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TITLE: Discovery of a First-In-Class MPP8 Antagonist to Reverse Lineage Plasticity in Treatment-Resistant Prostate Cancer

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14. ABSTRACT
The overarching goal of this proposal is to better understand the role of MPP8 in lineage plasticity and EMT in mCRPC and NEPC, discover a potent first-in-class antagonist of MPP8, and evaluate MPP8 chemical antagonism as a therapeutic strategy for EMT reversal. During this reporting period, we showed that MPP8 knockdown by siRNAs inhibits proliferation and cell motility, and cell invasion. We also showed that MPP8 knockdown reduces SIRT1 and vimentin protein (but does not increase E-cadherin). Using UNC7713 and second-generation compound UNC8850, we completed labeling studies and showed UNC8850 has 3.5-fold increased labeling compared to UNC7713. We performed whole-genome proteomic analysis of cells treated with UNC7713 and confirmed MPP8 is a target of UNC7713 while identifying 15 additional novel targets related to MPP8 antagonism. We confirmed that UNC7713 potently inhibits cell proliferation and clonogenic potential, inhibits cell migration and invasion, and causes apoptosis. We also discovered that UNC7713 is lethal to mice upon repeat dosing, but when formulated within liposomes is not immediately lethal to mice.

15. SUBJECT TERMS
Covalent antagonist, drug development, epigenetics, MPP8, neuroendocrine, prostate cancer

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1. INTRODUCTION

In the U.S., prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related death among men. Secondary resistance to treatments occurs frequently in metastatic castration resistant prostate cancer (mCRPC). Acquisition of alternative lineage programs, or lineage plasticity, can occur in approximately 20% of treatment-resistant mCRPC patients where the luminal epithelial identity of cells is reprogrammed so that the cells assume a non-luminal basal or mesenchymal identity, exhibit increased markers of small cell neuroendocrine prostate cancer (NEPC), and are treatment-refractory. This complex, multi- step process is known as epithelial-to-mesenchymal transition (EMT). Importantly, NEPC is a particularly lethal phenotype of prostate cancer, there are poor therapeutic options for NEPC, and clinical trials have not adequately addressed lineage plasticity or NEPC. Thus, there is an unmet clinical need to develop novel therapeutics that reverse lineage plasticity in patients before progression to NEPC. 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 from treatment-resistant mCRPC to NEPC. 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 mCRPC and NEPC, discover a potent first-in-class antagonist of MPP8, and evaluate MPP8 chemical antagonism as a therapeutic strategy for EMT reversal.

2. KEYWORDS:

1. Covalent antagonist
2. Drug development
3. Epigenetics, MPP8
4. Methyl-lysine reader
5. Neuroendocrine
6. Prostate cancer

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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

Major Task	Milestone	Proposed Timeline	% Completed	Notes
Evaluate MPP8 protein levels in prostate cancer TMA samples available through the PCBN.	Confirm that MPP8 protein expression increases with disease severity.	Months 1-9	10%	Antibodies and other reagents validated. But, PCBN lost funding. Still determining if collaboration with PI who took over the repository is possible.
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 inducible shRNA systems for MPP8 in LNCaP, PC3 and C4-2B cells. We also have inducible and non-inducible versions with an mCherry reporter

				included. We plan to incorporate into our enzalutamide and abiraterone-resistant lines, as well as into NCI-H660 lines in the coming year.
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	75%	We have not completed the H4K16ac and H3K9me2 and me3 work, but those will happen in the coming weeks. Recently, we completed co-IP work showing the relationships between SIRT1, MPP8, TASOR and Zeb 1/Zeb 2 (but not TWIST1/2). We have not begun to evaluate MPP8-DNMT3a and SETDB1 in the coming year.
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 mCRPC and NEPC three cell lines.	Months 1-18	75%	All proposed experiments have been completed in the LNCaP, PC3 and C4-2B lines using siRNAs. They all show that loss of MPP8 leads to decreased motility and invasion. In the coming year, we will confirm using our shRNA systems, and in our enzalutamide and abiraterone-resistant lines

Aim 2. Design and synthesize potent MPP8 antagonists.

Major Task	**Milestone**	**Proposed Timeline**	**% Completed**	**Notes**
Using UNC7713 as a starting point, employ iterative rounds of medicinal chemistry and compound screening.	Identify analogues to UNC7713 with optimize MPP8 labeling and minimize off-target labeling; confirm by DSF; confirm covalent adduct binding by mass spectrometry.	Months 1-15	75%	A total of 41 active second-generation MPP8 antagonists identified; 79 inactive compounds identified. We generated data that demonstrated second-gen compounds were better at labeling MPP8 than the original UNC7713 compound. For example, UNC8850 was 3.5-fold more

				efficient at labeling MPP8.
Evaluate how covalent ligands to engage and react with endogenous MPP8 in cells and selectively label MPP8 within the proteome.	Achieve a submicromolar cellular TE_{50} for UNC7713 or the optimized compounds. Then confirm selective labeling of MPP8 within the proteome due to the unique mode of reversible binding.	Months 6-24	50%	In HeLa cells treated for 24 hours, UNC7713 TE50 was confirmed to be 180 nM, and TE90 was 8 μM

Aim 3. Evaluate MPP8 antagonist effects on lineage plasticity and EMT-induced chemoresistance in prostate 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	60%	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 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	Months 6-36	80%	A majority of work has been completed using UNC7713 in LNCaP, PC3 and C4-2B cell. Experiments in NCI H-660 cell and our enza-resistant and abi-resistant LNCaP and C4-2B cells are ongoing.

	invasion among the three mCRPC/NEPC cell lines.			
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.

