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14. ABSTRACT

Purpose: The purpose of this project is to test a novel 2nd generation fluoropyrimidine (FP) polymer, CF10, for improved treatment of colon cancer with an emphasis on liver metastases, a primary cause of cancer mortality impacting mission readiness and the civilian population.

Scope: Studies test CF10 and CF10 in combination with oxaliplatin in the WAG/Rij rat model of liver metastases, in primary colon cancer models including patient derived organoids and patient derived xenografts, and in a syngeneic orthotopic colon cancer model for induction of immunogenic cell death and efficacy in combination with immune checkpoint blockade therapy

Major Findings: CF10 is well tolerated in rats and mice and displayed promising activity towards liver metastases in a pilot study in the WAG/Rij rat model. Strong activity much greater than 5-FU, is detected in primary colon cancer models. CF10 induced calreticulin translocation to the plasma membrane of cancer cells consistent with inducing immunogenic cell death.

15. SUBJECT TERMS
colon cancer, liver metastasis, fluoropyrimidine, 5-FU, thymidylate synthase, immunogenic cell death, replication stress

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1. Introduction

Our best option to treat colorectal cancer (CRC), besides surgery, is often cytotoxic chemotherapy with regimens that include fluoropyrimidine (FP) drugs, most frequently 5-fluorouracil (5-FU). FPs, while active in metastatic CRC (mCRC) are non-ideal and high mortality rates that affect mission readiness still occur. *A new FP-based therapy that reduces metastatic progression and decreases the lethality of mCRC, yet is well tolerated, would be highly impactful*. Our preliminary studies for this project demonstrated both improved anti-tumor activity and reduced systemic toxicity is possible using a 2nd generation FP polymer, CF10.

Our objectives in this DoD PRCRP IMPACT award are to advance CF10 towards the clinic. Since the initial clinical application for CF10 is anticipated to focus on treatment of liver metastasis, which is the primary cause of colon cancer mortality, our studies focus on demonstrating improved efficacy for CF10 in rat and mouse models of metastatic CRC (mCRC). Also, since immunotherapy is increasingly important for CRC treatment, we test if CF10 induces immunogenic cell death in CRC cells and works effectively in combination with immune checkpoint blockade.

Our Aim 1 studies (Major Tasks 1 & 2) utilize the WAG/Rij rat model of liver metastasis. This is a new model to our laboratory and we have overcome challenges related to rat breeding and formation of liver metastases that can be imaged to assess treatment effects. We initially proposed testing CF10 in this model as part of the DoD PRCRP IMPACT award but similar studies were also proposed in NIH-NCI R41 CA254834. To avoid overlap, we received permission to receive both awards and to focus on CF10 + oxaliplatin in the DoD award. We have now generated toxicity and some initial efficacy data for CF10 in this model. Encouraging data supportive of our hypothesis have been collected, but we have not yet initiated studies testing CF10+oxaliplatin. NIH-NCI CA254834 is now in a no cost extension.

Our Aim 2 studies (Major Tasks 2, 3, & 4) utilize primary CRC models to test CF10 efficacy. We had initially planned to transfer primary CRC models developed and characterized at TTUHSC to WFUHS, but transfer was not practical and these studies are primarily being undertaken under the direction of Dr. Palle at TTUHSC. Results to date are very encouraging and supportive our hypothesis that CF10 is superior to 5-FU.

Our Aim 3 studies (Major Tasks 5, 6, & 7) focus on CF10 activation of immunogenic cell death in human colon cancer cells and in a mouse orthotopic syngeneic model of colon cancer. We have initiated these studies and developed a flow cytometry assay to study translocation of calreticulin (CRT) to the plasma membrane and plan to proceed with testing as planned, including activity in combination with immune checkpoint blockade inhibitors.

Although there have been challenges, we have made excellent progress towards all of our Aims and Major Tasks and are on-track to complete studies as planned. Further, data generated in this project continue to support that CF10 is superior to 5-FU and likely to be much more effective at treating liver metastases consistent with the translational objectives of our project. We remain on-track to have a substantial impact on treatment of metastatic colon cancer within 5 years of the completion of this project.

