the Qiagen DNA FFPE Advanced kit. While we were able to acquire multiple pieces of tissue, the overall amount of tumor present in these slides limited the amount of material present for analysis. Because our sequencing collaborators indicated that the amount of DNA required to perform the epigenomic analysis was likely going to be more than we could realistically generate from our samples without fully expending the available tissue, we instead focused on RNA based assays. The use of formalin fixed paraffin embedded tissue for RNA sequencing has been demonstrated to be successful in many circumstances. Unfortunately, many of the tissues present in this study were cut from relatively older samples which likely further contributed to RNA degradation. Ultimately, it was decided that the RNA available from these tissues would provide useable sequencing data but was likely suboptimal for this purpose. Because of this, we instead turned to the Nanostring microarray technology, which is considerably more tolerant of smaller pieces of RNA and lower overall quality.

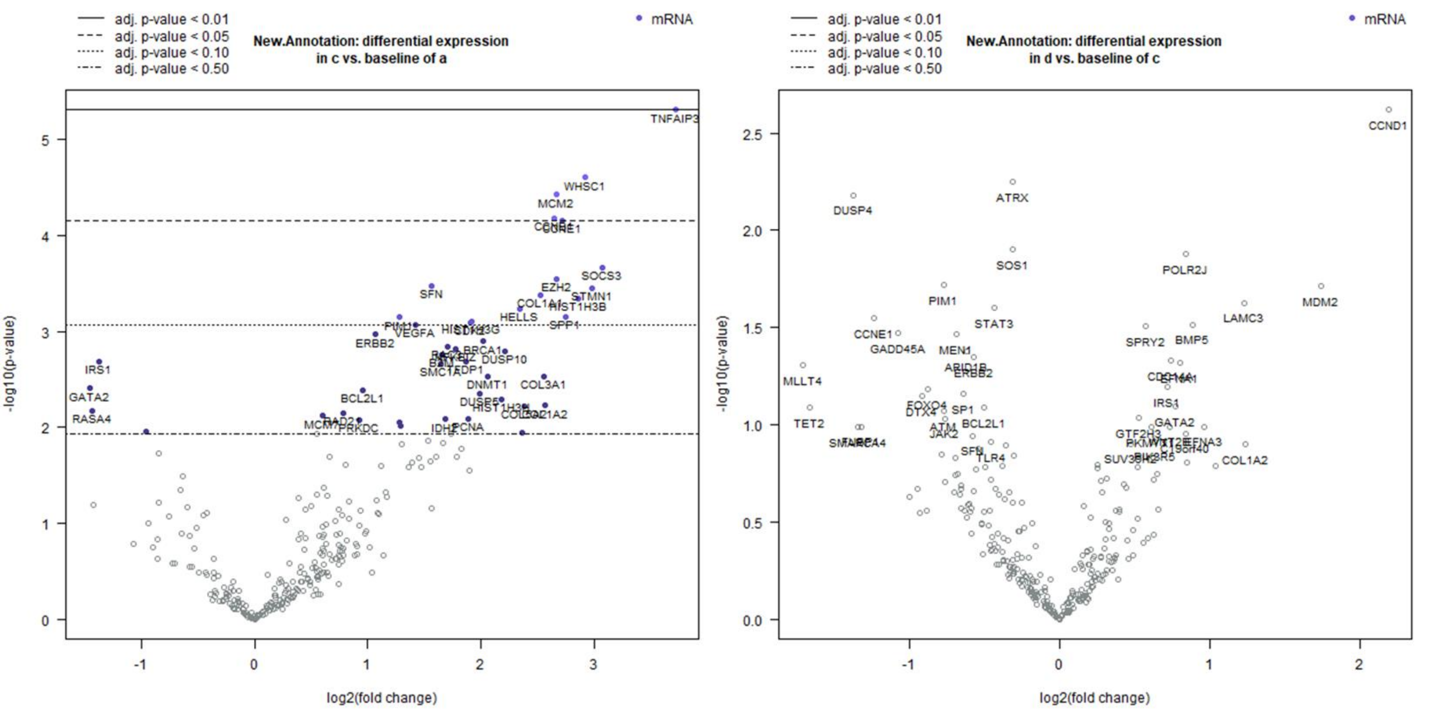

Figure 1: Differential gene expression in BCa tumor. Volcano plots of differentially expressed genes between high grade tumors from patients with Vietnam service (group c) versus low grade tumors with Vietnam service (group a) or high grade tumors without Vietnam service (group d).

This panel provides expression of hundreds of genes which have previously been related to cancer progression and development, and thus offers a holistic view of underlying gene changes.

We used the Nanostring nCounter Pancancer Pathways panel to evaluate differences between four groups of patients that had either: **A)** low grade tumors and Vietnam service (n=10), **B)** Low grade tumors without Vietnam Service (n=10), **C)** high grade tumors and Vietnam service (n=10), or **D)** high grade tumors without Vietnam service (n=16). We found substantial changes in raw p values between low and high grade tumors indiscriminate of whether Vietnam service had occurred (**Figure 1, Figure 2**). Adjusted p values indicated a much smaller number of genetic differences, although high and low grade tumors had clear difference in multiple genes such as *WHSC1*, *COL1A1*, *SOCS3*. Notably many of these genes were also statistically significantly upregulated in high grade tumors versus low grade tumors in the TCGA database (www.xenabrowser.com used to view data). We observed relatively minimal differences between patients with Vietnam service and patients without Vietnam service for both low grade and high grade tumors. While some genes were differentially expressed using raw p values, minimal numbers of genes were differentially expressed after adjusting p values for multiple comparisons.

