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14. ABSTRACT
Bladder cancer (BC) is a common and deadly disease, and despite recent treatment advances, metastatic BC (mBC) remains incurable. Mutations in genes that encode epigenetic/chromatin modifier proteins are common in mBC, with >90% of tumors harboring at least one inactivating mutation. The epigenetic reader protein MPP8 recognizes the histone-3-lysine-9-trimethyl (H3K9me3) region of target gene promoters, and recruits transcription factors associated with cell proliferation and metastasis. UNC7713 was developed as a covalent antagonist to disrupt MPP8 binding to H3K9me3. Here, we explored whether UNC7713 potently inhibits cell proliferation, migration, and viability in preclinical models of BC. During this reporting period, we demonstrated knockdown of MPP8 using siRNA (siMPP8) significantly limits cell proliferation, migration/invasion and viability in 5637, TCCSUP and UM-UC-3 bladder cancer cell lines. In addition, we were not able to show that siMPP8 impacts expression of SIRT1 and Zeb1, but we did demonstrate that MPP8 co-immunoprecipitated with TASOR, Zeb1, Zeb2, Snail, and Slug. This suggests interactions between MPP8 and these transcription factors can lead to repression of tumor suppressor genes. While we continue to struggle to link MPP8 knockdown or chemical antagonism with EMT, we did demonstrate that siMPP8 activates *IL6* and *IFNB1*. Last, during this period we were able to confirm data from the previous reporting period and showed that UNC7713 has submicromolar potency to inhibit proliferation, migration and viability in TCCSUP and UM-UC-3 cells. .

15. SUBJECT TERMS
Bladder cancer, covalent antagonist, drug development, epigenetics, MPP8

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1. INTRODUCTION:

Urothelial bladder cancer is a common and deadly disease. In bladder cancer, lineage plasticity that leads to epithelial to mesenchymal transition (EMT) has been associated with quicker disease progression, with treatment resistance, and worse survival for metastatic bladder cancer patients. An altered chromatin landscape is common in advanced bladder cancer, and epigenomic reprogramming has been associated with lineage plasticity and EMT. M-phase phosphoprotein 8 (MPP8) is a protein that recognizes the histone 3 lysine 9 trimethyl (H3K9me3) post-translational modification, and has been shown to repress tumor suppressor genes (**Figure 1**). It has also been shown to play a key role in the silencing of E-cadherin, a central modulator of EMT and metastatic spread. Utilizing structure-based design, we have discovered a lead MPP8 antagonist (UNC7713), which potently blocks H3K9me3 recognition by the MPP8 chromodomain. UNC7713 achieves its potency by using selective covalently labeling a cysteine in proximity to the H3K9me3 binding site. Therefore, the overarching goal of this proposal is to better understand the role of MPP8 in lineage plasticity and EMT in bladder cancer, discover a potent first-in-class antagonist of MPP8, and evaluate MPP8 chemical antagonism as a therapeutic strategy for EMT prevention or reversal in preclinical models of advanced and metastatic bladder cancer.

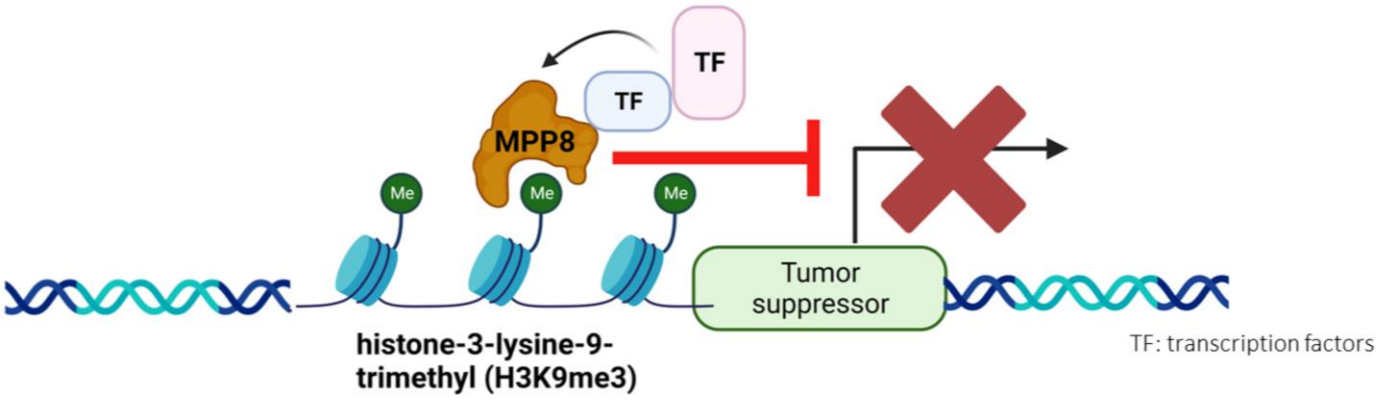

Figure 1. Mechanism of MPP8 antagonism in cancer.

2. KEYWORDS:

1. Bladder Cancer
2. Covalent antagonist
3. Drug development
4. Epigenetics
5. Epithelial to mesenchymal transition
6. Metastasis
7. Methyl-lysine reader
8. M-Phase Phosphoprotein 8 (MPP8)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Significant progress was made in Year 2 on two of the three Aims and the majority of major goals**.** Much of the focus of the work this year involved establishing MPP8 as an epigenetic regulator in bladder cancer (Aim 1), and further evaluation of UNC7713 (first-in-class MPP8 covalent antagonist; Aim 3). Efforts were made to move forward clinical pharmacology experiments; however, there were safety concerns with UNC7713 and second-generation antagonists, which halted pharmacokinetics (PK) – see section 5.

A complete synopsis of the major accomplishments in Year 2 are summarized in the table and narrative below.

Aim 1. Validate MPP8 as an epigenomic regulator of lineage plasticity and EMT in bladder cancer.

Major Task	Milestone	Proposed Timeline	% Completed	Notes
Generate an inducible MPP8 knockdown model.	Confirm that we have generated an IPTG-inducible shRNA knockdown model, and that MPP8 knockdown has effects on gene and protein expression of known EMT markers and known EMT inducers.	Months 1-15	90%	Recently completed. We now have shRNA and ITPG-inducible shRNA systems for MPP8 knockdown in 5637, TCCSUP and UM-UC-3 bladder cancer cells. This year we also have created ITPG-inducible and non-inducible versions in the same cell lines with an mCherry reporter included. These will serve as model systems for Aim 1 experiments, but also as controls for Aim 3.
Evaluate the effects of MPP8 knockdown on interactions with known inducers of lineage plasticity and EMT.	Confirm increased H4K16ac (but not H3K9me3) and decreased SIRT1 and ZEB1 binding at the *CDH1* promoter. Confirm disrupted SIRT1 and ZEB1, and DNMT3a and Snail interactions.	Months 1-18	65%	We have not completed the H4K16ac and H3K9me2 and me3 work, but those will happen in the coming weeks. We will also be evaluating the effects of MPP8 knockdown on deacetylation of H3K9. Recently, we completed co-IP work showing the relationships between MPP8 and the following: TASOR, Zeb 1/Zeb 2, TWIST1/2, Snail and Slug. We have not begun to evaluate MPP8-DNMT3a and SETDB1 in the coming year. We have also had a difficult time confirming the relationship between MPP8 and SIRT1 by co-IP in TCCSUP and UM-UC-3 cells, so we have a plan to use long cross linkers to better evaluate whether they can be pulled down together.
Evaluate *in vitro* effects of MPP8 knockdown on cellular phenotypes associated with lineage plasticity and EMT.	Demonstrate that MPP8 knockdown results in significantly less cellular motility and invasion among the bladder cancer lines (5637, TCCSUP and UM-UC-3).	Months 1-18	75%	All proposed cell proliferation, cell motility and invasion assays have been completed experiments have been completed in the 5637, TCCSUP, and UM-UC-3 bladder cancer cell lines using siRNAs as proof-of-concept. They all show that loss of MPP8 leads to significantly decreased proliferation, motility and invasion. In the coming year, we will confirm using our shRNA systems (both inducible and non-inducible).

Aim 2. Evaluate and optimize physiochemical properties of the novel MPP8 antagonists.