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 prostate 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 prostate cancer, such as reduction in proliferation and migration as well as an increase in cell death. In Aim 2, we proposed to build upon UNC7713 with a second-generation of MPP8 covalent antagonists.

In Aim 1, while we were developing our inducible shRNA models to be used during the upcoming year, we used with siRNA knockdowns in C4-2B, LNCaP and PC3 cells for proof-of-concept experiments. siRNAs against both MPP8 and SIRT1 to evaluate how loss of either of these key proteins impacts expression of EMT markers, and also impacts cell health phenotypes. When C4-2B cells were transfected with the siMPP8, we observed significant knockdown of MPP8 (as expected), but also SIRT1, and the mesenchymal marker vimentin at 48 h and 72 h (**Figure 1**). However, we did not observe increased E-cadherin expression. Then, when we transfected the C4-2B cells with siSIRT1, we observed the expected significant knockdown of SIRT1, but no knockdown of MPP8. In addition, while we still did not observe increased expression of E-cadherin, we did not observe vimentin loss secondary to SIRT1 knockdown like we did with MPP8 knockdown. Next, we used the siMPP8 in in all three cells lines, and demonstrated that a 48 h and 72 h siRNA transfection

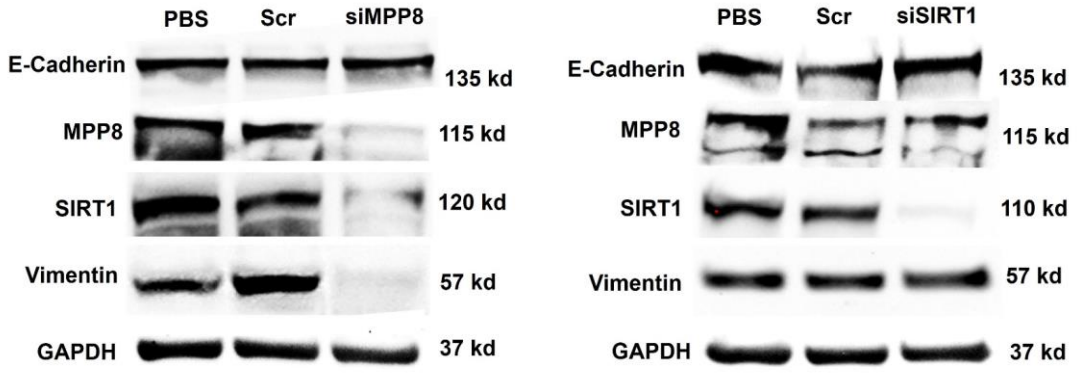


Figure 1. Effects of MPP8 and SIRT1 knockdown on EMT markers. C4-2B cells were transfected with siRNAs against MPP8 (left panel) and SIRT1 (right panel). Expression was compared against cells treated only with PBS and against a scrambled siRNA. Imaging took place 72 h after siRNA transfection, and GAPDH was used as a loading control.

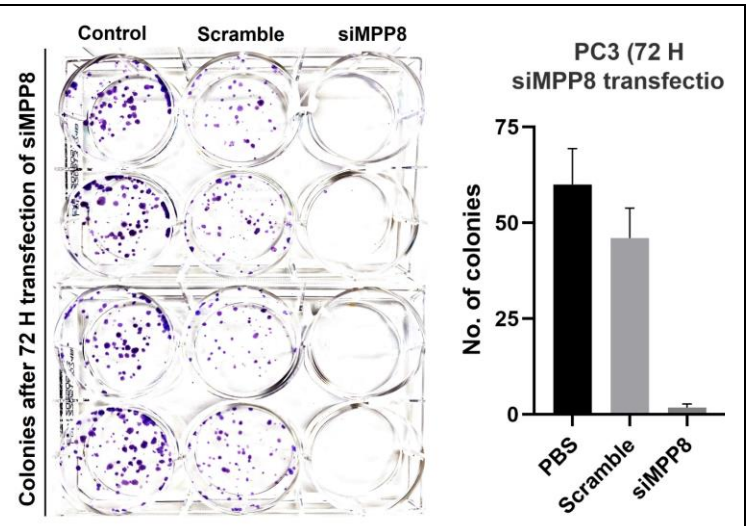


Figure 2. Clonogenic potential of PC3 cells transfected with siRNA against MPP8.

resulted in significantly reduced clonogenic potential when compared to PBS-treated cells and cells transfected with scrambled siRNA (**Figure 2**).