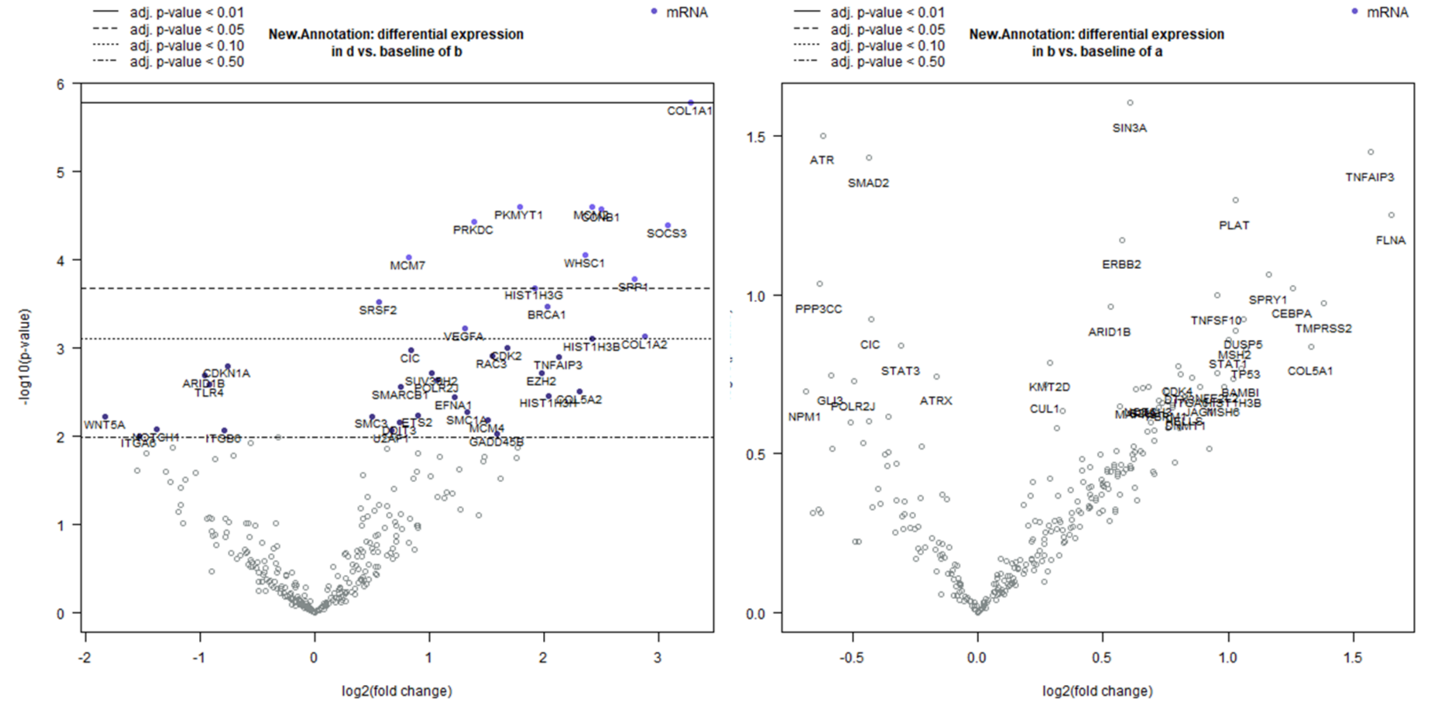

Figure 2: Differential gene expression in BCa tumor. Volcano plots of differentially expressed genes between high grade tumors from patients without Vietnam service (group d) versus low grade tumors without Vietnam service (group b) or low grade tumors with Vietnam service (group a)

Based on these results, we are largely confident in the underlying samples. Many of the genes measured had relatively low expression using the Nanostring system at the amount of RNA that we were able to use initially. We believe that if we concentrate our current RNA using traditional methods and repeat these assays, we might have a more robust set of genes to work from as many more genes with potential differential expression might be observed. Moreover, we can work with statistical collaborators to begin identifying potential gene sets that might differentiate between Vietnam service and no service. This might yield novel pathways to begin understanding if there are differences we haven't yet been able to quantify. We will pursue in new applications and through both startup and philanthropic funding. We can further expand our number of patients through our collaboration with the Joint Pathology Institute. While we initially were hoping for 20 patients per group, we ended up with less than this. Additional patients may help better define results in future studies.

Finally, we have a better understanding of the amount of tissue required for the individual assays proposed versus the quantity of nucleic acids that can reliably be obtained from older, formalin fixed paraffin embedded tissue. This will guide future studies in this area, and allow for better estimation of necessary materials.

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

The overall goal of this research is to understand TCDD/cacodylic acid induced changes on bladder cancer cells. We used both murine models and established cell lines to evaluate how TCDD/CA affect bladder cancer.

Major Task 1: Determine the impact of acute and long term low doses of TCDD and/or cacodylic acid on benign urothelial cell lines. We have fully completed our analysis of both cell lines treated with either TCDD or CA for extended periods. These cells were treated bi-weekly with the indicated concentration of TCDD or CA for the indicated period. Our primary means for assessing changes in proliferation is through use of a hexosaminidase assay. For the hexosaminidase assay, cells are plated in a 96 well plate and we use a simple colorimetric assay to assess the activity of hexosaminidase as a surrogate for the number of cells on the plate. We assess migration