Major Task	Milestone	Proposed Timeline	% Completed	Notes
Evaluate and optimize mouse live microsome (MLM) stability, plasma protein binding (PPB) and mechanisms of metabolism/transport.	Optimize the physiochemical properties of UNC7713 (or synthesized analogs). Characterize MLM stability, PPB, influx/efflux transport, and mechanism of metabolism.	Months 1-15	65%	ADMET Predictor and Simulations Plus modeling completed. MLM studies to determine Cl_{int} and $t_{1/2}$ have been completed. *In vitro* experiments to characterize metabolism and transport were on hold during Year 2 because of concerning *in vivo* safety data requiring us to formulate in lipid nanoparticles. Remaining clinical pharmacology experiments in Aim 2 will be completed using formulated UNC7713 in Year 3.
Evaluate in vivo pharmacokinetics (PK).	Characterize single-dose PK for UNC7713 (and additional analogs) after IV, IP and PO administration. Identify a compound with a favorable in vivo PK profile (%F by IP or PO >30%, C_{max} ≥50 ng/mL, t1/2 >2 h, CL <30 mL/min/kg, AUC >3000 ng*h/mL).	Months 6-24	40%	We obtained approval from ACURO to initiate *in vivo* studies. Initial single-dose *in vivo* PK work had already been completed as part of our preliminary data. However, because of concerning *in vivo* safety data that arose during the initial efficacy studies, requiring us to formulate UNC7713 and second-generation compounds in lipid nanoparticles. Remaining in vivo PK work in Aim 2 (i.e., steady-state PK for UNC7713 and PK characterization of second-generation antagonists) will be completed using formulated compounds during Year 3.

Aim 3. Evaluate MPP8 antagonist effects on lineage plasticity and EMT-induced chemoresistance in bladder cancer.

Major Task	Milestone	Proposed Timeline	% Completed	Notes
Evaluate *in vitro* effects of MPP8 antagonists on markers of EMT and known inducers of	Confirm MPP8 chemical antagonism effects gene and protein expression of known EMT	Months 6-36	25%	We continue to generate confounding data where certain EMT markers have not reduced when treated with UNC7713. For instance, we expect that inhibition of MPP8 would alleviate the

lineage plasticity and EMT.	markers and known EMT inducers; Confirm increased H4K16ac, as well as decreased SIRT1 and ZEB1 binding at the CDH1 promoter. Confirm MPP8 knockdown disrupts SIRT1 and ZEB1 interactions, and DNMT3a and Snail interactions.			repressive H3K9me3 mark on *CDH1*, thereby allowing increased E-cadherin expression. But, we cannot make E-cadherin expression budge. However, we do observe decreased N-cadherin and decreased vimentin. Even using optimized systems like TGF-β to induce EMT, and use collagen-coated plates that have EMT, we still observe confounding data that does not align with our phenotypic data.
Evaluate *in vitro* effects of MPP8 chemical antagonism on cellular phenotypes associated with lineage plasticity and EMT.	Achieve a submicromolar cellular IC_{50} for UNC7713 and any optimized compounds from Aim 2. Demonstrate that MPP8 chemical antagonism results in significantly less cellular motility and invasion among the three bladder cancer lines (5637, TCCSUP and UM-UC-3).	Months 6-36	80%	The majority of work has been completed using UNC7713 in the 5637, TCCSUP and UM-UC-3 cells.
Evaluate *in vivo* effects of MPP8 antagonism.	Characterize the *in vivo* of the effects of MPP8 chemical antagonism using mouse xenograft models.	Months 1-36	20%	ACURO approved our protocol, and we began mouse studies. But, we had some issues related to UNC7713 formulation and lethality in mice – see below in Section 5. We believe that we have overcome that barrier by using liposomal formulations of UNC7713 and second-generation MPP8 covalent antagonists. *In vivo* efficacy studies will continue using formulated compounds in Year 3.

What was accomplished under these goals?

During this reporting period for Year 2 of the award, despite minor barriers identified in Section 5 of this report, major achievements were made in all three Specific Aims, as highlighted in the previous three tables. Briefly, as a reminder, Aims 1 and 3 focus on evaluating the role of MPP8 as an epigenomic regulator in preclinical models of advanced bladder cancer. For these two Specific Aims, we hypothesize that either knocking down MPP8 (Aim 1) or chemically antagonizing MPP8 with UNC7713 (Aim 3) will prevent and/or reverse the lineage plasticity that results in epithelial to mesenchymal transition (EMT). Therefore, if we could prevent or

reverse EMT, then we would observe advantageous phenotypic changes in the bladder cancer, such as reduction in proliferation and migration as well as an increase in cell death.

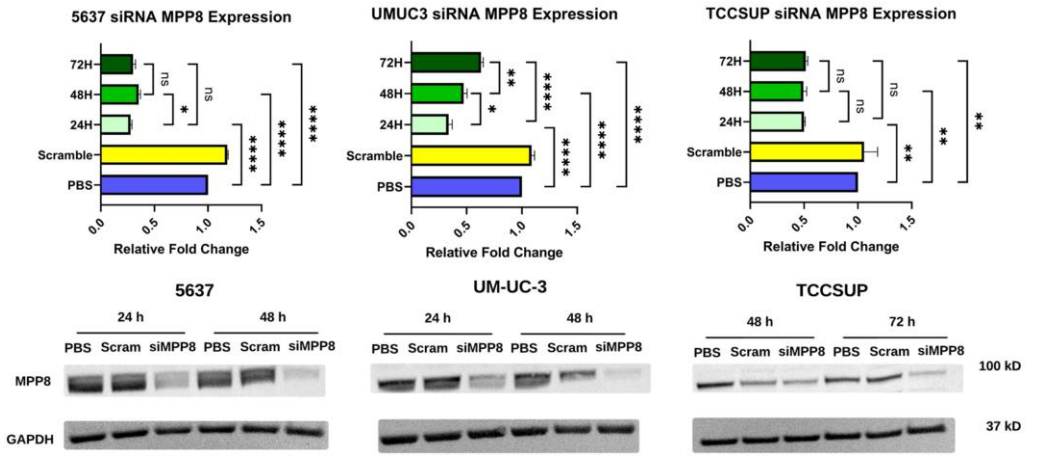

Figure 2. MPP8 gene and protein expression in 5637, TCCSUP and UM-UC-3 cells after transfection with siRNA against MPP8 for 24 and 48 hours.

In Aim 1, while we continued development of our ITPG-inducible and non-inducible MPP8 shRNA models in 5637, TCCSUP and UM-UC-3 bladder cancer cells lines, we used with siRNA knockdowns of MPP8 (siMPP8) and the NAD-dependent deacetylase sirtuin-1 (siSIRT1) in for proof-of-concept experiments. siRNAs against both MPP8 and SIRT1 were used to evaluate how loss of either of these key proteins impacts expression of EMT markers, and also impacts cell health phenotypes. When all three bladder cancer cell lines were transfected with the siMPP8, we observed significant (yet expected) knockdown of *MPP8* gene expression at 24, 48, and 72 hours when compared to PBS-treated cells and cells transfected with scramble RNA. We also observed significantly reduced MPP8 protein expression at 24 and 48 hours in the 5637 and UM-UC-3 cells; whereas, we observed reduced MPP8 expression at 72 hours in the TCCSUP cells (**Figure 2**). This aligns well with observations that TCCSUP doubling time (i.e., proliferation rate) is substantially slower than our other two bladder cancer cell lines. After confirming significant knockdown using the siMPP8, we continued to determine how MPP8 knockdown impacts different measures of cell health, including cell proliferation, migration and invasion as a proxy for metastasis, and viability. To evaluate MPP8 knockdown on proliferation, colony forming assays were performed. First, cells were plated, and then incubated with siMPP8 (or PBS, or scrambled RNA) and the transfection reagent lipofectamine for 24, 48 and 72 hours. Then, siRNA/lipofectamine was removed and replaced with fresh media for 10 days. After 10 days, drug-free media was removed, and cells were stained with crystal violet, and effects on proliferation were analyzed by Fiji. After 10 days >90% fewer colonies were detected in all three cell lines exposed to the siMPP8 when compared to PBS + lipofectamine (P<0.0001) (**Figure 3**). However, there was an anti-proliferative effect observed in the scrambled + lipofectamine-treated cells, and will definitely repeat these in our shRNA MPP8 knockdown lines in the coming year. And, if we decide to repeat using siMPP8, we have already purchased a new universal scramble RNA.

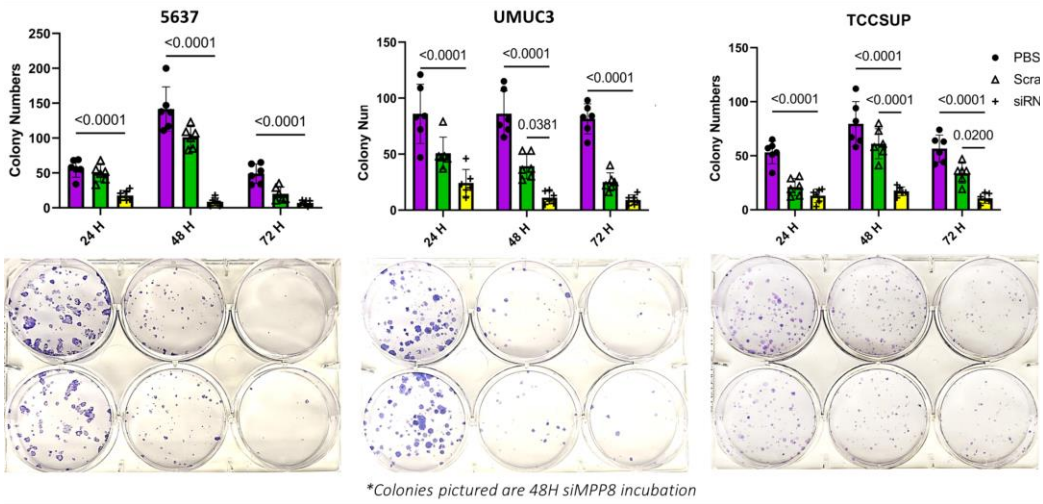

Figure 3. Colony forming assay results in 5637, TCCSUP and UM-UC-3 bladder cancer cell lines incubated with siRNA against MPP8 for 24, 48, and 72 hours.