2. Keywords

colon cancer, liver metastasis, fluoropyrimidine, 5-FU, thymidylate synthase, immunogenic cell death, replication stress

3. Accomplishments

Specific Aim 1. Demonstrate improved anti-metastatic activity for CF10 in a rat syngeneic colorectal cancer (CRC) liver metastasis (met) model

Major Task 1. Test if continuous i.v. infusion enhances CF10 liver uptake and GI-tumor localization and can be combined with oxaliplatin without causing hepatic or GI-tract toxicity.

Subtask 1.1. Submit documents for animal care and use committee (ACUC) approval

Accomplishment 1.1: Project approved by ACUC at both WFSM (Gmeiner) and TTUHSC (Palle)

Subtask 1.2. Submit ACUC documents for ACURO review and approval

Accomplishment 1.2: Project approved by ACURO

Subtask 1.3: Establishing tolerated CF10 and 5-FU dosing in combination with oxaliplatin with infusion and intra-venous (i.v.) administration (WFSM/Gmeiner)

Accomplishment 1.3: We have established that CF10 is better tolerated than 5-FU in both mouse and rat models using intravenous (i.v.) administration. We are extending these studies to studies to CF10+oxaliplatin and 5-FU+oxalipltin.

We conducted a study in n=5 female WAG/Rij rats in which rats were treated with i) vehicle; ii) 5-FU; iii) CF10. The 5-FU dose was based on literature precedent (50 mg/kg) while CF10 was administered to deliver equivalent fluoropyrimidine content based on UV absorbance at 260 nm. Rats were dosed 1x per week for 4 weeks, i.v. by tail vein injection. Studies revealed significant weight loss for 5-FU-treated mice vs vehicle while CF10 treatment did not induce weigh loss (**Figure 1**).

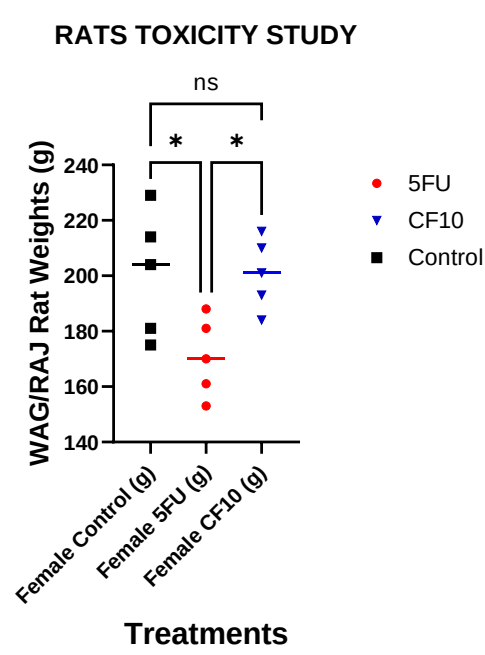

Figure 1. Treatment-induced weight changes with 28 day treatment (1x/week for 4 weeks). 5-FU and CF10 were dosed to deliver equivalent fluoropyrimidine content based on 5- mg/kg 5-FU with equivalence determined based on UV A260 absorbance. Rat weight at day 0 were not significantly different between groups

Subtask 1.4: Time-dependent effects of CF10 and 5-FU in combination with oxaliplatin on liver function, biodistribution, and other endpoints (WFSM/Gmeiner)

Accomplishment 1.4: We collected ultrasound imaging at baseline and at the completion of dosing at the 28-day toxicity testing to monitor potential cardiotoxicity. We are in the process of analyzing the ultrasound for potential cardiotoxic effects related to 5-FU treatment and to determine if this is less with CF10 treatment.