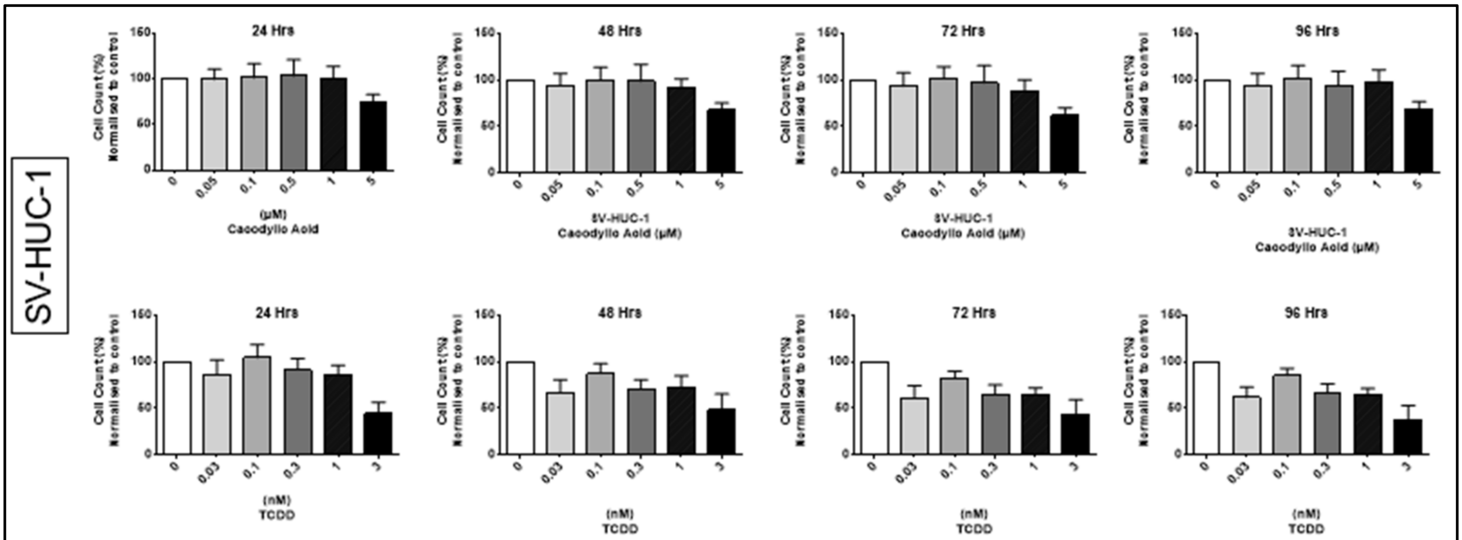

Figure 3: Cell proliferation in SV-HUC-1 cells exposed to CA or TCDD: SV-HUC-1 Cells were grown at specified concentrations of Cacodylic acid (µM) or 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (nM) for 3 months and proliferation were assayed at different time points (24, 48 , 72 and 96 Hrs) after plating using a hexosaminidase assay,

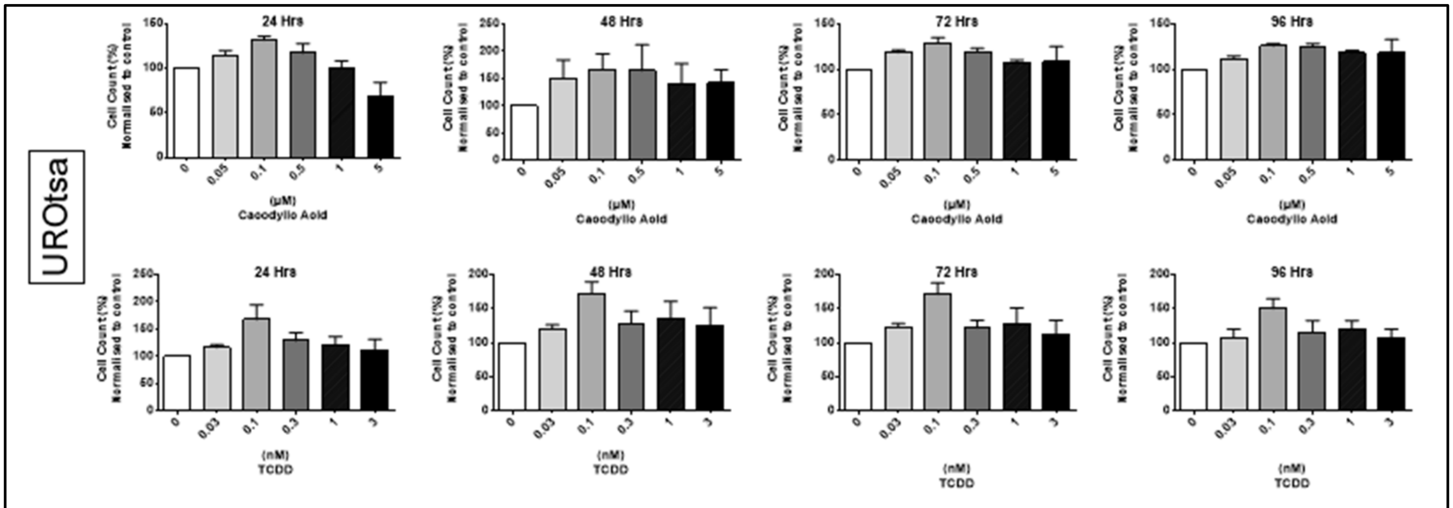

Figure 4: Cell proliferation in UROtsa cells exposed to CA or TCDD: UROtsa Cells were grown at specified concentrations of Cacodylic acid (µM) or 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (nM) for 3 months and proliferation were assayed at different time points (24, 48 , 72 and 96 Hrs) after plating using a hexosaminidase assay,