Following on these proliferation experiments, we conducted transwell migration assays to test for cell chemotaxis or migration in response to a chemical gradient. Briefly, cells that have a potential to migrate/invade will move from their original transwell were seeded and across a membrane where they have access to media with nutrients. Cells were seeded in the transwell and then incubated with siMPP8 (or PBS, or scrambled RNA)

and the transfection reagent lipofectamine for 24, 48 and 72 hours. Of note, we also included a PBS sample without lipofectamine to ensure the observed effects on migration and invasion were not due to toxic side-effects of lipofectamine transfection. In the TCCSUP and UM-UC-3 cell lines, there were significantly fewer cells that migrated when treated with siMPP8 when compared to cells treated with PBS alone or PBS + lipofectamine (**Figure 4**). And, there was a trend in both cell lines towards less migration in siMPP8-treated cells versus scramble + lipofectamine. But, we still have plans to repeat these experiments early in 2024 because, again, we think there was an issue with our scramble RNA and have purchased a new universal scramble. We also plan to recapitulate these data in cells with our MPP8 shRNA.

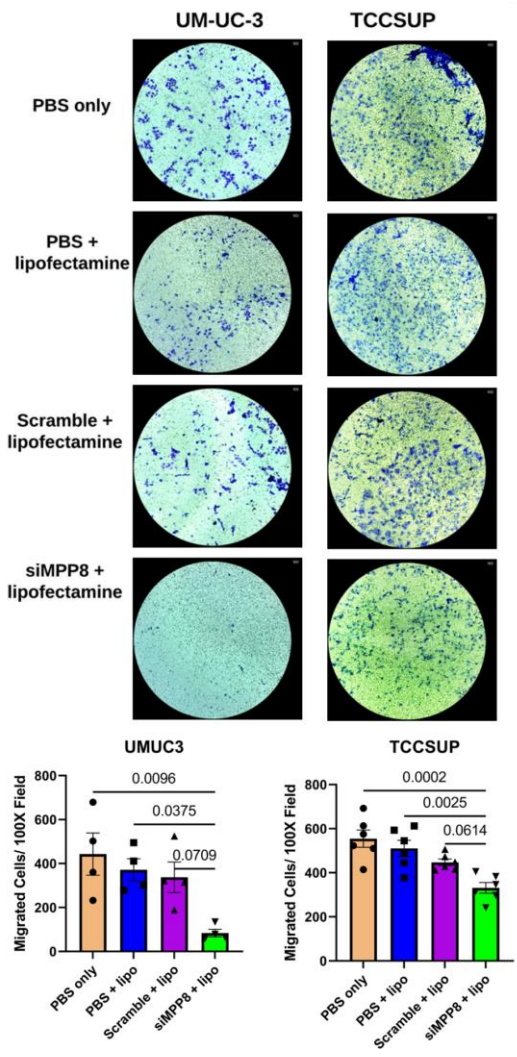

Figure 4. Migration/invasion assay results in TCCSUP and UM-UC-3 bladder cancer cell lines incubated with siRNA against MPP8 for 48hours.

We also wanted to investigate whether siMPP8 had an effect on limiting cell viability in these three bladder cancer lines. In Year 1, we demonstrated that covalent antagonism of MPP8 with our first-in-class compound (UNC7713) inhibits viability in all three cell lines with submicromolar potency. Similar to previous experiments, incubated with siMPP8 (or PBS, or scrambled RNA) and the transfection reagent lipofectamine for 24, 48 and 72 hours, and then evaluated for viability using CellTiter-Glo™. In **Figure 5**, it is apparent that siMPP8 has an adverse effect on viability of all three bladder cancer cell lines, particularly by 72 hours. Cells were normalized to PBS + lipofectamine (blue line), and by 72 hours the data shows the viability was reduced by at least 50% in cells with siMPP8 (green line; approximately 50% TCCSUP cells, 65% in 5637 cells, and over 80% in UM-UC-3 cells). Again, there was an unexpected effect of the scramble RNA on viability (purple line), especially in the TCCSUP cells, which will require us to repeat these experiments as well with our new universal scramble and in cells with our MPP8 shRNA.

Next, we wanted to nail down molecular mechanisms that might explain the associations we have observed between MPP8 knockdown and effects on cell health phenotypes in our three bladder cancer cell lines. While our early work showed that MPP8 knockdown can increase certain epithelial markers (e.g., ZO-1) and decrease certain mesenchymal markers (e.g., Vimentin), we continue to struggle with linking MPP8 knockdown to decreased proliferation, migration/invasion and viability as a function of inhibiting EMT (**Figure 6 – see Appendix 1**). Because MPP8 is such a new target in cancer, there are not many publications linking its knockdown to changes in genes or pathways. So, we discussed our issues with our co-investigator, Dr. Lindsey James, and she provided us with unpublished data from a different grant. In her work, they seek to determine how MPP8 antagonism impacts MYC-hyperactivated triple negative breast cancer (TNBC). Briefly, mammary cells were treated with a dTAG protein degrader of MPP8. They showed that inactivation of MPP8 leads to induction of antiviral immune pathways in MYC-hyperactivated HMEC cells by GSEA analysis of bulk RNA-seq data. But perhaps more importantly for us, they showed that, at a gene level, TNF-α and IL-6 are activated

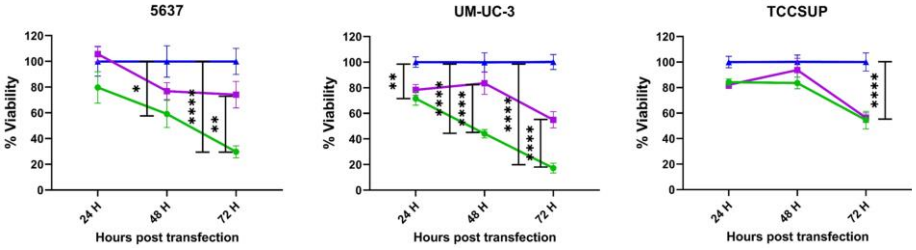

Figure 5. Cell viability is evaluated by Cell-Titer-Glo in cells treated with siMPP8, PBS + lipofectamine and scramble + lipofectamine for 24-72 hours.

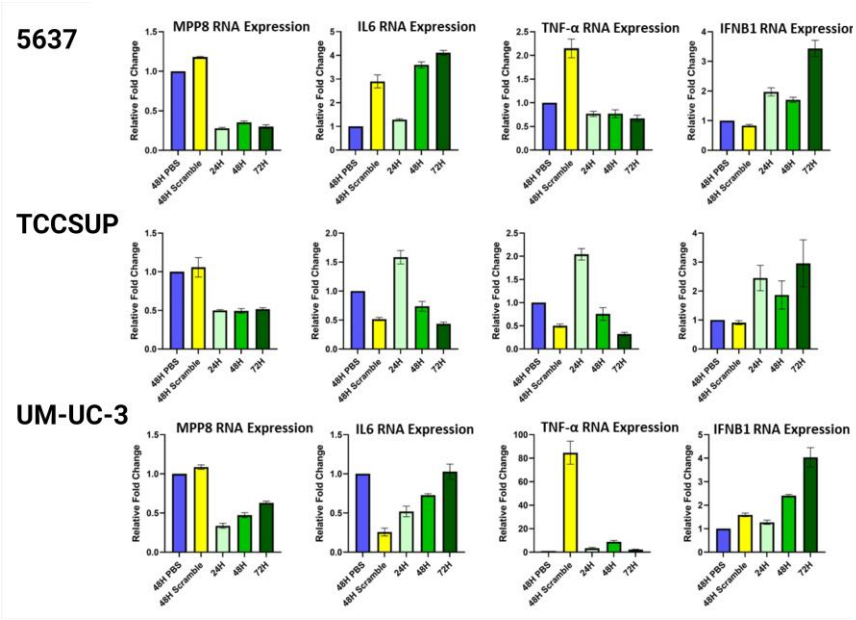

Figure 8. MPP8 knockdown and gene expression of immune markers. Cells were incubated with siMPP8 (or PBS, or scramble RNA) + lipofectamine for 24, 48 and 72 hours. *MPP8* gene expression, and expression of genes involved in antiviral immune response (*IL6*, *TNFA*, and *IFNB1*) were evaluated using RT-PCR.