Subtask 1.5: Assess liver toxicity from liver function enzymes and tissue pathology and assess toxicity in other tissues for CF10 and 5-FU treatment in combination with oxaliplatin. (WFSM/Gmeiner)

Accomplishment 1.5: We collected blood and tissues from rats at the conclusion of the 28-day dosing study. We fixed liver, heart, intestines, and other organs from the rats and have sent the tissue to our pathology core lab for sectioning and staining. Slides will be reviewed by co-I Dr. Caudell when available. We sent blood and plasma from drug-treated rats to Idexx for analysis of changes in liver enzymes and also changes in blood counts. Unfortunately, there were some problems with sample preparation and not all samples were analyzed. Among samples analyzed there were no noted significant changes in blood counts or liver enzymes attributable to drug treatment.

Major Task 2. Evaluate if CF10 infusion inhibits progression of liver metastasis.

Subtask 2.1. Demonstrate that CC531 cells are responsive to CF10 and undergo replication stress and apoptotic cell death in response to CF10. (WFSM/Gmeiner & TTUHSC/Palle)

Accomplishment 2.1: We performed initial dose response studies with CC531 cells that showed replication stress upon CF10 treatment (**Figure 2**).

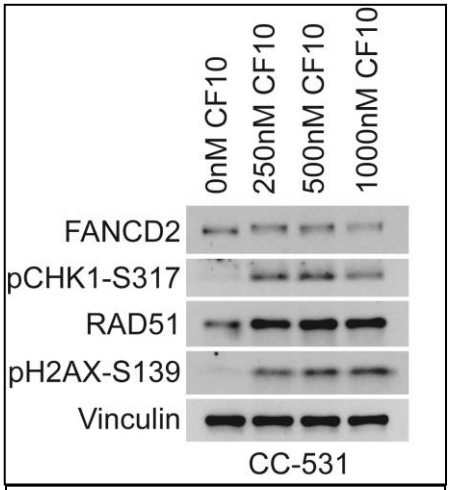

Figure 2. CF10 induces replication stress markers in CC-531 cells

Subtask 2.2. Develop CC531-luc cells by transduction with a luciferase-expressing vector (WFSM/Gmeiner)

Accomplishment 2.2: We completed viral transduction of CC531 cells using a RFP-luc expressing vector. We purchased the plasmid from Addgene, and it was amplified and inserted into lentivirus by the CSER shared resource of our Cancer Center. We then tested different ratios of lentivirus in the transduction and after antibiotic selection with puromycin we identified optimized conditions for luciferase expression in CC531 cells (**Figure 3**).

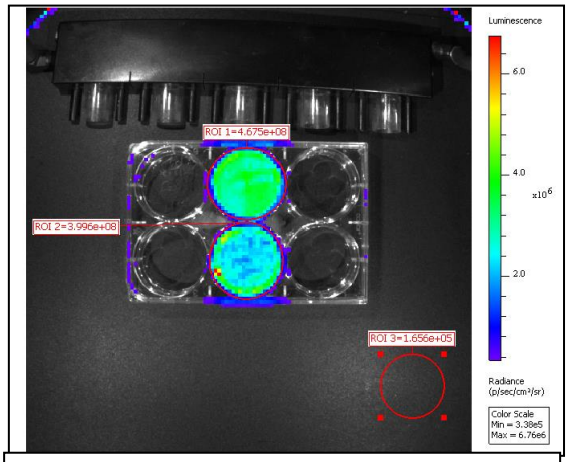

Figure 3. Validation of luciferase expression in CC531-luc cells. The cells robustly express luciferase *in vitro*.

Subtask 2.3. Assess if CF10+oxaliplatin inhibits progression of liver metastases (WFSM/Gmeiner)

Accomplishment 2.3: We demonstrated the ability to form liver metastases by direct injection of CC531-luc cells into the hepatic lobe of WAG/Rij rats. We proceeded to test if CF10 was more effective than 5-FU at inhibiting metastatic progression (**Figure 4**). Studies were conducted in a limited number of rats, n=5 total with vehicle (n=2; M/F), 5-FU (n=2; M/F), and CF10 (n=1; F). Due to loss of luciferase signal by CC531-luc cells after tumor formation *in vivo*, we imaged tumor growth *in vivo* using a fluorescent peptide that includes an RGD motif and is very specific for binding of tumor tissue through selective expression integrin $\alpha_v\beta_3$.