Next, we performed a scratch assay with cells transfected with siMPP8 and siSIRT1 to evaluate to what extent knockdown of these two genes resulted in reduced migration as a surrogate for metastatic potential (**Figure 3**). Here, we were able to show that knockdown of MPP8 and SIRT1 curtailed the ability of cells to fill in the original scratch, when compared to PBS-treated cells and cells transfected with scrambled siRNA, which indicated that knockdown impeded cellular mobility and metastatic potential. To confirm these results, we performed an invasion assay in C4-2B cells with siMPP8 and siSIRT1 (experiments in LNCaP and PC3 cells are ongoing). These data suggest that MPP8 and SIRT1 knockdown may reduce the ability of cells to invade as another surrogate for metastatic potential (**Figure 4**). However, at present these data are a little less convincing than the scratch assay data and need to be repeated.

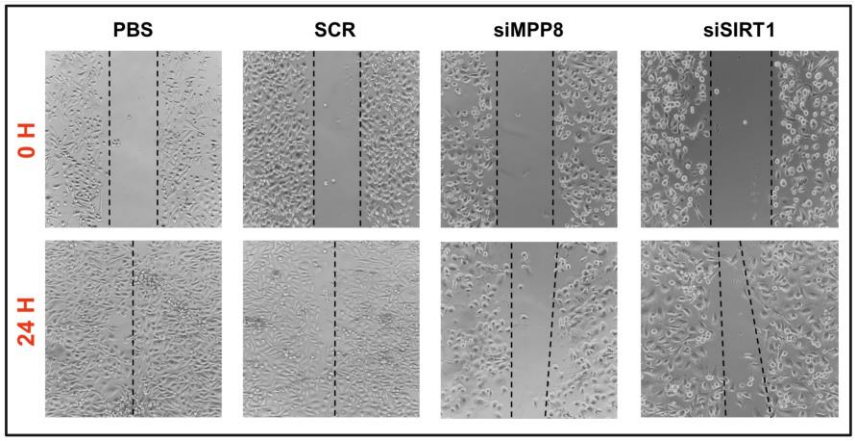


Figure 3. Cell migration in cells with siMPP8 and siSIRT1. C4-2B cells were transfected with siRNAs and then a standard scratch assay was performed.

In Aim 2, we performed additional *in vitro* profiling of second-generation MPP8 covalent antagonists. Notably, we had previously identified UNC8850 as one of our most promising second-generation compounds based on preliminary MPP8 labeling studies via mass spectrometry, and increased cell death in HeLa cells. During this reporting period, we performed full kinetic analyses of our lead second-generation candidates, including UNC8850. First, recombinant MPP8 is incubated with various concentrations of a given inhibitor over time. Because time ranges are highly compound dependent, on the scale of minutes, multiple timepoints are required. Then, observed covalent labeling is measured by mass spectrometry. During this reporting period, we confirmed that UNC8850 was approximately 3.5-fold more efficient at labeling MPP8 than UNC7713 with a k_{inac}/K_I ratio of 3200 ± 700 M^{-1} s^{-1} versus 960 ± 50 M^{-1} s^{-1} (**Figure 5**). This confirms the preliminary data from the previous reporting period, which revealed UNC8850 to have approximately 91% labeling of MPP8 chromodomains.

Additionally, we overcame a major barrier during this reporting period and recently experienced success in profiling the proteome-wide selectivity of UNC7713. Working with the UNC Michael Hooker Proteomics Core and Dr. Laura Herring (https://www.med.unc.edu/proteomics/), we performed a genome-wide proteomics screen in an attempt to identify targets of UNC7713 and proteins associated with MPP8. Cells were treated

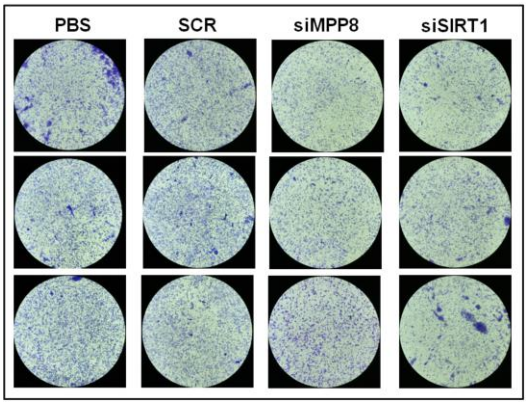


Figure 4. Cell invasion in cells with siMPP8 and siSIRT1. C4-2B cells were transfected with siRNAs and then a standard invasion assay was performed.