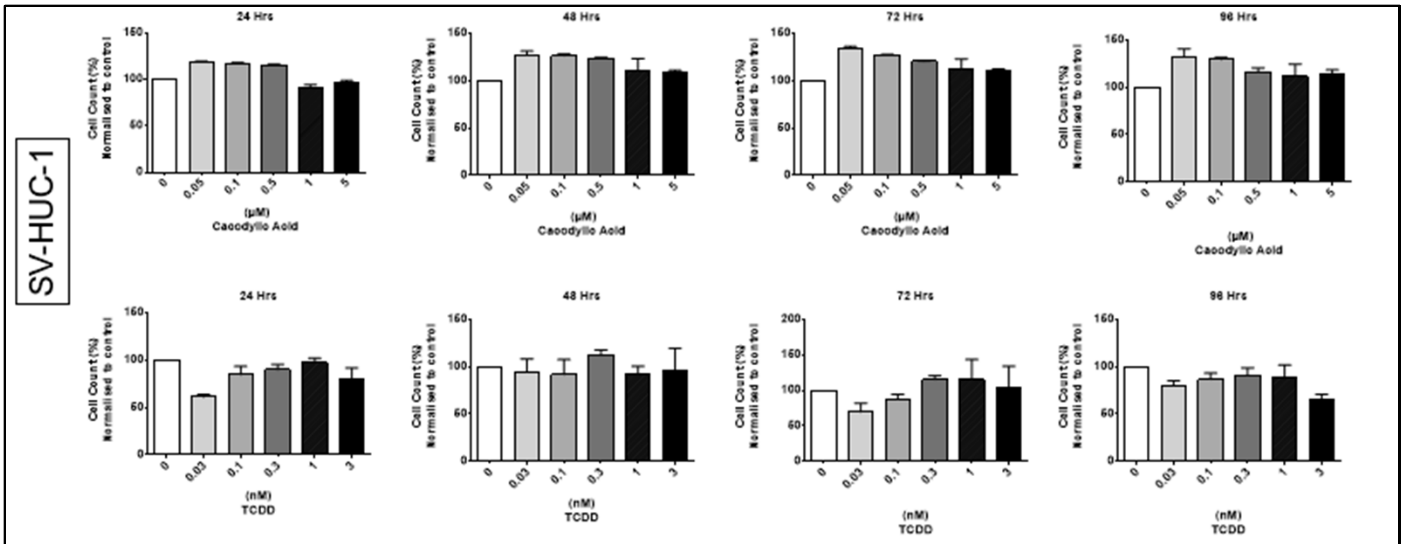

Figure 5: Cell proliferation in SV-HUC-1 cells exposed to CA or TCDD: SV-HUC-1 Cells were grown at specified concentrations of Cacodylic acid (µM) or 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (nM) for 6 months and proliferation were assayed at different time points (24, 48 , 72 and 96 Hrs) after plating using a hexosaminidase assay,

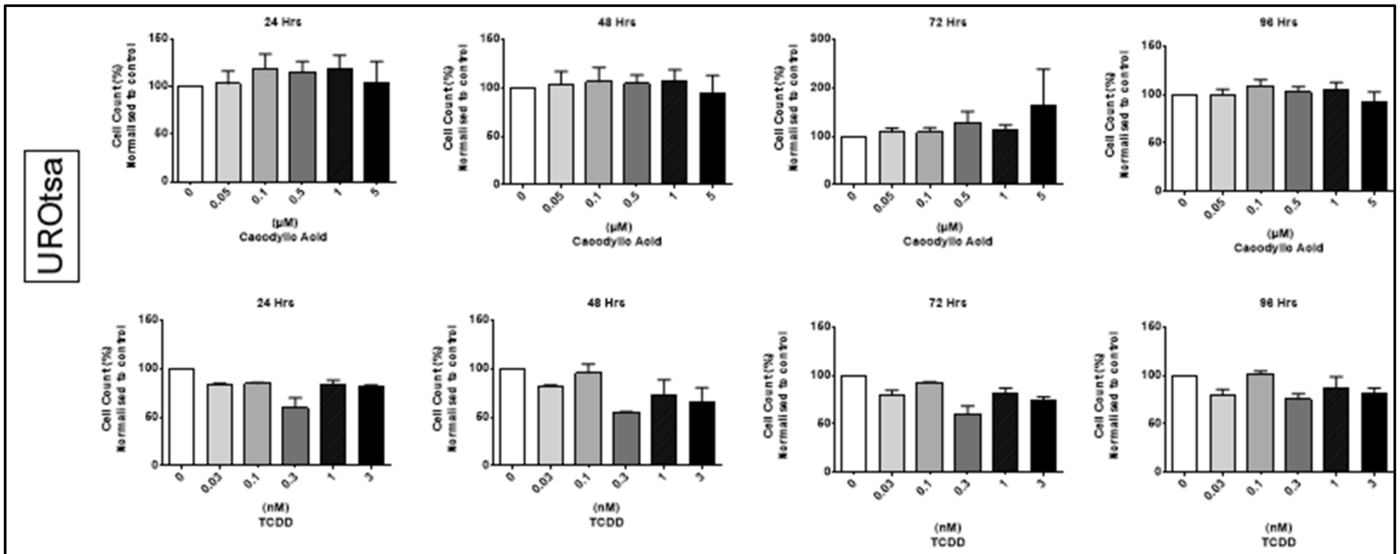

Figure 6: Cell proliferation in UROtsa cells exposed to CA or TCDD: UROtsa Cells were grown at specified concentrations of Cacodylic acid (µM) or 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (nM) for 6 months and proliferation were assayed at different time points (24, 48 , 72 and 96 Hrs) after plating using a hexosaminidase assay,

using a standard scratch assay wherein a scratch is made across a line of confluent cells and we quantify how fast the scratch fills over time using microscopy. We did not observe changes in proliferative potential at either three (**Figure 3/4**) or six (**Figure 5/6**) months. Even out to 12 months we did not observe a difference in proliferation in these cell lines (**Figure 7**), nor did we detect any major differences in cellular migration after three months (**Figure 8/9**). The lack of dose response limited our interpretation of establishment of an overall phenotypic change.