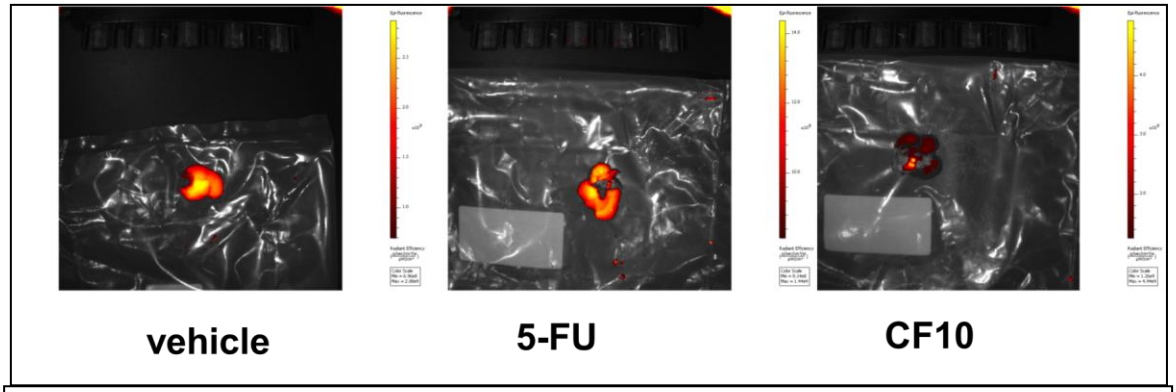

Figure 4. Imaging of liver metastases in female rats after 4 weeks of the indicated treatment. Liver metastases were formed by injecting CC531-luc cells into a hepatic lobe of WAG/Rij rats. After 4 weeks of treatment (1x/week via i.v. dose) with vehicle, 5-FU (50 mg/kg), CF10 (identical to 50 mg/kg 5-FU dose in FP content based UV rats were imaged using a fluorescent RGD peptide. Rats were then euthanized, and livers were extracted and imaged ex vivo. Tumor remained localized in one lobe with CF10 treatment while vehicle and 5-FU-treated rats displayed tumor progression.

Subtasks 2.4 – 2.5. Not yet initiated. (WFSM/Gmeiner)

Specific Aim 2: Demonstrate CF10 displays improved anti-metastatic activity in patient-derived organoid (PDO) and patient-derived xenograft (PDX) models.

Major Task 3. Test CF10 for improved efficacy in primary CRC models

Subtask 3.1. Test primary CRC models for response to CF10 and 5-FU induction of replication stress (TTUHSC/Palle)

Accomplishment 3.1: We performed a study in CRC primary colon organoids which shows increased replication stress in CF10-treated cells compared to the same dose of 5-FU (**Figure 5**).