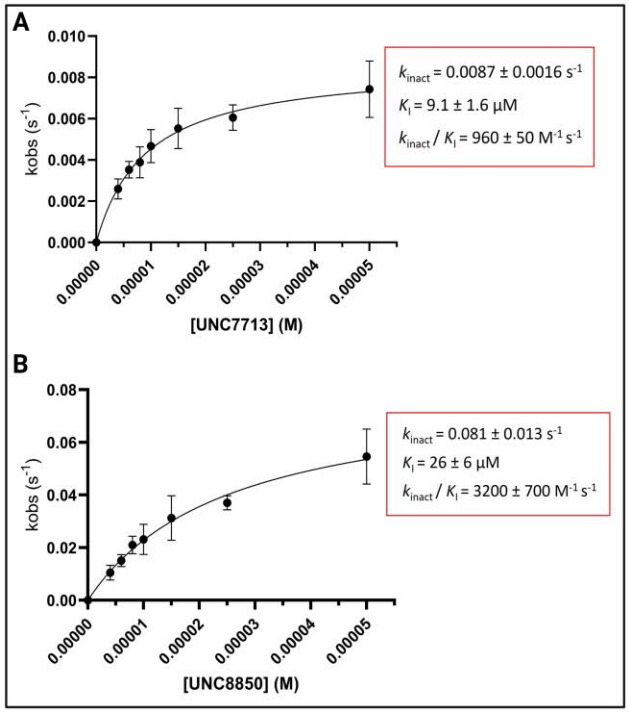


Figure 5. Results from labeling experiments of UNC7713 (top panel) versus UNC8850.

for 4 h with 1 µM UNC7713 or 0.1% DMSO. We selected a short incubation timepoint for this initial experiment in an attempt to limit any protein abundance changes due to MPP8 antagonism has on the transcriptome. We were able to identify 16 proteins that were targets of UNC7713, including MPP8 (**Figure 6** and **Table 1**). Interestingly, the two proteins that appear to be the top targets of UNC7713 in cells are SELENOH and TXNRD1, which are two antioxidant selenoproteins that contain catalytic selenocysteine residues. TXNRD1 has been previously targeted by covalent inhibitors to inhibit antioxidant activity in cancer cells. In addition, STAT2 and TRIM13 have been investigated for their role in prostate cancer pathogenesis, and CTNND1 could be a regulator of EMT that is affected by MPP8 loss. Each of these hits represent potential therapeutic targets. Because these data are so new, we have not had the chance to confirm these proteomics results with an orthogonal assay yet. However, we plan to perform follow-up experiments on these exciting results during the next reporting period, and importantly, these data show MPP8 is a target of UNC7713.

Table. 1. Proteomics results from cells treated with UNC7713.

Target	Coverage [%]	# Peptides	# Unique Peptides	Total Abundance	MW [kDa]	Log2 fold change	p-value
SELENOH	65	12	12	57	13.4	-6.10	0.00019774
ACOT8	18	3	3	21	35.9	-3.19	0.00944803
TXNRD1	63	29	29	224	70.9	-2.88	0.000027516
EHD4	15	9	5	24	61.1	-2.21	0.00100029
STAT2	2	2	2	2	97.9	-2.18	0.00332169
FEN1	31	11	11	55	42.6	-2.11	0.00195554
RTCA	15	5	5	8	39.3	-2.07	0.00348458
CIP2A	29	25	25	108	102.1	-2.04	0.00709446
TXN	57	7	7	82	11.7	-1.90	0.00179026
TBRG4	30	14	14	46	70.7	-1.76	0.00536271
UNC13D	33	25	25	110	123.2	-1.74	0.00555542
TRIP13	29	10	10	40	48.5	-1.65	0.00860319
CTNND1	20	15	15	76	108.1	-1.63	0.00767429
UGGT2	6	9	6	34	174.6	-1.62	0.0038718
DNAAF5	38	24	24	77	93.5	-1.61	0.00751992
MPHOSPH8	26	16	16	74	97.1	-1.56	0.00641278

In Aim 3, we continued experiments with UNC7713 (and the negative control compound UNC7716). Building on the data from the previous reporting period, and because we continue to generate confounding results about MPP8's link to EMT in advanced prostate cancer, we focused on evaluating how UNC7713 impacts pertinent cancer phenotypes (e.g., proliferation, migration, invasion, etc.) in C4-2B, LNCaP and PC-3 cells. Similar to the work described in Aim 1, we performed colony forming assays to evaluate how MPP8 antagonism decreases clonogenic potential. In all three cell lines, we were able to show a dose-dependent decrease in colony

formation after 48 h incubations with UNC7713, but not in cells treated with PBS or UNC7716 (**Figure 7**). For C4-2B and LNCaP cells, UNC7713 inhibited colony formation at concentrations ranging from 25 nM to 150 nM, while concentrations ranging from 100 nM to 400 nM were sufficient to inhibit colony formation in PC-3 cells. Next, we performed scratch assays similar to those described above. In C4-2B cells, we showed that 50 nM and 100 nM of UNC7713 were able to maintain a significantly greater proportion of the original scratch area at 24 h and 48 h when compared to 0.1% DMSO and 5 µM UNC7716 controls (**Figure 8**). Similarly, in PC-3 cells we found that 400 nM and 500 nM of UNC7713 were able to maintain a significantly greater proportion of the original scratch area at 24 h and 48 h when compared to 0.1% DMSO and 5 µM UNC7716 controls.

