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TITLE: Discovery of a First-in-Class MPP8 Antagonist to Reverse Lineage Plasticity in Bladder Cancer

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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. UNC7713 achieved submicromolar potency for reduction of cell viability in 5637 cells and was nearly 200x more potent than negative control UNC7716 (IC50: 0.28 µM vs. 56.03 µM). UNC7713 as low as 75 nM caused over 2.5x greater apoptotic signaling than 20 µM UNC7716 after 48 h. 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.

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 histone 3 lysine 9 trimethyl (H3K9me3) post-translational modification, and has been shown to play a key role in the silencing of E-cadherin, a central modulator of EMT, metastatic spread and transition to metastatic bladder cancer. 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.

2. KEYWORDS:

1. Bladder Cancer
2. Covalent antagonist
3. Drug development
4. Epigenetics, MPP8
5. Methyl-lysine reader

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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	50%	Inducible shRNA development underway, but in the meantime we have worked with siRNAs as proof-of-concept.
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	0%	Work has not begun yet, but will begin in 2023.

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-9).	Months 1-18	40%	Cell motility assays going well; transwell assay invasion assays to begin in 2023. We also added colony-forming assays to evaluate the effects of MPP8 knockdown on cell proliferation, and Annexin V/propidium iodide assays to evaluate MPP8 knockdown on apoptosis. These experiments are ongoing, but have yielded promising results.

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 Clint and t1/2 have been completed In vitro experiments to characterize metabolism and transport to be completed in Year 2
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%, Cmax ≥50 ng/mL, t1/2 >2 h, CL <30 mL/min/kg, AUC >3000 ng*h/mL).	Months 6-24	xx%	We have obtained IACUC approval from UNC; awaiting approval from ACURO to initiate in vivo studies – see below in Section 5.

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 lineage plasticity and EMT.	Confirm MPP8 chemical antagonism effects gene and protein expression of known 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.	Months 6-36	25%	A significant amount of work has been performed using UNC7713 in 5637 cells; however, we have confounding data where EMT markers have not reduced when treated with UNC7713. We hypothesize we need to further optimize our experimental systems (e.g., treat with TGF-β to induce EMT, and/or use collagen-coated plates). Work will start in TCCSUP and UM-UC-9 cells during the next reporting period.
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-9)..	Months 6-36	30%	A majority of work has been completed using UNC7713 in 5637 cells; still need to conduct experiments in TCCSUP and UM-UC-9 cells during the next reporting period. Cell motility assays going well; transwell assay invasion assays to begin in 2023. We also added colony-forming assays to evaluate the effects of UNC7713 on cell proliferation, and Annexin V/propidium iodide assays to evaluate UNC7713 on apoptosis. These experiments are ongoing, but have yielded promising results.

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	10%	We have obtained IACUC approval from UNC; awaiting approval from ACURO to initiate in vivo studies – see below in Section 5.

What was accomplished under these goals?

During this reporting period, despite barriers identified in Section 5 of this report, major achievements were made in all 3 Aims as highlighted in the previous three tables. In Aims 1 and 3, we evaluated the role of MPP8 as an epigenomic regulator in preclinical models of advanced bladder cancer with the hypothesis that either knocking down MPP8 (Aim 1) or chemically antagonizing MPP8 with UNC7713 (Aim 3) would result in reduction in epithelial to mesenchymal transition (EMT). And, if we could prevent or reverse EMT, then we would see a reduction in proliferation, migration, and ultimately increased cell death.

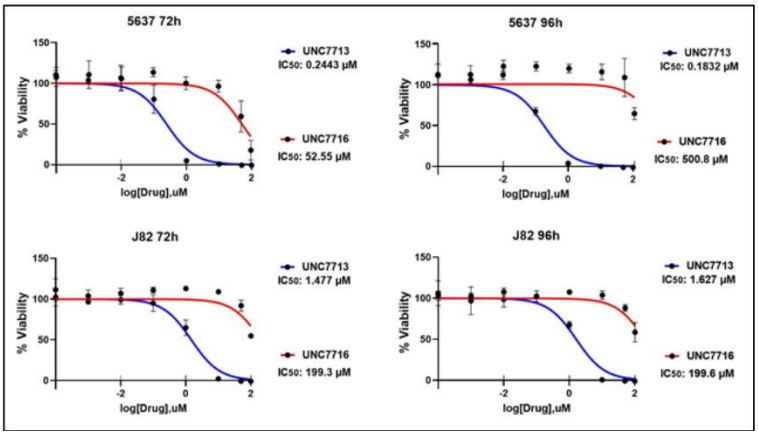

Figure 1. UNC7713 and UNC7716 dose response curves in 5637 and J82 cells at 72 and 96 hours.