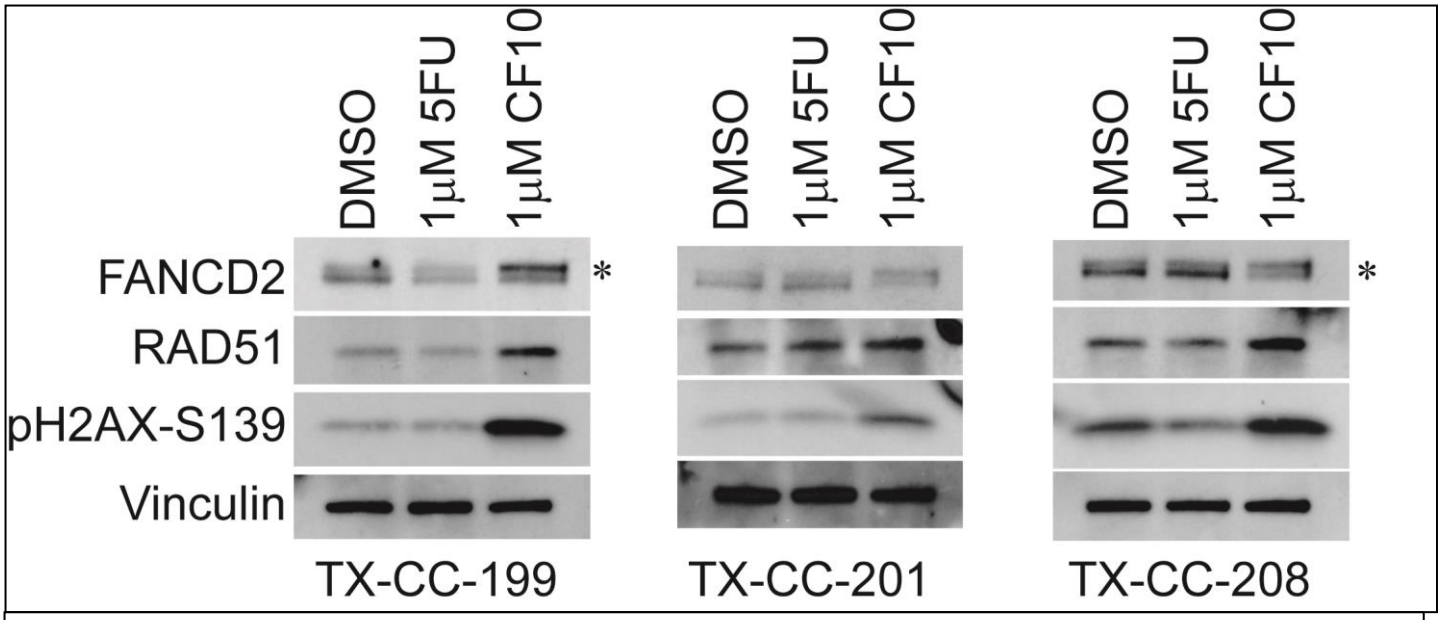

Figure 5: Primary colon organoids treated with CF10 show more replication stress compared to the same dose of 5FU treatment.

Subtask 3.2. Test CF10 for inhibiting migration in scratch and transwell assays (TTUHSC/Palle)

Accomplishment 3.2: Not yet initiated

Major Task 4. Test CF10 for improved efficacy in patient-derived organoid (PDO) models (TTUHSC/Palle)

Subtask 4.1. Develop PDOs from primary CRC cell models (TTUHSC/Palle)

Accomplishment 4.1: Not yet initiated

Subtask 4.2. Test CF10 for improved activity under normoxic and hypoxic conditions (TTUHSC/Palle)

Accomplishment 4.2: Not yet initiated

Subtask 4.3. Test if DNA damage as assessed by gammaH2AX foci in PDO models is a suitable pharmacodynamic (PD) activity for response to CF10. (TTUHSC/Palle)

Accomplishment 4.3: Not yet initiated

Major Task 5. Test CF10 for improved anti-metastatic activity relative to 5-FU in patient-derived xenograft (PDX) models

Task not yet started. (TTUHSC/Palle)

Specific Aim 3. Test if CF10 stimulates immunogenic cell death and synergizes with immune checkpoint blockade

Major Task 6. Test if CF10 infusion stimulates dendritic cell (DC) activation and an anti-tumor T-cell response

Subtask 6.1. Test if CF10 induces secretion of damage-associated molecular patterns (DAMPs) from mouse and human CRC cells (WFSM/Gmeiner)

Accomplishment 6.1: We are developing a flow cytometry assay to detect calreticulin (CRT) on the plasma membrane of drug-treated CRC cells. CRT plasma membrane localization is a DAMP that stimulates immune cell recruitment to tumors. We demonstrated CRT plasma membrane localization with oxaliplatin which is our positive control (**Figure 6**). We are now optimizing conditions for detecting CF10-induced plasma membrane localization. Once those conditions are optimized, we will test for other DAMPs (e.g. HMGB1, HSP70) using ELISA, Western blot or other methods.