as a result of MPP8 degradation (**Figure 7 – see Appendix 2**). Following on these data from the James Lab, we then incubated with siMPP8 siMPP8 (or PBS, or scrambled RNA) and the transfection reagent lipofectamine for 24, 48 and 72 hours. Then, we performed quantitative real-time polymerase chain reaction (RT-PCR) to determine if MPP8 knockdown activates *IL6*, *TNFA*, or *IFNB1*. While we still have issues with our universal scramble and will repeat our initial data suggests that IL6 is activated in 5637 and UM-UC-3, but not TCCSUP, cells. Unfortunately, we were not able to replicate the James lab data with respect to TNFA activation, and these experiments need to be repeated. Finally, we did observe striking activation of IFNB1, which is the gene that encodes interferon β1 (**Figure 8**). These data require validation by additional experiments (e.g., Western Blotting) and alternative gene knockdown systems (e.g., shRNA). But, these exciting preliminary data could open future lines of research inquiry because they suggest MPP8 knockdown, or ideally chemical antagonism with UNC7713, could be paired with FDA approved immunotherapies (e.g., pembrolizumab) as a combination strategy in bladder cancer. Ultimately, these data are still ambiguous, and we are still left wondering whether our failures to observe changes in EMT proteins (e.g., E-cadherin and N-cadherin) represent deficiencies in experimental designs or whether MPP8 inhibition is simply not linked to EMT in bladder cancer. Therefore, in the coming year, we are prepared to quickly pivot to bulk RNA-seq (and possibly CUT&RUN) to help determine genes and pathways affected by MPP8 loss or chemical antagonism with UNC7713. These technologies will invariably help us identify genes and pathways affected by MPP8 knockdown or antagonism, should our EMT hypotheses continue to be problematic.

Last, as part of Aim 1, we began to really dig deeper into the mechanisms underlying MPP8 as a potential epigenetic regulator of proliferation and migration/invasion in bladder cancer. Previously published work in prostate cancer has linked MPP8 to the deacetylase SIRT1, and we also know that SIRT1 has been linked to transfection factors implicated in EMT like Zeb1/2, TWIST1/2, Snail and Slug in previous breast cancer and osteosarcoma studies. First, we used siMPP8 and siSIRT1 knockdown models in an attempt to try to understand how knockdown of MPP8 impacts SIRT1 and Zeb1 expression, or alternatively how SIRT1 knockdown impacts expression of MPP8 and Zeb1. Cells were incubated with siMPP8 or siSIRT1 (or PBS, or scrambled RNA) and the transfection reagent lipofectamine for 24, 48 and 72 hours. Then they were lysed, and standard Western Blotting was performed. As **Figure 9** shows, we were able to knockdown MPP8 in siMPP8-treated cells, and SIRT1 in SIRT1-treated cells. However, knockdown of MPP8 did not

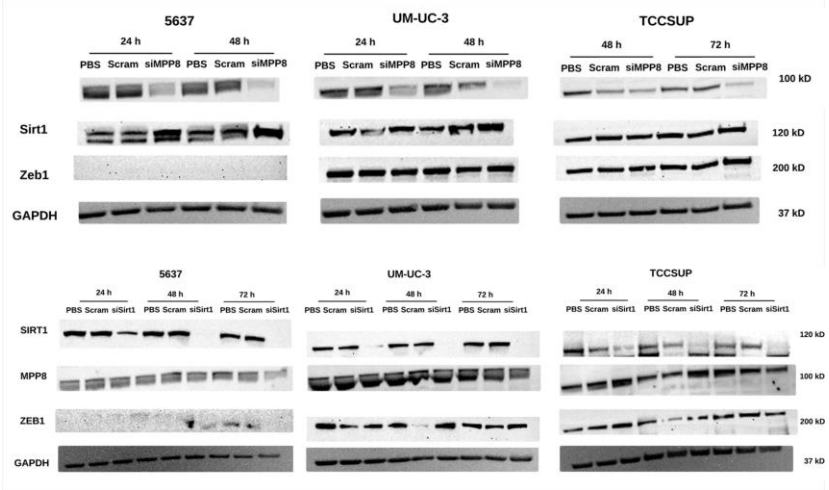

Figure 9. Western blots of MPP8, SIRT1 and Zeb 1 in bladder cancer lines treated with siMPP8 (top panel) and siSIRT1 (bottom panel).

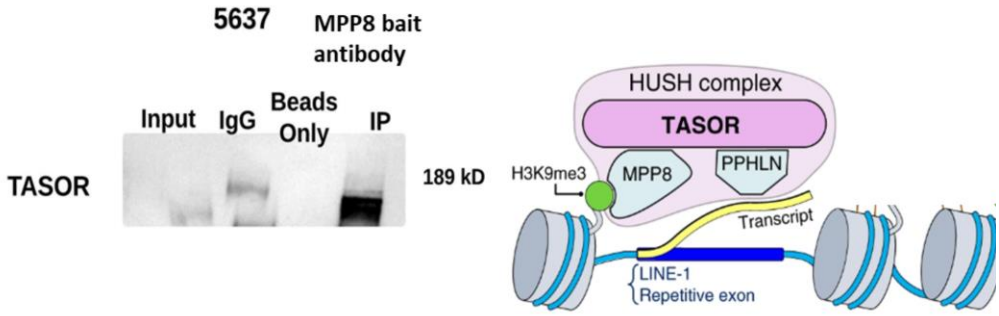

Figure 10. Co-immunoprecipitation between TASOR and MPP8 with MPP8 as the bait in 5637 cells.

seem to affect expression of SIRT1 or Zeb1 in any of the three cell lines. Similarly, knockdown of SIRT1 did not alter MPP8 or Zeb 1 expression. So, our next step was to perform co-immunoprecipitation (coIP) experiments to determine if we could pull down SIRT1, Zeb1/2, TWIST1/2, Snail and Slug with MPP8 as bait. Because we know that MPP8 is part of the HUSH complex with TASOR and periphilin, we first performed a coIP and showed that TASOR is indeed pulled down with MPP8 (**Figure 10**). Then, we performed similar experiments to determine if we could pull down SIRT1 and the aforementioned transcription factors. We were again able to pull down TASOR as a positive control, as well as Zeb 1, Snail, Slug, and possibly Zeb2 (but not TWIST1 or TWIST 2) with MPP8 (**Figure 11**). Further, we confirmed the interaction between MPP8 and Zeb1 in a follow-up experiment where Zeb1 was the bait and pulled down MPP8 (**Figure 12 – see Appendix 3**). Interestingly, we were not able to pull down SIRT1 with MPP8 (**Figure 11**). But, we know from previously published manuscripts that MPP8 binds H3K9me2 and H3K9me3 marks, that when MPP8 is bound at those loci it binds SIRT1, and ultimately SIRT1 deacetylates H3K9ac and H4K16ac causing a more packed heterochromatic state that leads to gene repression. Upon troubleshooting these data, we have hypothesized that the interactions between MPP8 and SIRT1 are so quick that it would be unlikely to pull down MPP8 and SIRT1 together under our normal experimental conditions. Thus, we have already begun experiments using a long crosslinker called dithiobis(succinimidyl propionate) or DSP. We hypothesize that using DSP will crosslink MPP8 and SIRT1 (should they interact with one another), and when crosslinked we will be able to pull them down together.