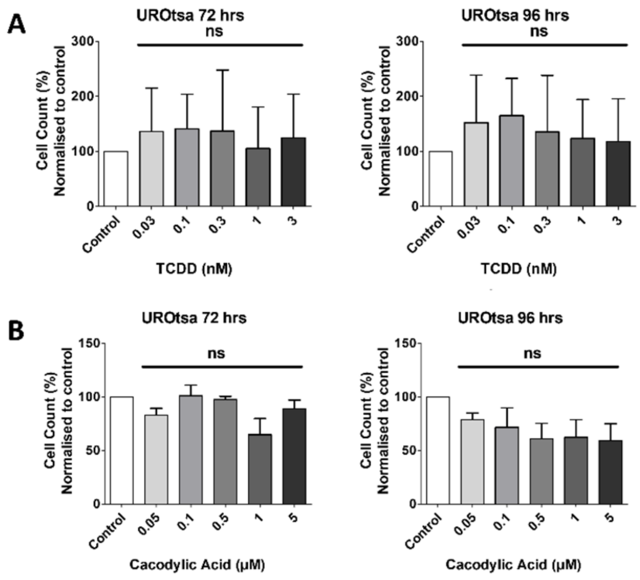

Figure 7: UROtsa cells treated with DMSO control or TCDD (A) (0.03 to 3nM) or cells treated with control media or media containing CA (0.05 to 5μM) (B) bi-weekly for one year were assessed for proliferative ability. Data are from n=3 separate experiments.

Moreover, we consistently observed that TCDD occasionally exerts some toxicity against the cells that resulted in us removing TCDD as a stimulus for brief periods. There are a number of possible reasons for this, including the fact that TCDD is highly lipophilic, and thus may either lose some solubility and/or directly induce toxicity as our higher doses have occasionally all but halted cellular proliferation. Because of this, it was necessary to remove the TCDD and allow the cells to regrow and then add the TCDD back to continue these experiments. This had led to periods where the cells were *not* exposed to TCDD for up to a week; however, continued exposure would have resulted in total cell death in all likelihood. Since this would have ended the experiment, we did not view this as feasible and instead chose to briefly remove the stimulus and continue to try and transform cells.

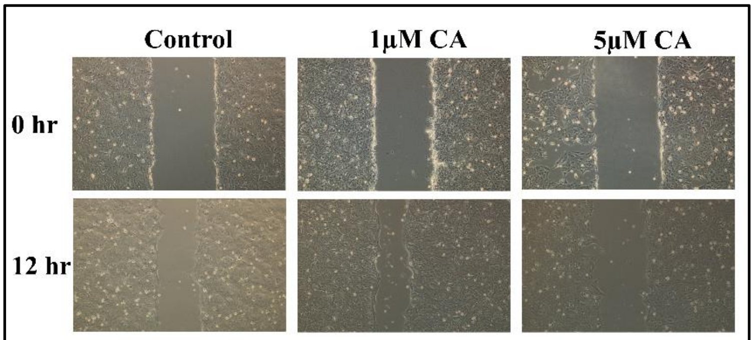

Figure 8: Cell migration in SV-HUC-1 cells exposed to CA : SV-HUC-1 Cells were grown at specified concentrations of cacodylic acid (μM) for 3 months and migration was assayed using a scratch assay.

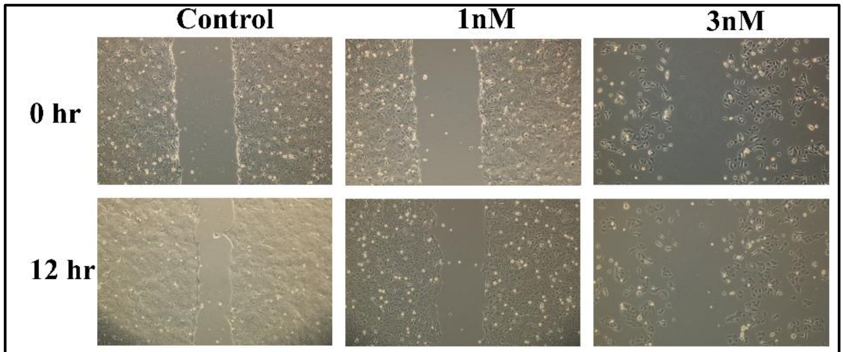

Figure 9: Cell migration in SV-HUC-1 cells exposed to TCDD: SV-HUC-1 Cells were grown at specified concentrations of TCDD (nM) for 3 months and migration was assayed using a scratch assay.

The benign cell lines used in our study did not undergo obvious malignant transformation in the presence of either TCDD or CA. Cellular proliferation and migration 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 10**). 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 (Ishida, 2010; PMID 19755661; however, these cells are already transformed cancer cells whereas we had hypothesized TCDD would transform benign cells.

To further confirm our findings, 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. 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 at large. 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 the human data in the coming months. We might be able to correlate changes in gene expression. The general lack of major changes partially supports the idea that these cells were minimally transformed. Currently, we do not feel the phenotype is sufficient to produce any sort of demonstrable and ultimately biologically meaningful change. Importantly, we have frozen aliqouts of cells from dozens of time points and if necessary, can re-evaluate these in a separate context or continue experiments using altered protocols.

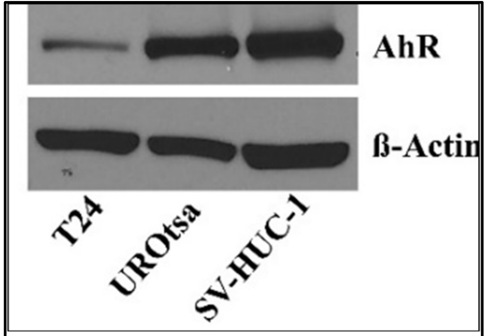

Figure 10: Total cell extracts cell lines were analyzed by western blotting with antibodies against AhR (Cell Signaling 83200) or ß-actin (4970).

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.

Overall, the results obtained indicated cells were minimally transformed in the presence of long term CA or TCDD. Interestingly, TCDD actively began killing cells at higher concentrations which might be worth investigating as a mechanism. While TCDD itself has no potential as a therapeutic, the use of a selective AhR agonist might be both useful and possible for augmenting bladder cancer if TCDD's toxic actions work through AhR.