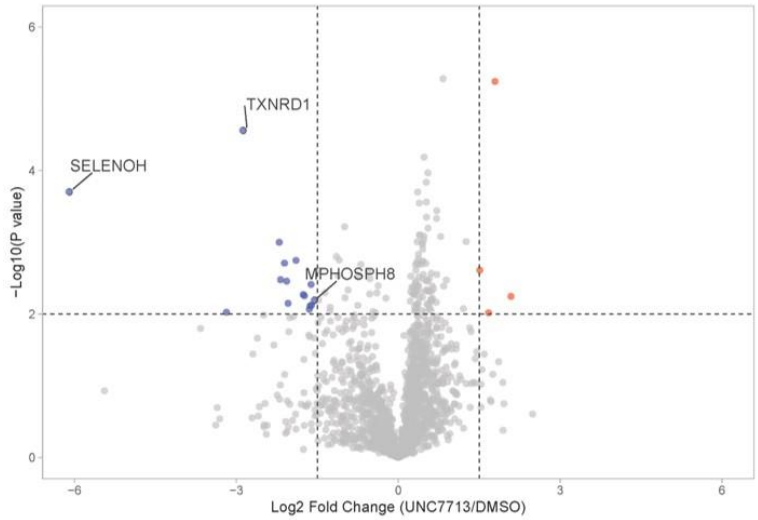


Figure 6. Volcano plot showing top hits from proteomics screen.

Finally, cells were treated with UNC7713 four ascending doses of UNC7713, or 0.1% DMSO and 5 µM UNC7716 controls and incubated for 48 and 72 hours. Then, they were stained with acridine orange to detect viable cells and ethidium bromide to evaluate whether cells were going through apoptosis. We showed, at concentration ranges identical to those that inhibited colony formation, that UNC7713 causes apoptosis (**Figure 9; see Appendix 1 below in Section 9**). In the upcoming reporting period, we plan to confirm these results using an Annexin V/propidium iodide assay and flow cytometry so that we can quantify to what extent UNC7713 causes early apoptosis versus late apoptosis and necrosis at a given concentration.

Overall, there were goals that were not met during this reporting period, and these are explained below in Section 5. But briefly, we still have not been able to secure TMA from PCBN (Aim 1). During this reporting period, we successfully established our collaboration with the UNC Pathology Services Core (https://unclineberger.org/pathologyservices/), primarily with PI Dr. Sarah Wobker and core director Gabriela de la Cruz. Per PCBN guideline, we validated all of the antibodies and other reagents we proposed to use on existing TMA samples from UNC (e.g., MPP8 antibody, E-cadherin antibody, etc.) (**Figure 10; see Appendix 2 below in Section 9**). However, when it was time to submit our application to obtain TMA samples form PCBN, we were informed that PCBN had lost funding. but I have been put in contact with the PI who took over the PCBN samples. In the coming months, I will try to secure TMAs from the PI and perform the proposed work. 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

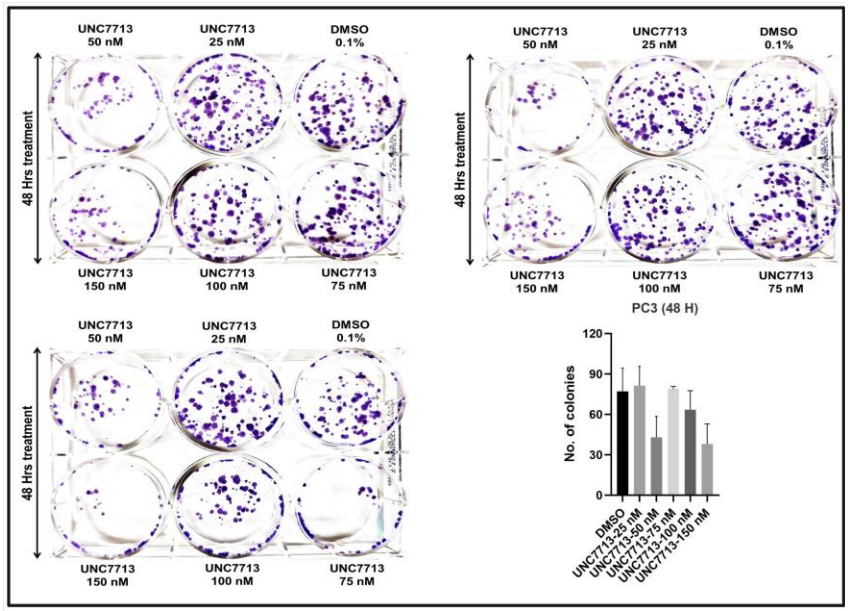
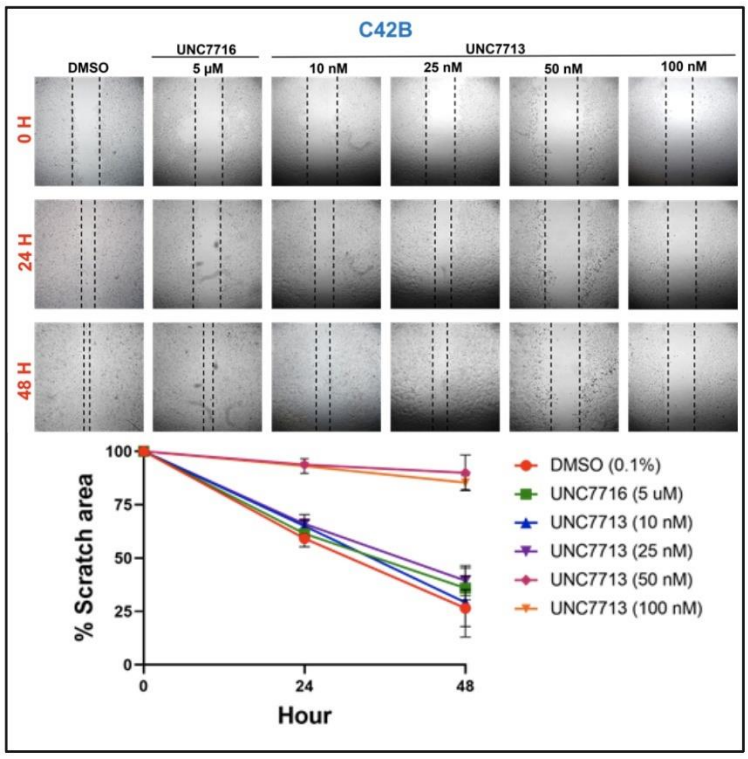