Subtasks 6.2 – 6.5. Studies not yet initiated (WFSM/Gmeiner)

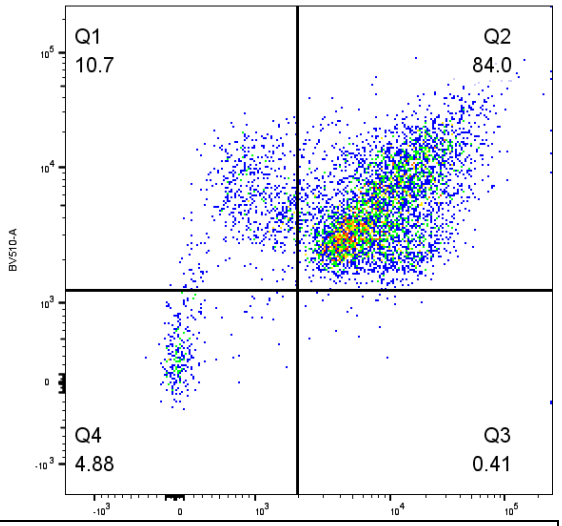

Figure 6. Plot of cell distribution for TS knockdown HCT-116 cells treated with oxaliplatin for 48h. Calreticulin (CRT) expression is the X-axis and "live/dead" stain is the Y-axis. Cell undergoing immunogenic cell death (ICD) are characterized by high CRT and low "live/dead". We plan to use this assay to screen for conditions in which CF10 induces ICD.

Major Task 7. Evaluate if CF10 decreases myeloid-derived suppressor cells (MDSCs), stimulates programmed cell death protein 1 ligand (PD-L1) expression on CRC cells, and sensitizes to anti-PDI therapy

Subtask 7.1. Develop luciferase-expressing MC-38-luc cells (WFSM/Gmeiner)

Accomplishment 7.1: We have generated MC-38-luc cells with similar luciferase expression as shown for CC531-luc cells in **Figure 2**.

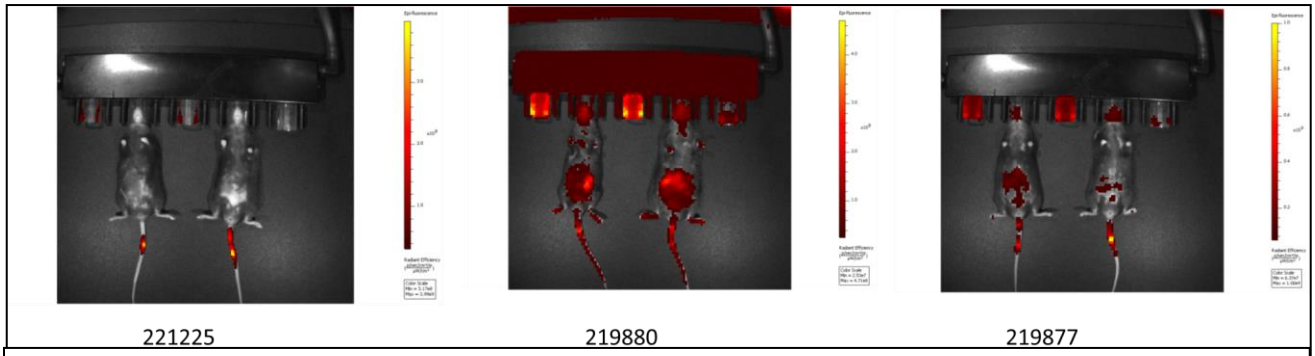

Figure 7. Imaging with a fluorescent RGP petide demonstrates formation of orthotopic colon tumors following intra-cecal injection of MC38-luc cells in C57Bl/6 mice. 4/6 of injected mice formed tumors.

Subtask 7.2. Form orthotopic MC-38-luc colon tumors and test CF10 effects on MDSCs, T-cells, and PD-1 expression. (WFSM/Gmeiner)

Accomplishment 7.2: We have formed orthotopic MC-38-luc colon tumors (**Figure 7**) but have not initiated testing of CF10 or its effects on MDSCs, T-cells, and PD-1 expression.

