AWARD NUMBER: W81XWH-18-1-0447

TITLE: Sensitization of Therapeutic-Resistant Pancreatic Cancer by Cancer Cell-Specific Drug Delivery

PRINCIPAL INVESTIGATOR: Stephen J Pandol, MD

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
8700 Beverly Blvd
Los Angeles, CA 90048

REPORT DATE: DECEMBER 2021

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only; proprietary information (DECEMBER 2021). Other requests for this document shall be referred to U.S. Army Medical Research and Development Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
DECEMBER 2021

2. REPORT TYPE:
Final

3. DATES COVERED
15AUG2018 - 14AUG2021

4. TITLE AND SUBTITLE:

Sensitization of Therapeutic-Resistant Pancreatic Cancer by Cancer Cell-Specific Drug Delivery

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0447

6. AUTHOR(S):
Stephen Pandol, MD (PI)
E-Mail: Stephen.Pandol@cshs.org

James Tomlinson, M.D, Ph.D (Partnering PI)
E-Mail: jtomlinson@mednet.ucla.edu

5d. PROJECT NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES):

Dr. Stephen Pandol
Cedars-Sinai Medical Center, VA Greater LA Healthcare System, 8700 Beverly Boulevard, Davis 3100, Los Angeles, CA 90048

Dr. James Tomlinson
University of California Los Angeles, Department of Surgery
10833 Le Conte Avenue, Los Angeles, CA 90095

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT

12. DISTRIBUTION / AVAILABILITY STATEMENT: Distribution authorized to U.S. Government agencies only; proprietary information (DECEMBER 2021). Other requests forthis document shall be referred to U.S. Army Medical Research and Development Command, 504 Scott Street,

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In the third year of this project, we have completed most of the tasks following the timeline of our Statement of Work, although the COVID-19 pandemic severely affected progress in the second year of the project by interrupting the proposed animal studies. We have partially characterized the two new pancreatic cancer cell lines by verifying their tumorigenicity in athymic mice, and confirmed dose-dependent killing of all the six pancreatic cancer cell lines by HMCD-SIM, all in doses below 12.5 μM. In the first test, HMCD-SIM inhibited UN-KPC-960 intrasplenic tumor growth and prolonged 129 mice host survival. Modified experimental protocol will be used in repeated studies to consolidate effect of the conjugate in immune intact mice. In GASP-1 ELISA assays with a wide spectrum of patient samples, it is revealed that substantial amount of GASP-1 was detected in pancreatitis, indicating that the GASP-1 biomarker lacks tumor cell-specificity. We have now confirmed that HMCD-SIM could indeed kill pancreatic cancer cells in vitro and inhibit pancreatic tumor growth in mouse, and completed a manuscript to report this finding. Additionally, with the support of the ongoing DoD PRCRP TTSA Award, we have identified a new entity from pancreatic cancer patient blood samples.

15. SUBJECT TERMS- Pancreatic ductal adenocarcinoma, heptamethine carbocyanine, simvastatin, conjugate, anti-tumor therapy, chemotherapeutic sensitization

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	**b. ABSTRACT** Unclassified	**c. THIS PAGE** Unclassified	Unclassified	61	**19b. TELEPHONE NUMBER** ***(include area***

TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	13
5. Changes/Problems	14
6. Products	16
7. Participants & Other Collaborating Organizations	18
8. Special Reporting Requirements	20
9. Appendices	20

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

This project was based on our previous finding that a specific group of heptamethine carbocyanine dyes (HMCD) has tumor cell-specificity. When it was synthesized as chemical conjugate with simvastatin, the HMCD-SIM became a highly tumor-specific cytocidal agent. In the proposed project, HMCD-SIM will be used as an anti-tumor agent and a sensitizer in the treatment of pancreatic cancers. We hypothesized that the tumor-specific HMCD-SIM targets pancreatic cancer cells through abnormally expressed OATP channel proteins on cancer cell surface. Inside cancer cells, HMCD-SIM is localized in subcellular organelles including mitochondria, where HMCD-SIM impairs mitochondrial integrity to cause organelle leakage, and apoptotic cell death. We proposed to validate HMCD-SIM as a promising new drug for pancreatic ductal adenocarcinoma (PDAC) targeting and therapeutic sensitization; and to determine the mechanisms of HMCD-SIM-mediated cancer cell killing and therapeutic sensitization. Pancreatic cancer cells will be subjected to HMCD-SIM treatment in the presence or absence of other conventional chemotherapeutic agents to evaluate therapeutic efficacy. A series of molecular and cellular studies will be used to elucidate the mechanism of HMCD-SIM action. Xenograft tumor formation, patient derived xenograft tumor formation, and KPC transgenic pancreatic cancer models will be used to validate therapeutic efficacy of the HMCD-SIM.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Pancreatic ductal adenocarcinoma, heptamethine carbocyanine, simvastatin, conjugate, anti-tumor therapy, chemotherapeutic sensitization

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Major goals of the project are: 1) To validate HMCD-SIM as a new drug for PDAC targeting and therapeutic sensitization; and 2) To determine the mechanisms of HMCD-SIM for cancer cell kill and sensitization.