The bladder cancer cell lines 5637 and J82 were treated with ascending doses of the covalent MPP8 antagonist UNC7713, incubated for 48-96 hours, and then evaluated for viability using Cell Titer Glo. 5637 and J82 cells were treated with ascending concentrations of UNC7713 (0.1 nM–100 µM), or negative control. Cell viability was measured using CellTiter-Glo™ after 72 h and 96h incubations. IC_{50} values were calculated using a four-parameter non-linear regression model using SAS JMP v15. 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) (**Figure 1**).

Next, 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. 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; **Figure 2A** and **Figure 2B**). We were also able to show that there was significantly more active apoptosis in UNC7713 treated cells than in 5637 cells treated with our 0.1% DMSO negative control (**Figure 2C**) and our 10% DMSO positive control (**Figure 2D**). Next, to evaluate effects on cell proliferation, colony forming assays were performed. 5637 cells were plated, incubated with four ascending concentrations of UNC7713 (25 nM–100 nM), UNC7716 20 µM, or 0.1% DMSO control for 48 hours. Then, drug 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 (25 nM–100 nM) when compared to 20 µM UNC7716 and

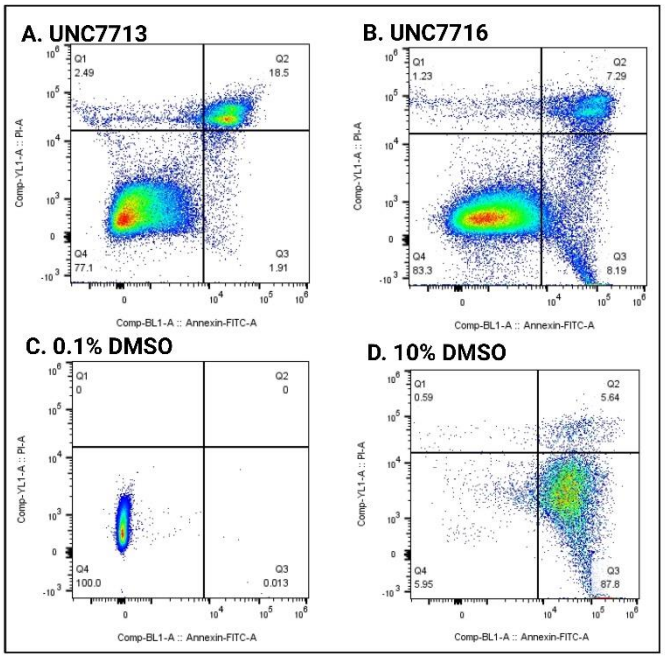

Figure 2. Annexin V/Propidium Iodide flow cytometry in 5637 cells to evaluate UNC7713-induced apoptosis.

0.1% DMSO controls (P<0.0001). Last, we conducted traditional wound healing assays (also known as "scratch" assays) to evaluate cell migration. Cells were placed in 12 well plates at and agitated to distribute cells throughout well. 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 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. 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).

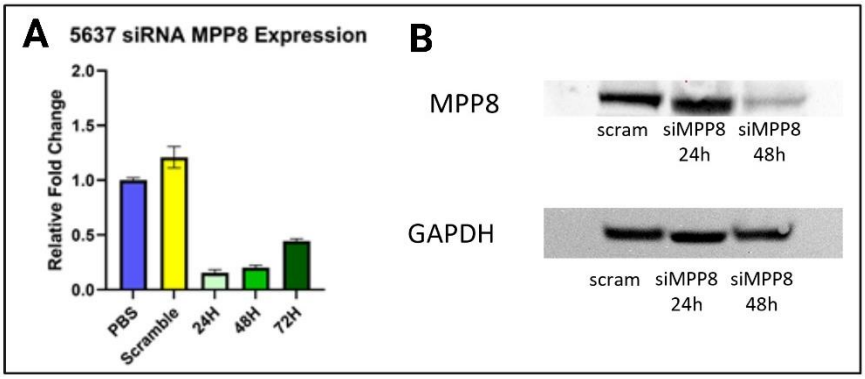

Figure 3. MPP8 gene **(A)** and protein expression **(B)** after transfection with MPP8 siRNA.