Major Task 2: Assess tumor development and global transcriptomic and epigenetic changes in a murine model of carcinogen induced BCa exposed to arsenic. We have completed all currently proposed animal experiments. Much of the data has been analyzed. We have surprisingly found the opposite of our initially proposed hypothesis, but we believe we may have some understanding of why this has occurred.

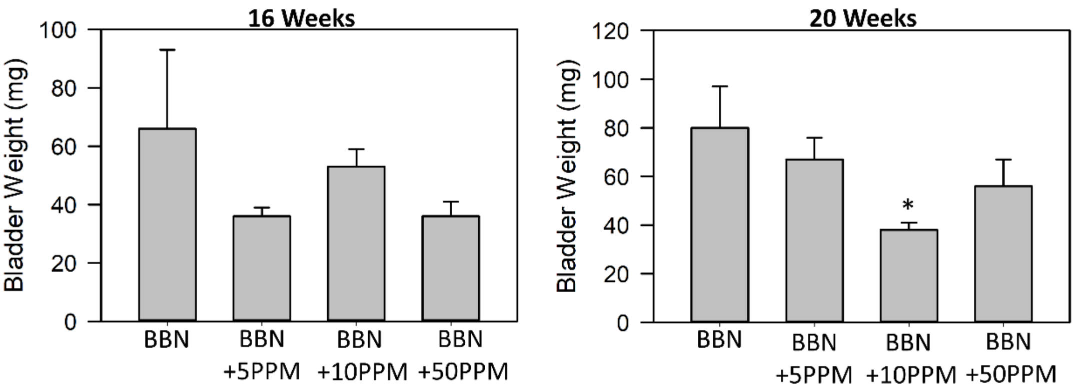

Figure 11: Tumor sizes in mice treated with BBN or BBN + cacodylic acid (CA). Bladder weights were assessed at euthanasia. * $p<0.05$ versus BBN alone. Note – one mouse in the 16w BBN group was dramatically larger than the rest of the group (bladder weight 426mg) which mediated the majority of the effect.

We initially treated animals with BBN, 0.05% in drinking water for 4 weeks followed by the addition of 5, 10 or 50 PPM cacodylic acid in drinking water for 4 weeks per the statement of work. At this point we ceased CA, and then allowed the experiment to finish.

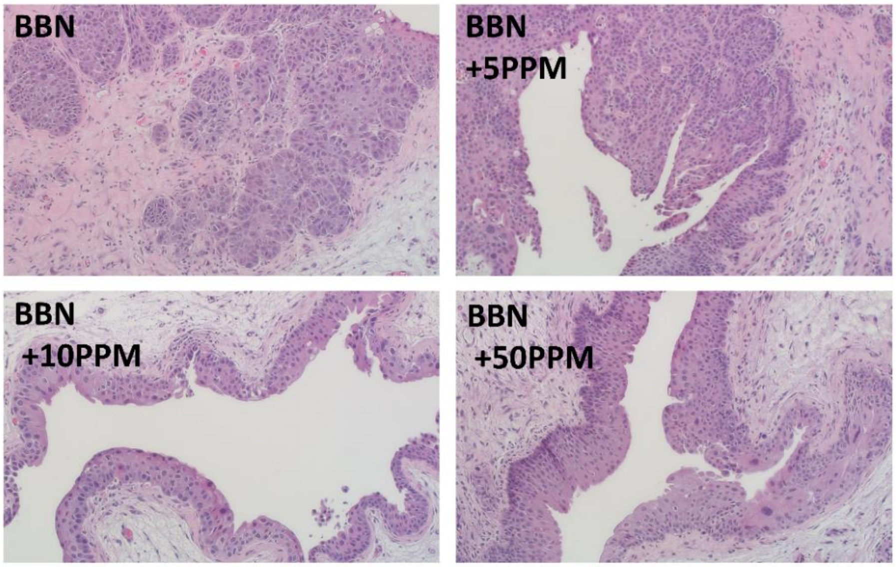

Figure 12: BBN induced carcinogenesis in the bladder. Representative H&E stains from animals treated with the indicated compounds.

Our hypothesis was that CA would enhance tumor formation rates. Surprisingly we found that the opposite occurred (**Figure 11, Figure 12**). At 16 weeks, there was no difference in tumor size or stage. In contrast, at 20 weeks we found that the 10 PPM group had significantly *smaller* tumors than the BBN alone group. The 50PPM group trended strongly in the same direction, but the presence of a single mouse with a large tumor precluded a statistically significant decrease. Tumor stage was assessed by a Board Certified Pathologist. ANOVA analysis using the Kruskal-Wallis test indicated there was a significant difference across groups, but post-hoc analysis failed to identify any specific difference when p values were adjusted for multiple comparison. These data indicate there was a statistically significant trend towards a generalized reduction in tumor stage with increasing CA values. While these data were opposite to our hypothesis, they align well with data from other labs indicating that arsenic, as a nuclear factor erythroid 2-related factor 2 (Nrf2) activator, can potentially have anti-tumorigenic effects on chemical carcinogen models. Given that the large body of evidence suggesting bladder cancer has high mutational rates and is linked to exposure to environmental factors, this might be highly relevant and suggest a context dependent role for arsenic in the promotion of bladder cancer.

We next assessed transcriptomic changes using RNA-sequencing of our 20-week sacrifice group (**Figure 11/Figure 13**). We omitted the 16-week groups as we did not observe changes in either bladder weight or tumor stage. Because of this, the likelihood that we would observe major and significant changes in RNA expression is minimal. Due to the high experiment cost, it does not make fiscal sense to continue with experiments that are unlikely to produce meaningful results.