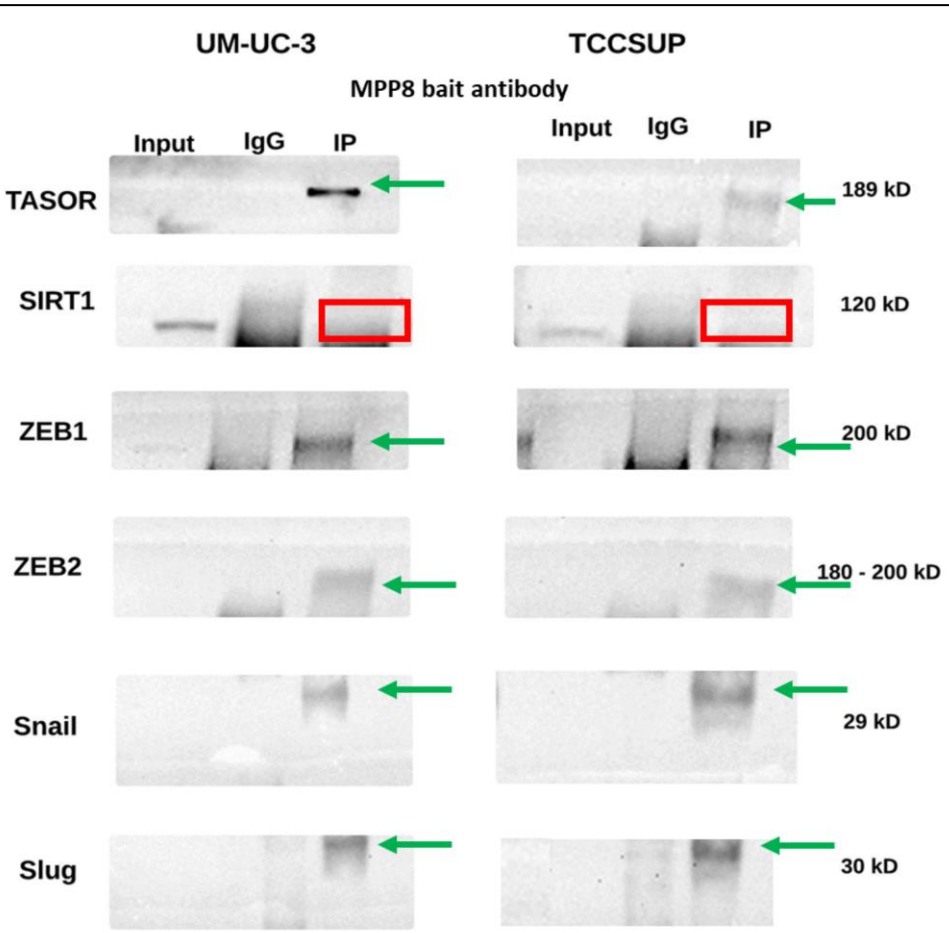

Figure 11. Co-immunoprecipiration between MPP8 and Zeb1, Zeb2, Snail and Slug (but not SIRT1, TWIST1 or TWIST2) with MPP8 as the bait in TCCSUP and UM-UC-3 cells.

In Aim 3, we focused on continued development of our first-in-class MPP8 covalent antagonist in bladder cancer. In the previous year, we showed that bladder cancer cell lines 5637 and J82 UNC7713 achieved submicromolar potency when limiting cell viability, and that UNC7713 was nearly 200x more potent than UNC7716 at 48 h (IC_{50}: 0.28 μM vs. 56.03 μM). Potency was also maintained at 96 h (0.18 μM). During this

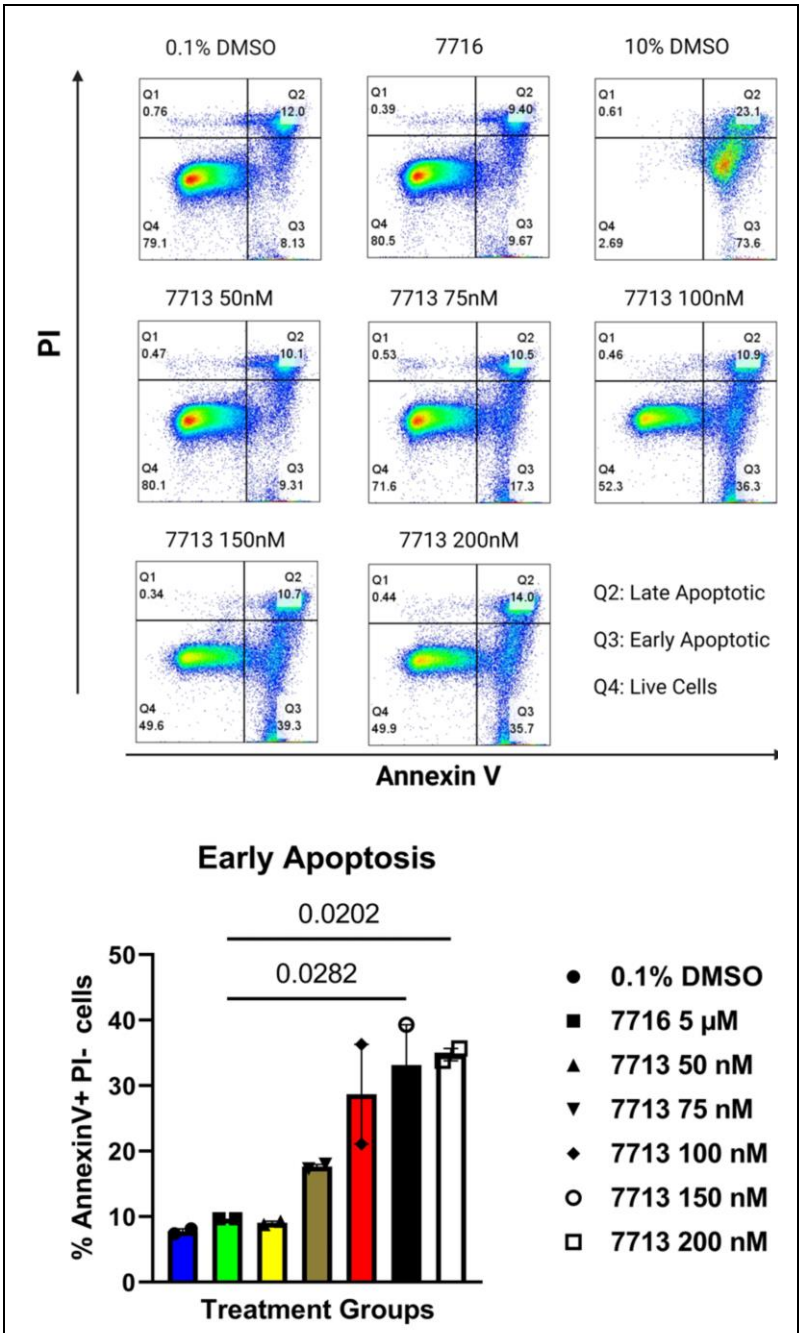

Figure 13. UNC7713 triggers apoptosis in TCCSUP and UM-UC3 cells (TCCSUP shown here).

reporting period, we followed up and confirmed UNC7713 in TCCSUP and UM-UC-3 cell lines. Briefly, the two lines were each treated with ascending concentrations of UNC7713 (0.1 nM–100 µM), or negative control compound UNC7716. Cell viability was measured using CellTiter-Glo™ after 72- and 96-hour incubations. IC_{50} values were calculated using a four-parameter non-linear regression model using SAS JMP v15. Similar to 5637 cells, UNC7713 potently inhibited cell viability in TCCSUP and UM-UC-3 lines as early as 48 h (IC_{50}: 0.76 µM and 0.34 µM, respectively). Next, we also conducted confirmatory studies in the two additional lines to what extent UNC7713 triggers apoptosis versus necrosis. Cells were stained with Annexin V and propidium iodide (PI) to evaluate apoptosis versus necrosis signaling by flow cytometry after 48 h incubation of UNC7713. Flow cytometry experiments were performed on a Thermo Attune NxT, and data were analyzed using FlowJo. Similar to the previous year in 5637 cells, concentrations of UNC7713 as low as 75 nM caused approximately 2-times greater apoptotic signaling than 20 µM of the negative control UNC7716 after 48 h (17.3% vs. 9.7% in TCCSUP cells). But, at only one dose level higher, 100 nM caused approximately 4-times greater apoptotic signaling than 20 µM UNC7716 (**Figure 13**).

Again, similar to the previous reporting period in 5637 cells, we confirmed UNC7713's anti-proliferative potential using colony forming assays. 5637 cells were plated, incubated with four ascending concentrations of UNC7713 (10 nM–75 nM), UNC7716 5 µM, or 0.1% DMSO control for 48 hours. Then, UNC7713 was removed and replaced with fresh media for 10 days. After 10 days, drug-free media was removed, and cells were stained with crystal violet. and colony formation was evaluated after 10 days using crystal violet and analyzed by Fiji. After 10 days >90% fewer colonies were detected in cells treated with UNC7713 (at doses ranging from 10-75 nM) when compared to 20 µM UNC7716 and 0.1% DMSO controls (**Figure 14 – see Appendix 4**). Last, we conducted traditional wound healing assays (also known as "scratch" assays) to evaluate cell migration in TCCSUP and UM-UC-3 cells. Cells were placed in 12 well plates and agitated to distribute cells throughout well. The cells were grown in a monolayer, treated with UNC7713 (25 nM–100 nM), and then subjected to a wound healing assay to evaluate cell migration after 48 hours. For wound healing, images were captured by an Olympus IX83 inverted microscope and analyzed by Fiji. After 48 hours, cells treated with UNC7713 did not migrate to initiate wound closure, but instead caused cell death, increasing the size of the initial wound. Both cell lines treated with UNC7713 at concentrations as low as 75 nM caused dramatic wound expansion compared to 0.1% DMSO and 20 µM UNC7716 (almost 50% wound expansion for UNC7713 at 100 nM versus 100% wound closure for both controls) (**Figure 15**).