Using 5637 cells treated with MPP8 siRNAs, we conducted quantitative real-time PCR to evaluate the effects on gene expression of *MPP8* and EMT effectors. MPP8 siRNA significantly reduced *MPP8* expression when compared to cells treated with an empty vector or scrambled RNA after 24, 48 and 72 hours (all P<0.0001, n=3) (**Figure 3A**). We also confirmed MPP8 siRNA decreased MPP8 protein expression (**Figure 3B**). As a result, we expected that reduced *MPP8* gene and MPP8 protein expression would lead to increased *CDH1*/E-cadherin expression and decreased expression of EMT markers, such as *CDH2*/N-cadherin and *VIM*/vimentin. However, for unexplained reasons, we have continued to observe unexpected results for downstream targets of MPP8 inhibition. After performing additional RT-PCR on the same C4-2B samples, we noticed that despite increases in *CDH1* gene expression (**Figure 4A**), we did not observe discernible increases in E-cadherin protein expression (Figure 4B). Similarly, we expected to see decreased CDH2/N-Cadherin and VIM/vimentin gene and protein expression after MPP8 knockdown. However, quite perplexingly, we instead observed increased gene and protein expression of these two EMT markers (**Figure 4**). Consequently, we have identified optimization of our *in vitro* platforms as a goal for the next reporting period. We hypothesize that perhaps the prostate cancer cells need to be grown on plates coated with collagen (or other similar substance) in an attempt to replicate extracellular matrix. Another idea for platform optimization will be to stimulate the cells with TGF-β to initiate active EMT prior to any evaluations with MPP8 genomic knockdown or chemical antagonism with MPP8. In addition, in the coming reporting period, we will focus on evaluating how UNC7713 (and second-generation antagonists) affect MPP8 gene and protein expression, as well as expression of genes and proteins central to EMT processes.

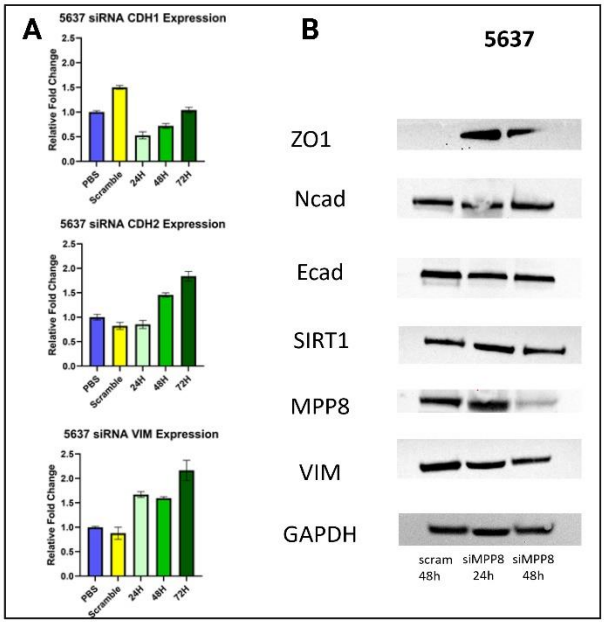

Figure 4. Gene **(A)** and protein expression **(B)** for EMT effectors after transfection with MPP8 siRNA.

In Aim 2, we conducted planned mouse liver microsome (MLM) studies on UNC7713 and active second-generation covalent MPP8 antagonist compounds identified by Dr. James and Mr. Buttery during a comprehensive medicinal chemistry campaign. The purpose of these MLM studies was to identify two physiochemical properties for UNC7713 and the second-generation compounds: Log P (a measure of lipophilicity) and intrinsic clearance (CL_{int}; an estimate of the intrinsic ability of the liver to metabolize and/or eliminate compounds in absence of restrictions imposed on drug delivery to the liver cell by blood flow or protein binding). **Table 1** details estimates of LogP and CL_{int} for UNC7713 and 17 active second-generation compounds.