Figure 7. Clonogenic assay results. PC3 cells were treared with 4 ascending concentrations of UNC7713, 5 µM UNC7716 or 0.1% DMSO control for 48 h. After 10 days, cells were stained with crystal violet. Quantification of number of colonies was performed by Fiji for Image J.



centered on formulated UNC7713. Third, an ongoing issue is related to our ability to conclusively establish the connection between MPP8 and EMT in models of advanced prostate cancer. While we have started to amass some really encouraging phenotypic data, we still continue to generate 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. As proposed in the previous report, we evaluated ways to attempt to replicate the extracellular matrix (e.g., using collagen-coated plates), but were unsuccessful. We continue to optimize conditions where TGF-β activates EMT, but these experiments are ongoing. Last, once we determined that the enzalutamide-resistant LNCaP and C4-2B lines we were given from a collaborator at UC Davis were likely not resistant up to 20 μM (as described in the previous report), we initiated creating our own enzalutamide lines. We also took the opportunity to establish more drug-resistant model systems, and began creating abiraterone-resistant LNCaP and C4-2B lines. While we have not yet used them experimentally, we have titrated doses of enzalutamide to 18 μM in both lines (target is 20 μM) and abiraterone to 4 μM (target is 5 μM). Thus, in the coming report period, we will have the opportunity to generate data about MPP8 knockdown and chemical antagonism in both drug-resistant systems.

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

Nothing to Report

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?

Nothing to Report

4. IMPACT:

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

Nothing to report

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 prostate 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 had **two** problems that have led to ongoing delays:

1. Problem: For one of our major tasks in Aim 1, we still have not been able to secure TMA from PCBN. As previously mentioned, we successfully collaborated with the UNC Pathology Service Core (https://unclineberger.org/pathologyservices/), primarily with PI Dr. Sarah Wobker and core director Gabriela de la Cruz. At the point where we had validated all of the antibodies and other reagents we proposed to use on existing TMA samples from UNC (e.g., MPP8 antibody, E-cadherin antibody, etc.), we were informed that PCBN had lost funding.

Problem Resolution: There has not been resolution yet, but I have been put in contact with the PI who took over the PCBN samples. In the coming months, I will try to secure TMAs from the PI and perform the proposed work. Should that tactic not prevail, I will attempt to procure prostate cancer TMAs from those banked at UNC as part of our internal LCCC 1212 protocol, which has allowed us to collect and process biospecimens from patients with genitourinary malignancy patients. I am a co-investigator on LCCC 1212, so I should be able to secure access to available TMA; however, our internal repository is not nearly as robust as the former PCBN (e.g., we do not have TMAs from NEPC patients), so the priority will be to work with the PIs at Johns Hopkins in an attempt to get specimens from those previously collected under the PCBN.

2. 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

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

None – we will continue to use UNC7713 as our lead compound with UNC7716 and UNC7715 as the negative control compounds. We will continue to use enzalutamide and abiraterone in development of our resistant LNCaP and C4-2B cell lines, and enzalutamide is being used in our mouse studies for one the control arms (standard of care).

6. PRODUCTS:

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

  Two publications in process, but none submitted yet (we hope both will go out for review before the end of 2023). One will focus on interactions between MPP8, SIRT1 and key transcription factors. The second will focus on UNC7713. We will also be submitting an abstract to AACR on UNC7713.

  Journal publications.

  Nothing to Report

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

  Nothing to Report

  Other publications, conference papers and presentations.