Foremost, differential gene expression analysis found a number of different genes that were substantially upregulated in our BBN group that were not present in our BBN plus arsenic group. As we have hypothesized arsenic might function as a epigenetic regulator, it is possible that it is regulating genes that actually promote tumor formation as well. Pathways associated with tumor formation that were downregulated in our BBN + 10PPM CA group or BBN + 50PPM CA group include a number of genes associated with RNA processing as well as genes associated with keratinization, VEGF signaling, mitosis and more. We are now particularly interested in a couple of different genes we believe may be involved including serine protease inhibitor kazal-type-1 (*SPINK*) genes associated with a broad number of tumors. Use of Ingenuity Pathway Analysis (**Figure 13**) indicated a number of pathways were significantly different, but these changes were relatively modest in context. Because none of the data acquired from RNA-sequencing yielded obvious and targetable pathways that were common between our 10ppm and 50ppm groups, we simultaneously began investigating other possible mechanisms through other assays.

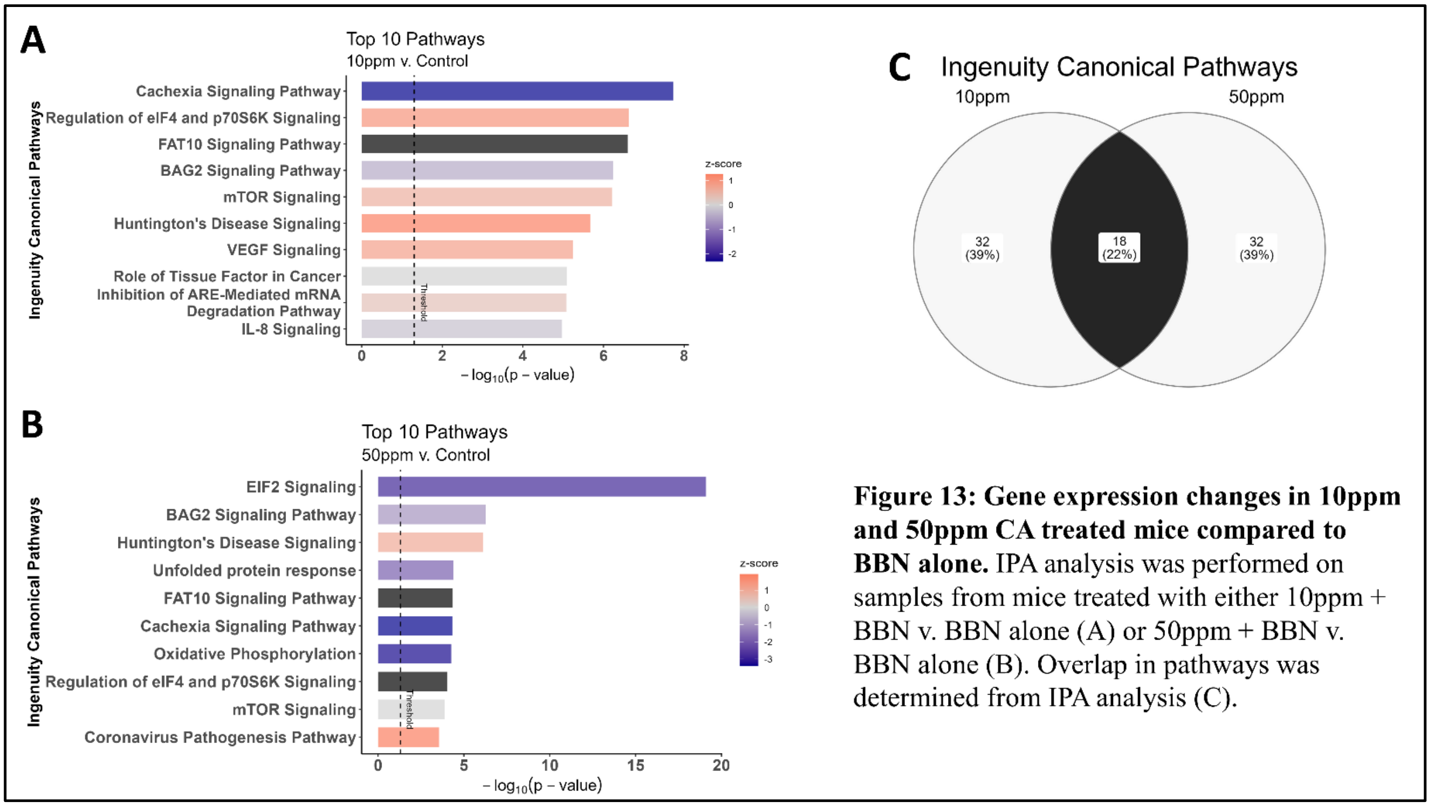

Figure 13: Gene expression changes in 10ppm and 50ppm CA treated mice compared to BBN alone. IPA analysis was performed on samples from mice treated with either 10ppm + BBN v. BBN alone (A) or 50ppm + BBN v. BBN alone (B). Overlap in pathways was determined from IPA analysis (C).

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 14**). 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