In addition, we made *in silico* predictions about the clinical pharmacology of UNC7713, based on its molecular structure using GastroPlus Version 9.8.1003. We also confirmed previous in vivo PK estimates from

Table 1. MLM Results

Compound	LogP	CL_{int} (mL/min/kg)
UNC7713	2.65	181.0
UNC8360	3.45	124.0
UNC8362	3.07	279.0
UNC8364	3.44	225.13
UNC8411	3.44	318.30
UNC8451	3.15	267.07
UNC8453	2.93	139.25
UNC8454	4.38	122.08
UNC8507	3.72	498.22
UNC8596	4.38	327.40
UNC8604	4.35	971.95
UNC8607	3.95	181.0
UNC8650	4.35	272.40
UNC8702	3.82	438.66
UNC8707	4.23	926.7
UNC8710	4.61	1068.97
UNC8712	3.93	431.29
UNC8716	4.24	1129.47
UNC8739	2.72	0

mouse data collected prior to the Award using non-compartmental analysis (NCA) and Phoenix 64 Version 8.3.5.340. These confirmed NCA results and the MLM data allowed us input initial PK estimates into GastroPlus to generate initial physiologically based PK (PBPK) model fit estimates (e.g., observed vs. predicted curves for mouse administered 3 mg/kg IV dose of UNC7713. These initial modeling and simulation data will be crucial for the *in vivo* clinical pharmacology work included in this Award.

First, **Table 2** depicts the clinical pharmacology for UNC7713 based on its chemical structure. It is a lipophilic compound, but does not have a LogP >5 which gives us confidence that it will still be orally bioavailable. This is confirmed by estimates of 30-35% oral bioavailability in both mice and humans. It is not predicted to freely cross the blood brain barrier, which limits CNS penetration. UNC7713 is predicted to be a substrate of CYP3A4, but its metabolism not predicted to produce an active metabolite. Predictions show UNC is a substrate for organic anion transporters OATP1B1 and 1B3, but an inhibitor of organic cation transporters OCT1 and OCT2. Last, it is a predicted to be both a substrate and inhibitor of the Pgp efflux transporter.

Table 2. ***In-silico predictions (based on structure) using GastroPlus Version 9.8.1003***

	Property	**Predicted Value**			
Physiochemical properties	LogP	4.47 (at pH = 1); 2.65 (at physiologic pH)			
	Solubility	0.21 (mg/mL at pH= 9.82)			
	Permeability in humans	0.4 (cm/s x 10^4)			
	Permeability in mouse (rat-like)	0.1913 (cm/s x 10^4)			
	Likelihood of BBB Penetration	Low (74%)			
		70Kg human	21g mouse		
Predictions for a compartmental pharmacokinetics	Blood/Plasma Conc Ratio	0.7			
	Adjusted fraction unbound percentage:	6.82%	7.92%		
	Clearance (Cl)	41.14 L/h			
	Volume of distribution (V_d)	8.43 L/Kg			
	Half-life ($T_{1/2}$)	9.94 h	NR		
	First pass extraction % by liver	65.05%			
	Oral bioavailability (F)	32.43%	33.74%		
CYP450 enzymes metabolism predictions	**Enzyme**	**V_{max}**	**K_m**	**Metabolite**	
	CYP3A4 (Gut/Liver)	0.31 mg/s or 0.000862 mg/s/mg-enzyme	3.95 (mg/L)	None predicted	
Transporter Interaction predictions	**Inhibitor of**	**Not inhibitor of**	**Substrate of**	**Not a substrate of**	
	OCT1 (94%)	OATP1B1 (94%)	OATP1B1 (90%) (K_m =1.22 uM)	OCT1 (89%)	
	OCT2 (92%)	OATP1B3 (48%)	OATP1B3 (82%) (K_m =0.42 uM)	OCT2 (50%)	
	Pgp (64%)	OAT1 (88%)	Pgp (93%)	OAT1 (92%)	

	BSEP (47%) (IC_{50} =22.46 uM)	OAT3 (94%)	BCRP (95%);	OAT3 (96%)
		BCRP (47%)		

Table 3 provides updated PK parameter estimates from in vivo mouse work conducted **prior** to this Award. These data confirm UNC7713 has properties that will allow us to develop an initial PBPK using GastroPlus, and give us confidence to conduct the next-step *in vivo* clinical pharmacology experiments detailed in Aim 2 during the next reporting period.