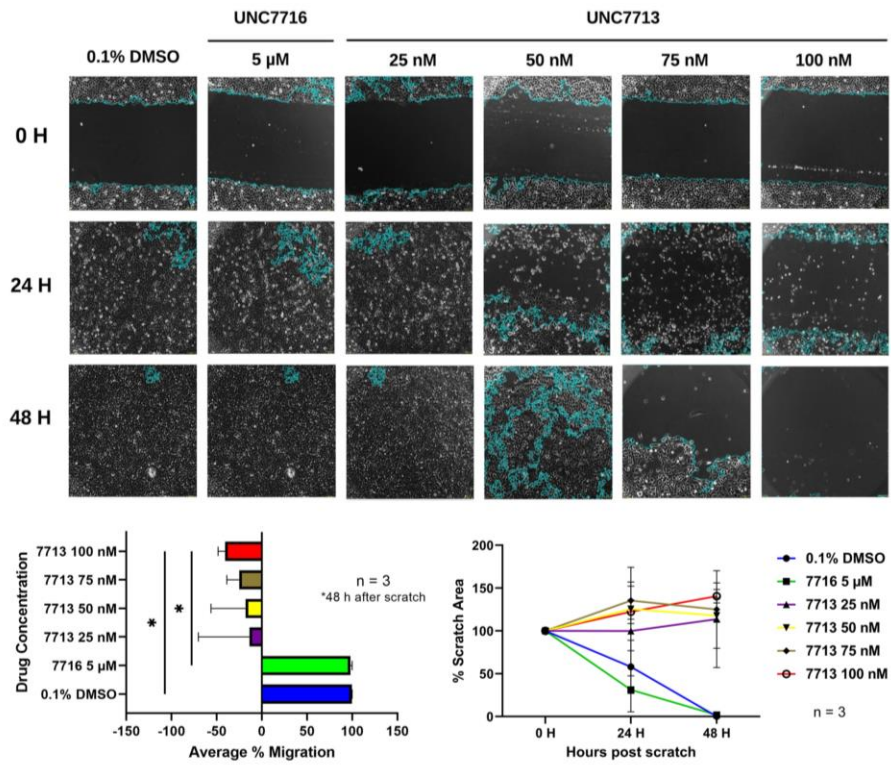

Figure 15. Scratch assay results show 50-100 nM UNC7713 were potent enough to prevent migration in TCCSUP cells.

Overall, there were goals that were not met during this reporting period. First, we continue to work on getting our ITPG-inducible shRNA system optimized. While there have not been technical barriers, the process of getting the system stably transfected has been lengthier than anticipated. But, as an unplanned byproduct of the process, we will also have created an inducible shRNA system that contains the fluorescent marker mCherry, which will allow us opportunity to perform more elegant imaging during our in vivo efficacy studies to come in the next reporting period. We also will continue to make headway on understanding MPP8's role as an epigenetic regulator by using siRNAs until our inducible system is optimized in the coming reporting period. Last, we have not made as much progress on our hypotheses related to MPP8 and EMT. While we have very encouraging phenotypic data (e.g., proliferation assays, migration assays, and cell death assays), we have generated confounding PCR and Western blotting data about the role of MPP8 knockdown or chemical antagonism and expression of *CDH1*/E-cadherin, *CDH2*/N-cadherin, etc. In the coming reporting period, we have already planned to evaluate changes to our experimental platforms. As previously mentioned, we still plan to investigate plates coated with collagen (or other similar substance) and/or stimulate the cells with TGF-β to initiate active EMT. We anticipate these modifications will help us to more closely recapitulate EMT within *in vitro* systems, which will lead to more rigorous and reproducible data moving forward. But, as previously mentioned, should we continue to fail to link MPP8 knockdown or chemical antagonism to EMT in bladder cancer, we will shift to bulk RNA-seq so that we can identify genes and pathways perturbed by MPP8 knockdown or chemical antagonism as possible explanations for the encouraging phenotypic data we continue to amass. Last, for our *in vivo* efficacy studies (Aim 3), we observed that UNC7713 and UNC8739 were prohibitively toxic to the mice, particularly after repeat dosing. Dr. James and I have worked with formulation experts at UNC, and found that a liposomal formulation of UNC7713 is not toxic to mice. As a result, we have started a new round of *in vivo* efficacy studies centered on formulated UNC7713.

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

Major Task 1: Career Development activities

Major Task	Proposed Timeline	Completed?	Notes
Attend BIOC 702, a class focused on chemical compounds that regulate chromatin, and novel epigenetic tools to understand chromatin function	Months 1-12	No	Will complete in Year 3; will also audit PHCO 750: Proteomics Methods and Applications in Spring 2024.

Attend short-courses and seminars on research ethics and responsible conduct of research from NC TraCS Institute	Months 1-15	Yes for Years 1-2 (ongoing)	Short course completed; will also attend available offerings in Y2-3.
Present research progress to Dr. Kim monthly; present research to UNC ESOP, UNC Lineberger Comprehensive Cancer Center faculty and to UNC Chromatin and Epigenetics Program faculty at least twice per year	Months 1-36	Yes for Years 1-2 (ongoing)	Present regularly at Kim Lab meeting; presented once in March 2022 and June 2023 to UNC Chromatin and Epigenetics Program and UNC LCCC faculty
Attend national conferences (e.g., AACR, ASCO GU Symposium) at least twice during the Award period; Attend epigenetics workshops (e.g., NIEHS epigenetics workshop)	Months 1-36	Ongoing	Abstract #1 presented to 2024 AACR Annual Meeting (Appendix 5). Abstract #2 to be submitted for the AACR "Special Conference on Bladder Cancer: Transforming the Field" in Charlotte, NC from May 17-20, 2024.

How were the results disseminated to communities of interest?

Nothing to Report

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

The following **three** major activities will be crucial towards successfully accomplishing goals in the next reporting period, as well as overall project success:

1. We will finalize optimization of our ITPG-inducible shRNA system for MPP8 knockdown (and our dCas9-VPR system to over-express MPP8 for rescue experiments). Despite having generated encouraging proof-of-concept data using siRNAs, these model systems will be crucial for us to confirm MPP8 as an epigenetic regulator in bladder cancer. It will also allow us to have control over timing of gene knockdown, which will be so important during the mouse efficacy studies (as a control for UNC7713 and/or second-generation compounds).

2. We will continue to determine if the phenotypic changes in proliferation, migration, invasion and viability are all secondary to the effects of MPP8 knockdown/chemical antagonism on inhibiting EMT. However, if issues persist confirming this as a major mechanism, we have plans to quickly pivot to bulk RNA-seq (and possibly CUT&RUN) to help determine genes and pathways affected by MPP8 loss.

3. Now that we know A) that unformulated compounds are lethal to mice within minutes after repeat dosing and B) that formulating them in lipid nanoparticles overcomes this lethal phenotype in experiments conducted by Dr. James and colleagues in xenograft models of TNBC, we will initiate mouse studies in support of work proposed in Aim 2 (remaining PK work) and Aim 3 (efficacy studies).

4. IMPACT:

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

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

None to report – but we are considering changes based on our continued negative data regarding EMT. We are considering whether we should conduct bulk RNA-seq (+/- CUT&RUN) on our bladder cancer lines treated with UNC7713 and UNC8850 (and other top second-generation compounds) to determine other pathways that are fundamentally changed when MPP8 is covalently antagonized. We have started amassing quite an impressive amount of phenotypic data showing that MPP8 antagonism prevents proliferation, migration and invasion. It also seems to cause DNA damage that leads to apoptosis. However, as previously mentioned, we continue to fall short linking MPP8 antagonism to inhibition of EMT that then ultimately causes phenotypic responses. Otherwise, we continue to make significant progress with ongoing experiments, and we look forward to the coming year for increased productivity.

Actual or anticipated problems or delays and actions or plans to resolve them

We encountered only **one** major problem that led to ongoing delays:

Problem: For our *in vivo* efficacy studies described in Aim 3, we found that UNC7713 and UNC8850 were prohibitively toxic to mice. For UNC7713, mice were either in profound distress or died within 15 minutes of administration after doses 2-4. As previously mentioned in the annual technical report from 2021-2022, UNC8739 is one of the second-generation MPP8 covalent antagonists that was developed to increase the drug-like properties of UNC7713. However, it was lethal to mice within 5 minutes of administration by multiple routes (IV, IP and oral).

Problem Resolution: Dr. James and I coordinated with faculty in the Division of Pharmacoengineering and Molecular Pharmaceutics (DPMP) at the UNC Eshelman School of Pharmacy, and they formulated the UNC7713 and UNC8739 compounds by encapsulating them in liposomes. While this approach had little effect on UNC8850 (and we continue ways to strategize delivery to mice), it significantly lessened UNC7713's lethality. Using another second-generation compound (UNC8864) that was selected because of improved labeling in recombinant MPP8 protein and efficacy in HeLa cells over UNC7713, Dr. James conducted PK studies outside of the scope of this award. Her group found they could conduct a maximum tolerated dose (MTD) study of liposomal UNC7713 and UNC8864, and found that both encapsulated compounds were well-tolerated up to 10 mg/kg (with repeat IV administration). They are currently conducting efficacy studies and are currently testing the liposomal formulations in xenograft studies of triple negative breast cancer (TNBC). In the coming year, we will proceed with similar studies to evaluate liposomal formulations of UNC7713 and UNC8364 (and possibly alternate formulations of UNC8850) in mice.