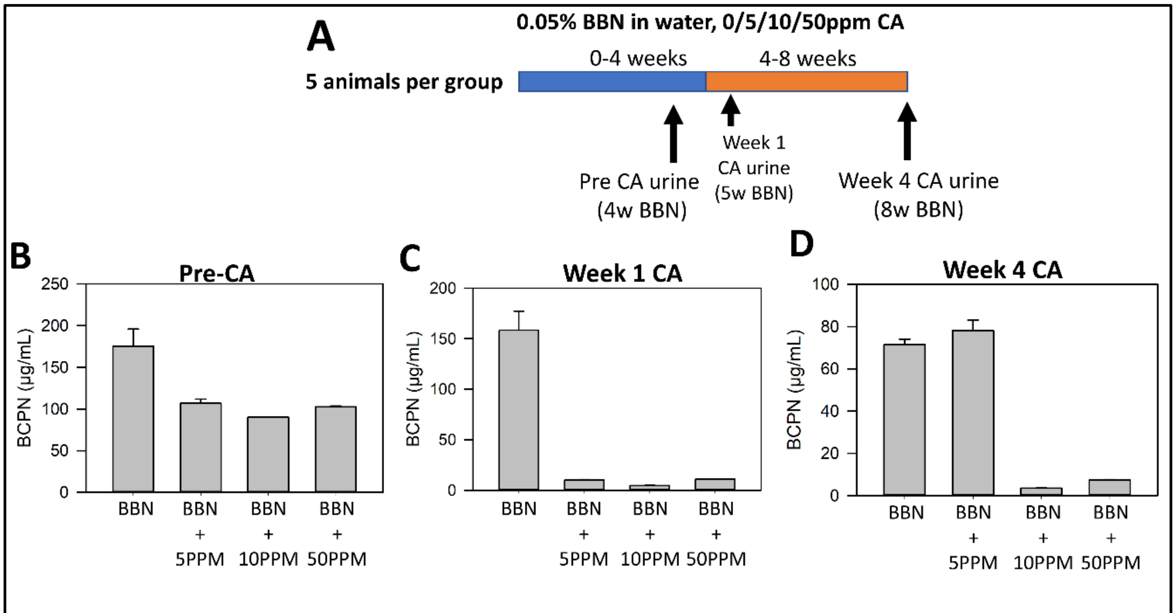

Figure 14: BCPN levels in mice given BBN or BCPN was measured via HPLC-MS. Dosing schematic in (A). BCPN levels prior to (B), 1 w after (C), or 4 w after (D) CA administration during BBN administration in urine of mice.

observed 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. 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 of BBN to its active compound. Importantly, the role of BBN metabolism in the model remains highly understudied. It is entirely possible that many of the effects reported thus far are actually a result of changes in metabolism of BBN. Our future plans include the use of this assay to better understand known differences in mouse models of BBN, which would be highly beneficial to the field.

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 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 in association with our statistical collaborators and this unique change was still present. We are still investigating this phenomenon. 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 models of BCa (Kim et al., 2011). TCGA analysis using the UALCAN database confirms that *RUNX3* is hypermethylated in human BCa tumors, even in 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* methylation 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

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.

methylated *RUNX3* DNA in plasma samples from human patients as a potential indicator of early disease formation.

In conclusion, the majority of the goals of the project were met. Our novel and valuable findings regarding changes in BCPN metabolism ultimately correlating with changes in tumor formation are potentially extremely valuable to the field. Moreover, these findings can be expanded on to better understand changes in multiple other scenarios. While differences in our primary hypothesis were not as robust as anticipated, we will continue to evaluate our human data, potentially repeating these experiments at higher RNA concentrations that might expand upon our results. The primary remaining goal is a comparison between human and murine data once we have fully evaluated our human samples. We anticipate future studies will address this.

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

The grant has offered training for both our prior post-doctoral fellow Dr. Ganesh Rajendran and our Research Assistant Professor Dr. Ben Woolbright. Dr. Rajendran had the opportunity to discuss sequencing methodology with our collaborators which offered a new breadth of information for him to understand.

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. 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. Dr. Woolbright has since moved on to an independent position as an Assistant Professor of Cancer Biology where he will continue to use this methodology to understand mechanisms of bladder cancer.

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, which we anticipate we will send to a cancer related journal within six months. 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.

[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?
N/A

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. The lack of focus on BBN metabolism has been an issue in the field in our opinion, as it may explain some phenomena that

are currently attributed to other factors. Our goal is to push the field to incorporate understanding BBN metabolism into the basic battery of tests that commonly accompany the model.

We believe the most impactful research is still yet to come. Further statistical analysis of our human data we hope will generate a clearer understanding of which genes are different. Moreover, we hope that we can develop a panel of genes that are mostly robustly effected in our Vietnam Service patient population, which we can follow up on in future studies.

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 as they may explain prior findings which yielded unanticipated answers.

What was the impact on technology transfer?
Nothing to Report. While we have established the BCPN assay in our lab, this was based on prior knowledge.

What was the impact on society beyond science and technology?
These studies may be useful for ultimately assessing risk of Vietnam Era service. Work in this area will require further investigation using additional samples and a broader investigation.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
While we originally planned to use RNA sequencing, we ultimately decided this would not produce high quality data. This was done alongside industry collaborators with expertise in sequencing. Moreover, because of the lack of DNA acquirable from the tissue specimens, we were unable to complete the epigenetic analysis. While this is unfortunate, per our original plan, we instead used a microarray to make assessments of genetic changes in these tissue. Using Nanostring technology which works far better with lower RNA inputs and lower RNA quality, we were still able to make assessments of the tissue provided. This tissue demonstrated some similarities between TCGA and our own findings, which gave some confidence that the tissue was useable.

Actual or anticipated problems or delays and actions or plans to resolve them
We experienced delay in acquisition of tissue from the JPC. This was due to the labor intensive issues with sample identification and processing. We remained in communication with our colleagues and have facilitated the material transfer from the JPC. This was eventually resolved and samples were used during the NCE. We appreciate the diligence of our collaborators.

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 was largely neutral and thus we completed the bulk of the goals of the project still.

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

No significant changes present.

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 during the second year of the application.

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?

NIH/NCI R21 CA277516-01 2/1/23-1/31/25
(PI: **Taylor**/Welch)
Regulatory Role of Mitochondrial DNA in Bladder Cancer Progression

BCAN Innovation Award 8/1/23 – 7/31/25
(PI: **Taylor**)
Exploring therapeutic vulnerability in a Downs Syndrome model for bladder cancer drug development.

NIH/NCI K22 7/1/2023 – 6/30/28
(PI: **Woolbright**)
Role of Gut-Immune Interactions in Aging-Associated Bladder Cancer

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