Subtask 7.3. Form orthotopic MC38-luc colon tumors and test CF10 efficacy in combination with anti-PD-1 therapy. Studies not yet initiated. (WFSM/Gmeiner)

Training and Professional Development

This project provides training for a PhD student, Taylor Monique Young. A 2nd PhD student, Charles Okechukwu also contributes to this project although his stipend is supported by another related grant to Dr. Gmeiner (NIH-NCI CA254834). A MS student in Dr. Gmeiner's lab, Ashmitha Suresh Kumar, also contributes to this project. All students have received training in special animal handling techniques from WFUSM animal resources program personnel. Both Ms. Young and Mr. Okechukwu are African American and Ms. Suresh Kumar is from Kenya,

Dissemination of Results

Taylor Monique Young, a PhD student working with Dr. Gmeiner on the project, was the direct supervisor for a minority (African American) high school student, Meera Bhakta, who worked in Dr. Gmeiner's laboratory during the Summer of 2022. The student performed a poster presentation on-line to NIH staff who sponsored the program.

Plans for Accomplishing Goals

Aim1: Major Tasks 1 & 2

We plan to continue to continue to work with the WAG/Rij rat mode of colorectal liver metastases and to extend studies to accomplish the stated objectives in subtasks 1.3, 1.4, 1.5 & 2.3

- Perform additional studies to establish significance for target advantages of CF10 relative to 5-FU
- Initiate testing of CF10+oxaliplatin vs 5-FU+oxaliplatin and demonstrate advantages for CF10
- Disseminate results of Aim 1 in abstract form and in a peer-reviewed publication

Aim 2: Major Tasks 3, 4, 5

We plan to continue to test CF10 in primary CRC models, PDOs and extend these studies to PDX models

- Perform additional studies in primary CRC lines and PDO establish significant advantages for CF10
- Initiate testing of CF10 in PDX models
- Disseminate results of Aim 2 in abstract form and in a peer-reviewed publication

Aim 3: Major Tasks 6 & 7

We plan to continue to test CF10 in primary CRC models, PDOs and extend these studies to PDX models

- Perform testing for DAMP secretion and activation of immunogenic cell death by CF10 in CRC cells
- Initiate testing of CF10-induced immunogenic cell death in C57BL/6 or iKAP model
- Disseminate results of Aim 3 in abstract form and in a peer-reviewed publication

4. IMPACT – nothing to report

5. Changes/Problems

Cost & Availability of CF10. CF10 has become exceedingly expensive commercially and difficult to access. The CF10 we used in the studies included in this report was purchased from ST Pharma in Korea and cost more than $27,000 per gram and took several months for shipment. Dr. Gmeiner is working through the company he founded, Deep Creek Pharma, to produce gram quantities of analytically pure CF10. As part of these efforts, the Gmeiner lab is evaluating some modifications in polymer design. For example, inclusion of ddC rather than AraC at the 3'-terminus to make the synthesis more straightforward. If this or other modifications result in an improved FP polymer that we intend to take into clinical trials instead of CF10 we will notify DoD and request permission to test such an improved compound instead of CF10.

Difficulty breeding rats and forming liver metastases. We had not previously bred rats for studies and getting the required number at the correct time for experiments has been challenging. We have made progress and are managing rat breeding colony and anticipate being able to generate rats needed to continue studies. We do anticipate reducing the number of rats used for studies. This is possible, in part, because of the large effect size observed for CF10 in our initial pilot study evaluating effects on liver metastases. If we are unable to complete studies using the WAG/Rij rat model proposed, we intend to perform studies in mice with syngeneic tumors using C57BL/6 mice, the animal model proposed for our Aim 3 studies. Alternatively, we recently obtained a genetically engineered mouse model of spontaneous colon cancer, the iKAP model, from Dr. DePinho at MD Anderson.

Difficulty in imaging tumors with luciferase-expressing cells. Although we have transduced CC531 and MC38 cells with a lentivirus to express luciferase and formed orthotopic tumors with these cells, tumors were detected in only a fraction of the expected numbers of rats and mice. Further, tumors did not persist based on IVIS imaging of luciferase and we concluded that are tumor forming procedures were not operative. An alternative possibility is that tumors formed but that the tumor cells stopped expressing luciferase. To test this, we synthesized a fluorescent peptide that includes an RGD motif and localizes to tumors. Using this peptide, we have successfully detected orthotopic tumors in both rats (**Figure 3**) and mice (**Figure 4**).