Changes that had a significant impact on expenditures

None to report

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

Not applicable

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

On the bright side, we received our ACURO approval in a letter from Dr. Krinon Moccia on January 23, 2023. Shortly thereafter, we began preliminary mouse work using UNC7713. Unfortunately, we found that repeat exposure to UNC7713 and second-generation MPP8 covalent antagonists cause rapid death (e.g., within 15 mins of administration on doses 2-4). Therefore, we suspended animal studies and worked with personnel at the UNC Eshelman School of Pharmacy to develop a system by which UNC7713, UNC8364 and UNC8739 are encapsulated in liposomes. In work by the James lab (not supported by this award), Dr. James and her group were able to demonstrate that encapsulated liposomal UNC7713 and UNC8364 do not cause immediate death in their PK studies, were able to complete an MTD study, and are currently testing the liposomal formulations in xenograft studies of TNBC. In the coming year, we will proceed with similar studies to evaluate liposomal formulations of UNC7713 and UNC8364 in mice.

Significant changes in use of biohazards and/or select agents

We will continue to use UNC7713 as our lead compound with UNC7716 and UNC7715 as the negative control compounds (in addition to prioritized second-generation MPP8 antagonists). However, for *in vivo* PK studies (Aim 2) and efficacy studies (Aim 3), we will formulate the compounds within lipid nanoparticles for the safety of the mice.

6. PRODUCTS:

- **Publications, conference papers, and presentations.**

  1. Abstract #1 (**Appendix 5**) was presented at the 2023 AACR Annual Meeting.

  Gonzalez Tineo S, Kemper RM, Tripathi SK, Buttery PH, Kim WY, James LI, Crona DJ. Evaluating the potency of a first-in-class covalent antagonist of the H3K9me3 reader protein MPP8 in bladder cancer. Abstract 6283. Cancer Research. 2023;83(7_Supplement):6283-6283.

  2. Abstract #2 (focused on interactions between MPP8, SIRT1, transcription factors and H3K9ac/H4K16ac) will be submitted prior to the February 27, 2024 deadline for the AACR "Special Conference on Bladder Cancer: Transforming the Field" in Charlotte, NC from May 17-20, 2024.

  Journal publications.

  Two publications in process, but none submitted yet (we hope at least one will go out for review before the end of 2023). The first will focus on interactions between MPP8, SIRT1, key transcription factors and chromatin marks (H3K9ac and H4K16ac). The second will focus on UNC7713.

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

  Nothing to Report

  Other publications, conference papers and presentations.

- **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?

1.

Name:	Daniel J. Crona, PharmD, PhD
Project Role:	Principal Investigator
ORCID iD:	0000-0003-3742-8863
Nearest Person Month Worked:	3
Contribution to Project:	Oversight of all activities in Aims 1-3 (Major Tasks 1-9); authored UNC IACUC protocol and UNC IRB for PCBN submission to obtain TMA samples; oversight and mentoring for Dr. Kardouh, Ms. Gonzalez Tineo, Mr. Buttery and Mr. Kemper.
Funding Support:	Department of Defense Award W81XWH2110748; NIH/NIGMS; American Cancer Society; UNC Eshelman School of Pharmacy (start-up funds)

2.

Name:	Lindsey I. James, PhD
Project Role:	Co-Investigator
ORCID iD:	0000-0002-6034-7116
Nearest Person Month Worked:	1
Contribution to Project:	Discovery of UNC7713 and UNC7716; oversight and mentoring of Mr. Buttery in Aim 2 and synthesis of UNC7713 and UNC7716.
Funding Support:	Department of Defense Award W81XWH2110748; NIH/NCI; UNC Eshelman School of Pharmacy and the Eshelman Institute for Innovation; Pinnacle Hill, LLC

3.

Name:	Stephany Gonzalez Tineo
Project Role:	Graduate Student
ORCID iD:	0000-0002-6229-3481
Nearest Person Month Worked:	9
Contribution to Project:	Activities described in Aims 1 and 3 (all Major Tasks).
Funding Support:	This award only

4.

Name:	Peter H. Buttery, BS
Project Role:	Graduate Student
ORCID iD:	0000-0001-7778-1552

Nearest Person Month Worked:	3
Contribution to Project:	Synthesis of UNC7713 and UNC7716; some preclinical pharmacology experiments detailed in Aim 2.
Funding Support:	Department of Defense Award W81XWH2110748; additional James Lab grants

5.

Name:	Ryan Kemper
Project Role:	Research Specialist
ORCID iD:	0000-0002-5468-7827
Nearest Person Month Worked:	3
Contribution to Project:	Select activities in Aims 1 and 3 in support of Ms. Gonzalez Tineo. Specifically, Mr. Kemper continues to work with her on optimizing the inducible shRNA system described in Aim 1 of the proposal.
Funding Support:	• American Cancer Society; NIH/NIGMS; Crona Lab start-up funds • Mr. Kemper is **not** funded by this award

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

COLLABORATIVE AWARDS:

Not applicable

9. APPENDICES:

APPENDIX 1. Figure 7 from Section 3: Accomplishments

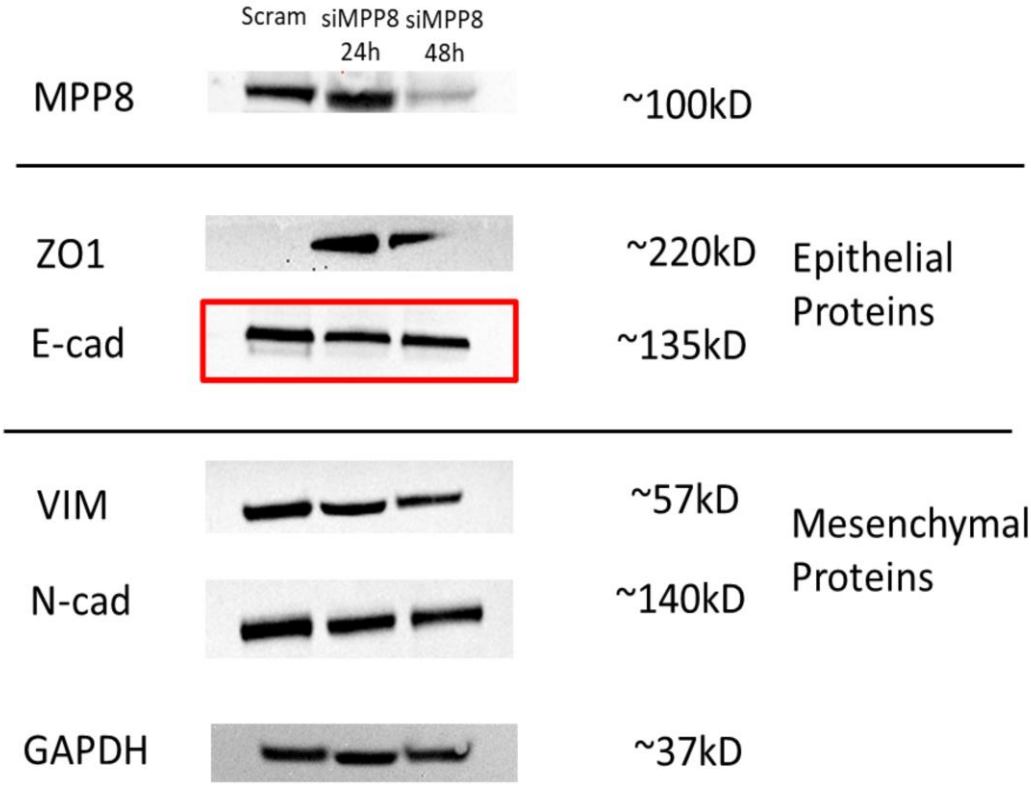

Figure 6. Effects of siMPP8 on EMT markers. Cells were incubated with siMPP8 (or PBS, or scramble RNA) + lipofectamine for 24 and 48 hours. MPP8 protein expression, expression of epithelial proteins involved in EMT, and expression of mesenchymal proteins involved in EMT were evaluated using standard Western Blotting techniques. Shown in this figure are 5637 cells.