  Nothing to Report

- **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. What individuals have worked on the project?

A.

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. Tripathi, Mr. Buttery and Mr. Kemper.
Funding Support:	Department of Defense Award W81XWH2110876; NIH/NIGMS; American Cancer Society; UNC Eshelman School of Pharmacy (start-up)

B.

Name:	Lindsey I. James, PhD
Project Role:	Co-Investigator
ORCID iD:	0000-0002-6034-7116
Nearest Person Month Worked:	3
Contribution to Project:	Discovery of UNC7713 and UNC7716; oversight and mentoring of Mr. Buttery in Aim 2 activities (Major Tasks 5-6)
Funding Support:	Department of Defense Award W81XWH2110876; NIH/NCI; UNC Eshelman School of Pharmacy and the Eshelman Institute for Innovation; Pinnacle Hill, LLC

C.

Name:	Surya K. Tripathi, PhD
Project Role:	Post-doctoral Fellow
ORCID iD:	0000-0002-9633-4405
Nearest Person Month Worked:	12
Contribution to Project:	Activities described in Aims 1 and 3
Funding Support:	This award only

D.

Name:	Peter H. Buttery, BS
Project Role:	Graduate Student

ORCID iD:	0000-0001-7778-1552
Nearest Person Month Worked:	3
Contribution to Project:	All activities described in Aim 2 of the grant (Major Tasks 5-6)
Funding Support:	Department of Defense Award W81XWH2110748 (this award) and Award W81XWH2110876; additional James Lab grants

E.

Name:	Ryan Kemper
Project Role:	Research Specialist
ORCID iD:	0000-0002-5468-7827
Nearest Person Month Worked:	1
Contribution to Project:	Select activities in Aims 1 and 3 in support of Dr. Tripathi. 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. Figure 9 from Section 3 (Accomplishments).

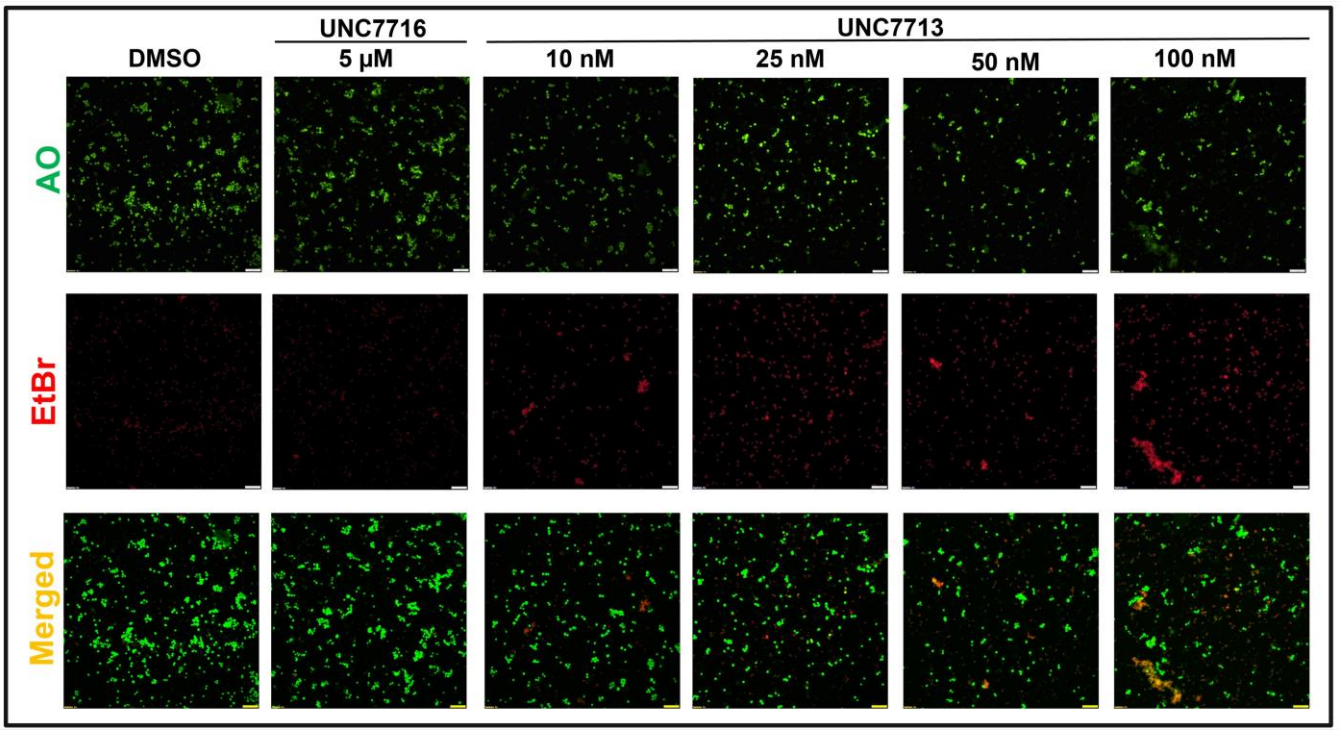


Figure 9. UNC7713 causes cells to undergo apoptosis. Cells were incubated with UNC713, negative control UNC7716 or a 0.1% DMSO control for 72 h. Then, cells were stained with acridine orange (green) to measure viable cells and ethidium bromide (red) to measure cells undergoing apoptosis.