Table 3. Non-compartmental analysis (NCA) using Phoenix 64 Version 8.3.5.340

IV Dose (mg/Kg)	**PK Obs**		**C_0 (ng/mL)**	**$T_{1/2}$ (hr)**	**AUC_{last} (hr*ng/mL)**	**$AUC_{INF\ pred}$ (hr*ng/mL)**	**$AUC_{\%Extrap\ pred}$ (%)**	**Cl_{pred} (mL/min/kg)**	**$V_{ss\ pred}$ (L/kg)**	
3	6		2703.39	1.76	453.85	456.80	0.65	109.46	3.14	
IP Dose (mg/Kg)	**PK Obs**	**T_{max} (hr)**	**C_{max} (ng/mL)**	**$T_{1/2}$ (hr)**	**AUC_{last} (hr*ng/mL)**	**$AUC_{INF\ pred}$ (hr*ng/mL)**	**$AUC_{\%Extrap\ pred}$ (%)**	**Cl/F_{pred} (mL/min/kg)**	**Vz/F_{pred} (L/kg)**	**F (%)**
10	6	0.05	1365.11	1.06	607.66	609.91	0.37	273.26	24.97	40%

Using these NCA parameters estimates from the IV dose and the IP dose to calculate the bioavailability of the IP dose: F = (AUC_{IP}/ AUC_{IV}) x ($Dose_{IV}$/$Dose_{IP}$) = (609.91 hr*ng/mL)/(456.80 hr*ng/mL)* (3 mg/Kg)/ (10 mg/Kg) = **0.40**. Therefore, bioavailability of the IP dose = **40%**. Using F, we calculated hepatic extraction ratio (ER): Extraction ratio (ER) = 1 - F = 1 - 0.4 = 0.6 Therefore, percentage of the drug extracted by the liver on first pass metabolism = 60%. Then, we used ER to calculate hepatic clearance (CL_H): ER = CL_H / Hepatic blood flow (Q), where hepatic blood flow for mice is about 90 mL/min/Kg. This yielded a Q of approximately 1.89 mL/min for a 0.021 Kg mouse, which led to an estimate of CL_H: Cl_H = ER x Q = 0.6 x 1.9 mL/min = 1.14 mL/min = 0.068 L/hr. Without previously described MLM analyses, we could have also used hepatic clearance to calculate CL_{int}. However, we used the MLM CL_{int} as we developed our initial PBPK model in GastroPlus.

Also, from our preliminary NCA data, CL_{Total} = 0.138 L/hr, which is a combination of clearance mechanisms for UNC7713, Cl_H), renal clearance (CL_R), and other clearance mechanisms (CL_{Other}): Cl_R + Cl_{Other} = 0.138 L/hr - 0.068 L/hr = 0.07 L/hr. Maximum Cl_R per GastroPlus physiology for a 21g mouse PBPK was 0.047 L/hr, thus leaving 0.023 L/hr attributed to CL_{Other}. Since the clearance of other organs is unknown, an attempt to assign the rest of clearance for "rest of the body" clearance was made. However, a maximum of 0.011 was allowed per physiology. Thus, total systemic clearance in the model used was 0.126 L/hr. Thus, the initial input into GastroPlus PBPK mode included: LogP = 2.65, CL_H = 0.068 L/hr, CL_R = 0.047 L/hr, and CL was capped at . 0.11 L/hr. Based on these inputs, initial PBPK model predictions were generated for a 21 kg mouse (**Table 4**).

Table 4. Predictions after the above was incorporated in a minimal PBPK 21g mouse model

	Property	**Predicted Value**
Predictions for a 21g mouse	Blood/Plasma Conc Ratio:	0.7
	Adjusted fraction unbound percentage:	8.84%
	First pass extraction % by liver	65.81%
	Oral bioavailability (F)	7.28%

Figure 5 depicts PBPK model observed versus predicted values for a 21 g mouse administered a single 3 mg/kg IV dose of UNC7713. From this analysis, the simulation included a total time of 24 hours, and generated a C_{max} discernibly higher than the NCA (9.5 x 10^4 ng/mL (compared to 2703.4 ng/mL from NCA IV). However, the PBPK $AUC_{0\text{-}inf}$ was similar to the NCA (501.78 versus 456.80 hr*ng/mL). Our initial model also predicted a hepatic C_{max} of 315.9 ng/mL.