APPENDIX 2. Figure 7 from Section 3: Accomplishments

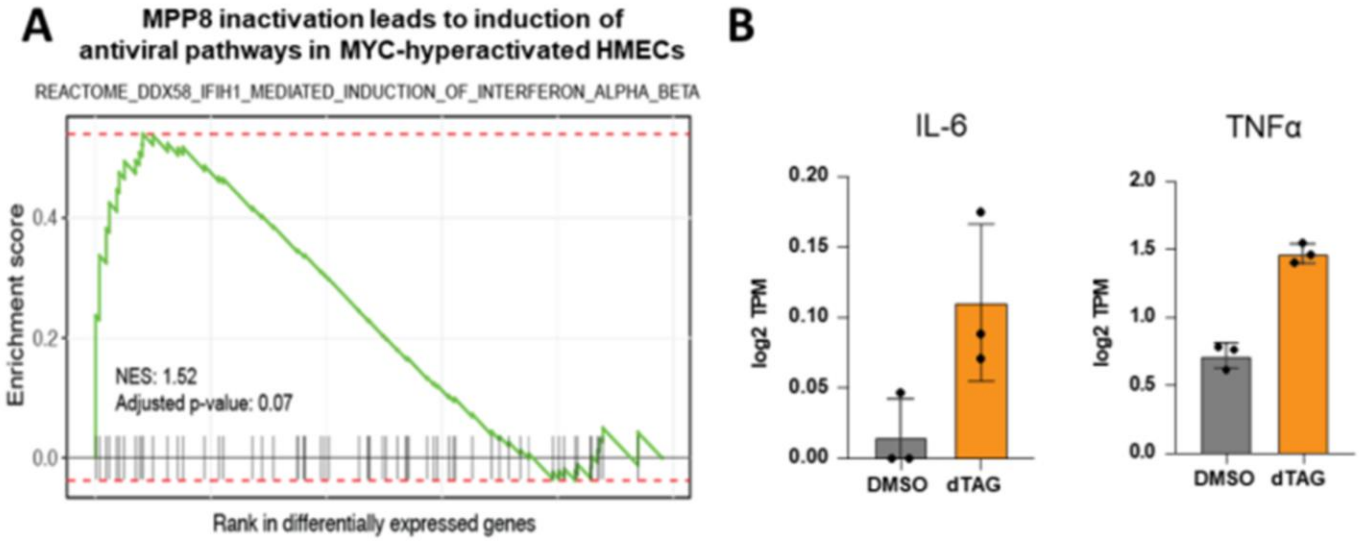

Figure 7. <u>Unpublished</u> work from the James Lab at UNC linking MPP8 and immune system markers. Mammary cells were treated with a dTAG protein degrader of MPP8. (**A**) Inactivation of MPP8 leads to induction of antiviral immune pathways in MYC-hyperactivated HMEC cells by GSEA analysis of bulk RNA-seq data. (B) TNF-α and IL-6 are activated as a result of MPP8 degradation. Barplots show increased mRNA expression upon dTAG-mediated MPP8 degradation in HMEC cells.

APPENDIX 3. Figure 12 from Section 3: Accomplishments

Figure 12. Co-immunoprecipitation experiments show MPP8 can be pulled down in bladder cancer cell lines when Zeb1 was used as the bait.

APPENDIX 4. Figure 14 from Section 3: Accomplishments

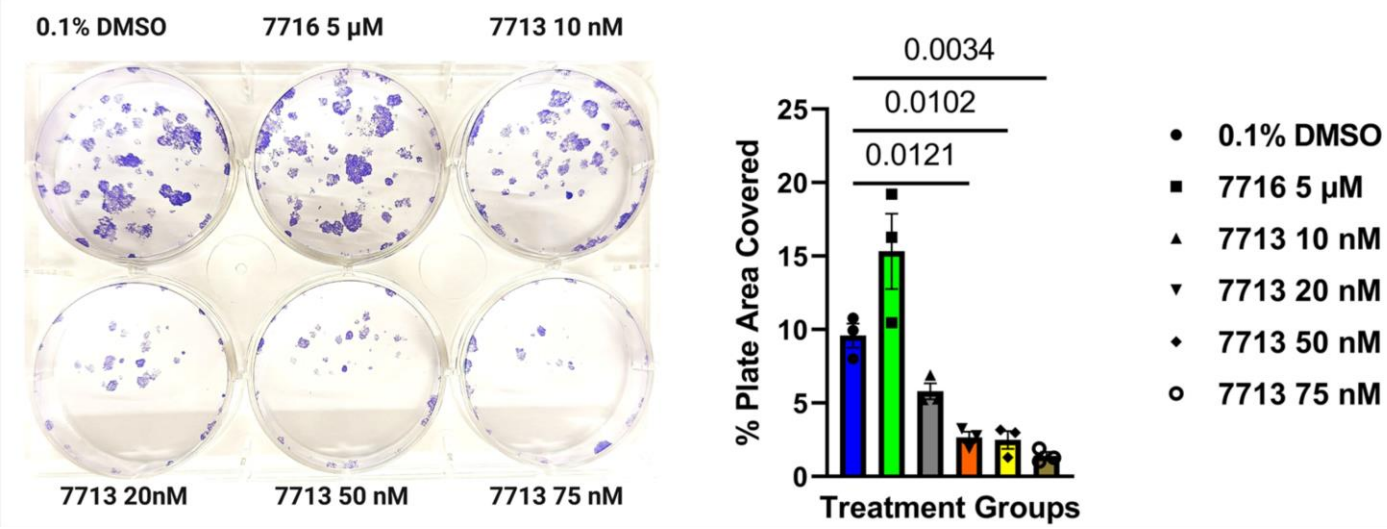

Figure 14. UNC7713 abrogates cell proliferation in TCCSUP and UM-UC-3 bladder cancer cells at doses ranging from 10-75 nM. Barplots represent n=3 biological replicates. TCCSUP cells shown in this figure.

APPENDIX 4. Abstract presented by Stephany Gonzalez Tineo the 2023 AACR Annual Meeting Gonzalez (*Cancer Research*. 2023;83(7_Supplement):6283-6283).

Title: Evaluating the potency of a first-in-class covalent antagonist of the H3K9me3 reader protein MPP8 in bladder cancer

Authors: Stephany Gonzalez Tineo,[1] Ryan M. Kemper,[1] Surya K. Tripathi,[1] Peter Buttery,[2-3] William Y. Kim,[4-7] Lindsey I. James,[2-4] Daniel J. Crona[1,4,8]

Affiliations:
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5 Division of Oncology, Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, NC, USA
6 Department of Genetics, School of Medicine, University of North Carolina, Chapel Hill, NC, USA
7 Department of Pharmacology, Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, NC, USA
8 Department of Pharmacy, University of North Carolina Hospitals and Clinics, Chapel Hill, NC, USA

Abstract

Background: Bladder cancer (BC) is a common and deadly disease, and despite recent treatment advances, metastatic BC (mBC) remains incurable. Mutations in genes that encode epigenetic/chromatin modifier proteins are common in mBC, with >90% of tumors harboring at least one inactivating mutation. The epigenetic reader protein MPP8 recognizes the histone-3-lysine-9-trimethyl (H3K9me3) region of target gene promoters, and recruits transcription factors associated with cell proliferation and metastasis. UNC7713 was developed as a covalent antagonist to disrupt MPP8 binding to H3K9me3. Here, we explored whether UNC7713 potently inhibits cell proliferation, migration and viability in preclinical models of BC.

Methods: 5637 cells were treated with ascending concentrations of UNC7713 (0.1 nM–100 μM), negative control compound UNC7716, or 0.1% DMSO negative control. Cell viability was measured using CellTiter-Glo™ after 48 h and 96h incubations. IC_{50} values were calculated using a four-parameter non-linear regression model in GraphPad. Cells were stained with Annexin V and propidium iodide (PI) to evaluate apoptosis versus necrosis signaling by flow cytometry after 48 h incubation of UNC7713. Flow cytometry experiments were performed on a Thermo Attune NxT, and data were analyzed using FlowJo. 5637 cells were grown in a monolayer, treated with UNC7713 (25 nM–100 nM), and then subjected to a wound healing assay to evaluate cell migration after 48 h. To evaluate effects on cell proliferation, 5637 cells were treated with four doses of UNC7713 (25 nM–100 nM), and colony formation was evaluated after 10 days using crystal violet and analyzed by Fiji. For wound healing, images were captured by an Olympus IX83 inverted microscope and analyzed by Fiji.

Results: UNC7713 achieved submicromolar potency for reduction of cell viability in 5637 cells and was nearly 200x more potent than UNC7716 at 48 h (IC_{50}: 0.28 μM vs. 56.03 μM). Potency was also maintained at 96 h (0.18 μM). Concentrations of UNC7713 as low as 75 nM caused over 2.5x greater apoptotic signaling than 20 μM UNC7716 after 48 h (18.5% vs. 7.3% Annexin V and PI positive cells). Next, cells treated with UNC7713 did not migrate to initiate wound closure, but instead caused cell death, increasing the size of the initial wound. Cells treated with UNC7713 at concentrations as low as 50 nM caused dramatic wound expansion compared to 0.1% DMSO and 20 μM UNC7716 (70% wound expansion for UNC7713 vs. 100% wound closure for both controls). Last, after 10 days >90% fewer colonies were detected in cells treated with UNC7713 (25 nM–100 nM) when compared to 20 μM UNC7716 and 0.1% DMSO controls.

Conclusions: These preliminary data support further inquiry into the role of *MPP8* in BC. Future studies will focus on identifying molecular mechanisms that underlie UNC7713's ability to inhibit cell proliferation, migration and viability in preclinical models of BC.