Appendix 2. Figure 10 from Section 3 (Accomplishments)

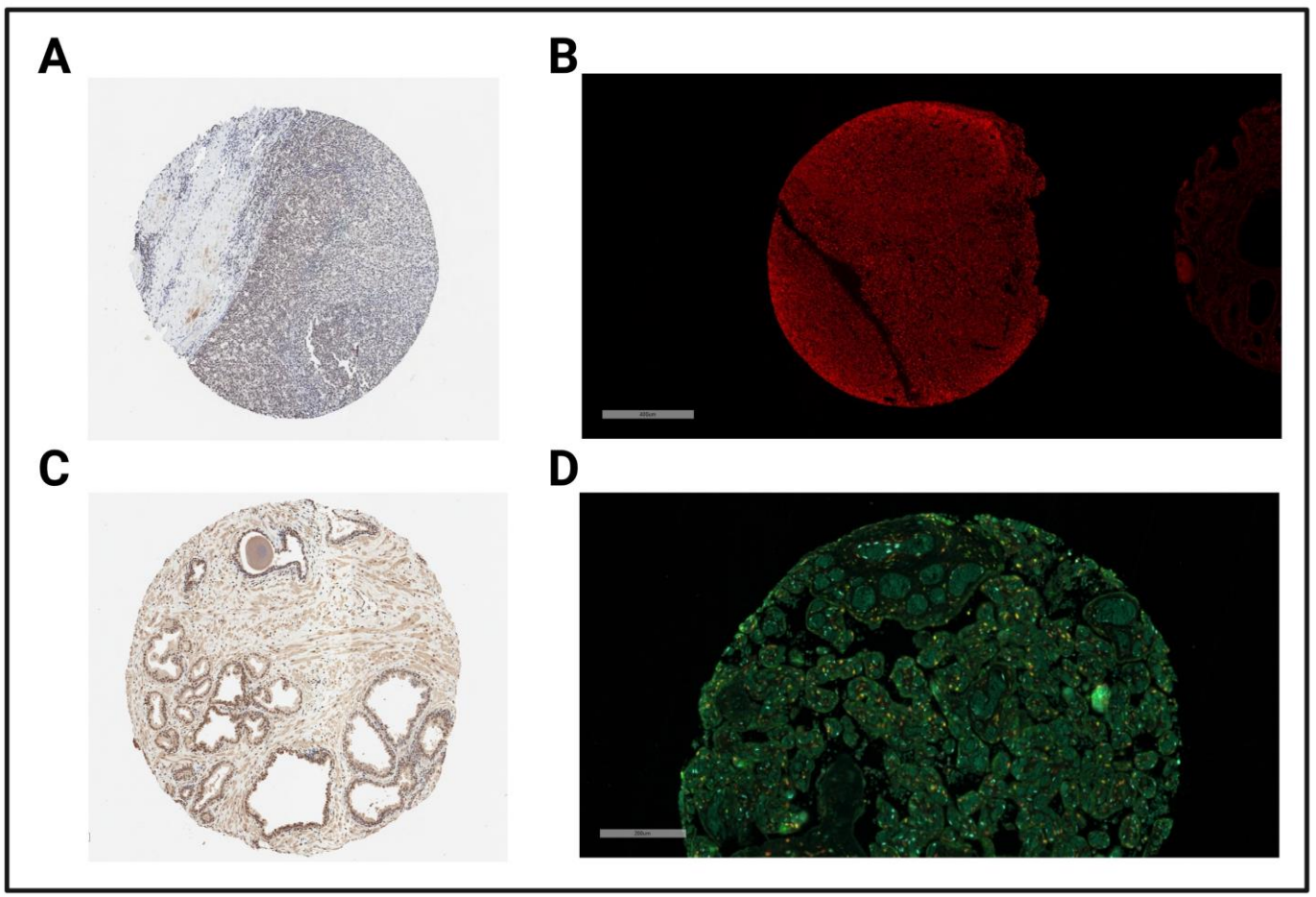


Figure10. Antibody validation for PCBN. Per PCBN guidelines, antibodies to be used in evaluation of their TMA samples required validation. Using UNC TMA samples, normal immunohistochemistry (IHC) antibodies and immunofluorescent (IF) antibodies were both validated. **Panel A** shows MPP8 expression by IHC in tonsil tissue, while **Panel B** shows MPP8 expression by IF in the tonsil tissue. **Panel C** shows MPP8 expression by IHC in a prostate adenocarcinoma, while **Panel D** shows a triple stain of MPP8 (red), SIRT1 (green) and ZEB1 (cyan) expression by IF in a prostate adenocarcinoma.