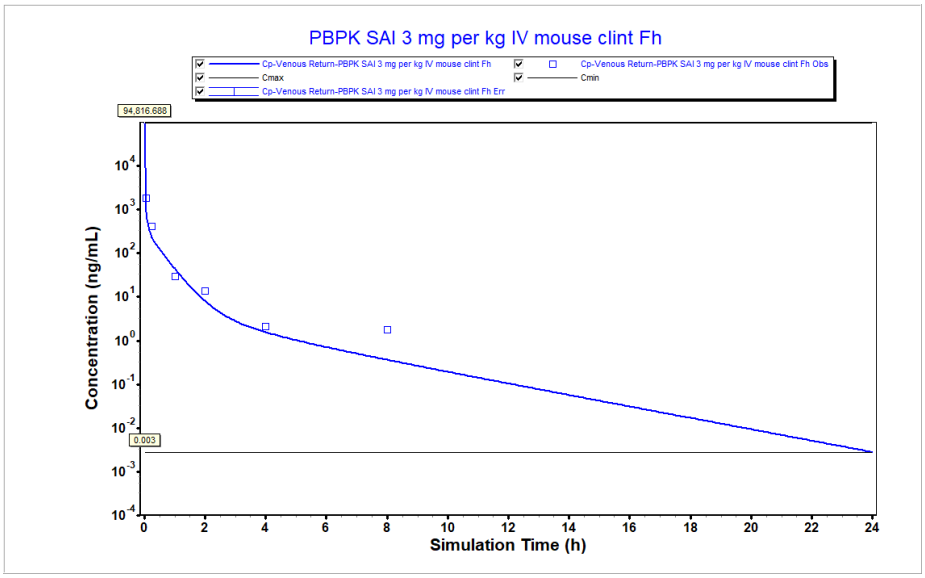

Figure 5. Initial PBPK Observed vs Predicted Concentrations.

Overall, there were goals that were not met during this reporting period. First, we continue to work getting on inducible shRNA system optimized. While there have not been technical barriers, the process of getting the system stably transfected has been lengthier than anticipated. We 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 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.

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 2; will also audit PHCO 750: Proteomics Methods and Applications in Spring 2023.
Attend short-courses and seminars on research ethics and responsible conduct of research from NC TraCS Institute	Months 1-15	Yes for Year 1	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	Ongoing	Present regularly at Kim Lab meeting; presented once in December 2021 to UNC Chromatin and Epigenetics Program 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 submitted to 2023 AACR Annual Meeting (Appendix 1).

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

1. Assuming our mouse protocol is approved by ACURO, we will initiate mouse studies in support of work proposed in Aim 3.
2. We will finalize optimization of our ITPG-inducible shRNA system so that we are able to continue to understand MPP8s's role as an epigenetic regulator. This will include ensuring that our system is stably transfected so that our planned experiments are not time-constrained like siRNA experiments.
3. We will continue to optimize our experimental platforms so that we are assured we have the correct experimental conditions to evaluate MPP8's role in EMT in preclinical models of advanced prostate cancer.
4. We will continue to optimize the drug-like characteristics of analogues to UNC7713 based on *in vivo* PK, as well as *in vitro* pharmacology experiments detailed in Aim 2.

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

Currently, we have not made any major changes to the approach. Despite having some small issues experimentally with confounding data to gene and protein expression of primary targets of MPP8 (e.g., *CDH1*/E-cadherin and *CDH2*/N-cadherin), we have made only small changes to the experiments to enhance cell culture conditions. We are moving along well with ongoing experiments and look forward to the coming year for increased productivity.

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

There were **two** main delays caused by barriers during the report period:

1. First, my application to ACURO was delayed by staffing issues at UNC. Most importantly, my inability to hire a post-doctoral fellow or research technician led to delays in some aspects of project. This included a delay in starting to author a mouse protocol to our internal UNC IACUC. In addition, staffing issues in our UNC Division of Laboratory Animal Medicine (DLAM) caused delays because there were fewer opportunities to work with their personnel during the protocol development period, as well as fewer times that IACUC personnel met to review and approve protocols. However, this delay should not negatively affect successful completion of the *in*

vivo activities proposed in Aim 3/Major Task 9 because we have established a new working relationship with DLAM personnel who have assured us that we can easily get back on the proposed timeline in the next reporting period.

Problem resolution: None - we have received UNC IACUC approval for our protocol, and are awaiting feedback from ACURO.