Formation of TS-overexpressing MC-38 cells (subtask 7.1). Overexpressing TS in MC-38 will require considerable work as apparently the mouse *TYMS* gene encoding TS is not cloned into a vector that would make it straightforward to introduce and overexpress mouse TS in the MC-38 mouse colon cancer cell line. To overcome this, we are developing 5-FU-resistant MC-38 cells. Generating a 5-FU-resistant MC-38 variant was the rationale for overexpressing TS so this cell model meets the design objective of our studies. While in most instances selection of colon cancer cells for 5-FU resistance results in selection of cells that overexpress TS we found our resistant cells did not have basal TS overexpression but 5-FU treatment induced TS overexpression. We think this will be a valid and very interesting model to use for this subtask.

Use of the iKAP model as an alternative to C57BL/6 and possibly WAG/Rij. Forming orthotopic colon tumors through intra-cecal injection or liver metastases through injection of tumor cells into a hepatic lobe requires animal surgery which is time consuming and expensive and uncomfortable for animals. We have obtained the iKAP GEMM from Dr. DePinho and this model, which is based on C57BL/6 mice, spontaneously forms tumors with injection of tamoxifen into the distal colon which does not require surgery. It is likely that the iKAP model will be used for Aim 3 studies evaluating immunogenic cell death and CF10 + immune checkpoint therapy and this model would be considered to be valid and more clinically relevant than C57bl/6 with MC-38 orthotopic tumors.

6. PRODUCTS – nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	William Gmeiner (WFUHS)
Project Role	PI
Researcher ID	
Person Months	4.8
Contribution	Designed experiments, trained personnel, troubleshooting, advising
Funding	This award

Name	Kumar Palle (TTUHSC)
Project Role	Co-PI
Researcher ID	
Person Months	1.2
Contribution	Designed experiments, trained personnel, troubleshooting, advising
Funding	This award

Name	Chinna Mani (TTUHSC)
Project Role	
Researcher ID	
Person Months	5.02
Contribution	Designed and performed experiments, interpreted results
Funding	This award

Name	Taylor Monique Young
Project Role	Graduate student (PhD)
Researcher ID	
Person Months	4
Contribution	Designed and performed experiments related to immunogenic cell death
Funding	This award

Name	Charles Chidi Okechukwu
Project Role	Graduate student (PhD)
Researcher ID	
Person Months	1
Contribution	Rat studies including, weigh, tail vein injection, euthanasia, tissue extract
Funding	NIH-NCI CA254834

Name	Ashmitha Suresh Kumar
Project Role	Graduate student (MS)
Researcher ID	
Person Months	1
Contribution	Imaging mice and rats
Funding	internal

Name	Ralph D'Agostino, Jr
Project Role	Co-I
Researcher ID	
Person Months	0.125
Contribution	Biostatistical analysis
Funding	This award

Name	David Caudell
Project Role	Co-I
Researcher ID	
Person Months	0.375
Contribution	Veterinary pathology
Funding	This award

Name	Boris Pasche
Project Role	Co-I
Researcher ID	
Person Months	0.125
Contribution	Clinical applicability of project
Funding	0.375

Name	Shay Soker
Project Role	Co-I
Researcher ID	
Person Months	0.125
Contribution	Organoid advice
Funding	This award

Name	David Soto-Pantoja
Project Role	Co-I
Researcher ID	
Person Months	0.375
Contribution	Immunotherapy advice
Funding	This award

Name	Xue (Amy) Ma
Project Role	Co-I
Researcher ID	
Person Months	0.375
Contribution	Animal surgery
Funding	This award

Change in Active Support for PI's or senior key personnel – nothing to report

Other organization involved as partners – nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards

Project is a collaboration between Gmeiner (WFUHS) and Palle (TTUHSC). A duplicate report from each PI will be submitted. Tasks are clearly marked with responsible PI and research site.

9. APPENDICES – not included