2. Second, my current annual report has been submitted woefully late because of COVID-related health issues that I experienced over the past 6 months. As a brief synopsis:

A. I contracted COVID-19 in July 2022 when traveling to NYC to help my sister recuperate from her ankle reconstruction surgery.
B. While in NYC, I experienced what likely falls into the "moderate" COVID symptom category (e.g., fever, night sweats, etc.), and I was prescribed paxlovid. After symptoms subsided, and after an appropriate number of days, I returned to North Carolina.
C. Shortly after my return, I started noticing symptoms classically associated with "long-hauler" COVID. These included extreme and worsening fatigue, memory issues, word-finding issues, and some dizziness if not well hydrated. Later, I also noticed symptoms not necessarily associated with COVID-19, including polyuria, polydipsia, and profound weight loss (approx. 30-35 pounds). All of these severely hampered my work productivity as I just tried to keep my head above water.
D. In an attempt to rule out diabetes, I finally checked my blood glucose on a home glucometer and surprisingly found my fasting blood glucose (BG) to be >400. I presented to the UNC emergency department the next day and was found to have a fasting BG of 474 and an A1c of 14.1 (keep in mind I had no history of diabetes or pre-diabetes before COVID).
E. I was able to see a UNC provider that week and was initiated on insulin and metformin. The good news is it helped get my BG from 474 down to 150s almost overnight and most all of the symptoms I attributed to COVID disappeared.
F. Unfortunately, that precipitous decline in BGs has left me with severe blurred vision for the past 2+ weeks that has made even routine work tasks almost impossible without help. An UNC ophthalmology appointment revealed no structural damage to my eyes (i.e., no retinopathy or macular degeneration), which is great. The ophthalmologist told me, however, that the rapid BG drop caused a change in osmotic pressure in my eyes that changed the shape of the lenses. The blurred vision that accompanies the change in lens shape is not permanent, but can take weeks or even a month to correct.

Problem resolution: None – I am now receiving care for a Type 2 diabetes diagnosis. The medications are working well, and all my symptoms have subsided. Now that my vision has returned, I was able to finally complete this report. Please be assured that this type of delay will not occur in the future annual reports or the final report.

Changes that had a significant impact on expenditures

Only one significant factor affected expenditures during this reporting period. I was unable to hire a post-doctoral fellow or research technician. As everyone in academia is experiencing, shallow applicant pools for post-doctoral fellows and technicians is complicating research progress. After receiving this award in September 2021, I placed an advertisement in October 2021, spent 4 months recruiting and ended up not being able to hire my preferred candidate because of J-1 visa issues. Similarly, after three separate advertisements, I was unable to hire a research technician with suitable skills for this work. Fortunately, my division recruited a graduate student with significant molecular biology background after having worked at Caris. She started in my group on August 1, 2022 and has since taken over the all aspects of the work in Aims 1 and 3, and has participated in modeling detailed in Aim 2.

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

No changes, significant or otherwise have been made to the vertebrate animal plan because we have not yet started the mouse work (waiting on ACURO approval presently). In addition, we have not made changes to the selected agents, and will continue to use UNC7713 as our lead compound with UNC7716 as the negative control. None of the research in this Award is human subject research.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

None – we have not yet begun vertebrate animal work (awaiting ACURO approval).

Significant changes in use of biohazards and/or select agents

None – we will continue to use UNC7713 as our lead compound with UNC7716 as the negative control.

6. PRODUCTS:

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

  Nothing to Report

  Journal publications.

  Nothing to Report

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

  Nothing to Report

  Other publications, conference papers and presentations.

  Abstract submitted in November 2022 to AACR Annual Meeting (**Appendix 1**)

- **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:	Mirmar Kardouh
Project Role:	Post-doctoral Fellow
ORCID iD:	0000-0002-6718-5623
Nearest Person Month Worked:	2
Contribution to Project:	Modeling and simulation activities described in Aim 2
Funding Support:	• Internal UNC Eshelman School of Pharmacy funding; Cetara • Dr. Kardouh is **not** funded by this award

3.

Name:	Stephany Gonzalez Tineo
Project Role:	Graduate Student
ORCID iD:	0000-0002-6229-3481
Nearest Person Month Worked:	5
Contribution to Project:	Activities described in Aims 1 and 3, with emphasis on early PCR and Western blotting, as well as phenotypic assays (e.g., colony forming proliferation assays, scratch assays, and cell death assays). Assisting Dr. Kardouh with modeling and simulation in Aim 2
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; 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:	1.5
Contribution to Project:	Select activities in Aims 1 and 3 in support of Ms. Gonzalez Tineo. Specifically, Mr. Kemper continues to work 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. Abstract submitted in November 2022 to the AACR Annual Meeting

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:
1 Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA
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4 Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA
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
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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.