Tasks	**Timeline (months)**	**Site 1 % Completion**	**Site 2 % Completion**	**Site 3 % Completion**
Specific Aim 1: To validate HMCD-SIM as a new drug for PDAC targeting and therapeutic sensitization.				
Major Task 1: Determining the therapeutic efficacy of HMCD-SIM.				
Local IRB/IACUC Approval	1 - 2	100%	100%	NA
HRPO/ACURO Approval	1 - 6	100%	100%	NA
Subtask 1: Conducting large scale synthesis and purification of HMCD-SIM conjugate.	1 - 6	100%	N/A	N/A

<table>
<tr><td>Subtask 2: Determining efficacy of HMCD-SIM on six cultured human PDAC cell lines of BXPC-3 (ATCC), MiaPiCa2 (ATCC), CS-P-2 (Chung), CS-P-26 (Chung), and two new lines to be established from clinical specimens; as well as two mouse PDAC cell lines of UN-KPC-960 (Pandol) and UN-KPC-961 (Pandol).</td><td>7 - 18</td><td>100%</td><td>N/A</td><td>N/A</td></tr>
<tr><td>Subtask 3: Acquiring fresh patient surgical specimens for ex vivo culture to establish and characterize two new human PDAC cell lines (assuming a rate of 1 cell line successfully established from every 6 specimens).</td><td>7 - 18</td><td>100%</td><td>100%</td><td>100%</td></tr>
<tr><td>Subtask 4: Determining anti-tumor efficacy of HMCD-SIM by treating human BxPC-3, MiaPaCa II, CS-P-2, and CS-P-26 and mouse UN-KPC-690 and UN-KPC-961 xenograft tumors, and spontaneous PDAC tumors in KPC mice.</td><td>7 - 18</td><td>50%
(We did this work with only 3 of the proposed 6 cell lines)</td><td>100%</td><td>50%</td></tr>
<tr><td>Subtask 5: Performing lymphocyte depletion in tumor-bearing mice to investigate the role of host immunity in HMCD-SIM-induced inhibition.</td><td>19 - 24</td><td>0%
(Not done because the mechanism of action showed direct killing of cancer cells</td><td>100%</td><td>N/A</td></tr>
<tr><td colspan="5">Milestone(s) Achieved:
1. A total of 5 g high purity HMCD-SIM is prepared, and two new human PDAC cell lines are established.
2. The efficacy of HMCD-SIM on human and mouse PDAC cells in vitro will be validated in 1.5 years.
3. The efficacy of HMCD-SIM on human and mouse PDAC tumors in vivo will be validated in 1.5 years.
4. Role of the host immunity in HMCD-SIM-induced tumor inhibition will be illuminated in 2 years.</td></tr>
<tr><td colspan="5">Major Task 2: Confirming the ability of HMCD-SIM to sensitize chemotherapies.</td></tr>
<tr><td>Subtask 1: Determining IC_{50} of GEM, PTX, CDDP, TKIs and mTOR inhibitors in the six cultured human PDAC cells as listed in Major Task 1, Subtask 2.</td><td>7 - 18</td><td>40%
(Only done with GEM and some TKIs showing no synergism)</td><td>N/A</td><td>N/A</td></tr>
<tr><td>Subtask 2: Obtaining reduced IC_{50} of GEM, PTX, CDDP, TKIs and mTOR inhibitors in combination treatment with HMCD-SIM using the six cultured human PDAC cells as listed in Major Task 1, Subtask 2.</td><td>7 - 18</td><td>40%
(Only done with GEM and TKIs because there was no synergism observed) treatments)</td><td>N/A</td><td>N/A</td></tr>
</table>

<table>
<tr><td>Subtask 3: Treating mice bearing human BxPC-3, MiaPaCa II, CS-P-2, and CS-P-26 tumors with GEM in combination with a low dose HMCD- SIM to improve animal survival.</td><td>13 - 24</td><td>25%
(Done with MiaPaCa II with GEM as this is the standard of care treatment)</td><td>N/A</td><td>N/A</td></tr>
<tr><td>Subtask 4: Treating GEM-resistant human MiaPaCa II xenograft tumors with HMCD-SIM to inhibit xenograft tumor growth and metastasis to improve animal survival.</td><td>19 - 30</td><td>50%
(as above in Subtask 3)</td><td>N/A</td><td>N/A</td></tr>
<tr><td>Subtask 5: Conducting histopathologic analyses and GASP-1 detection on HMCD-SIM-treated xenograft tumors to confirm the efficacy.</td><td>13 - 36</td><td>60%</td><td>100%</td><td>60%</td></tr>
<tr><td>Subtask 6: Using statistical analysis on data of xenograft tumor inhibition to confirm efficacy.</td><td>13 - 36</td><td>60%</td><td>100%</td><td>60%</td></tr>
<tr><td colspan="5">Milestone(s) Achieved:
1. Reduced IC_{50} for the GEM, PTX, CDDP and TKIs in combination with HMCD-SIM on the 6 human and 2 mouse PDAC cell lines will be determined within the first 18 months.
2. Sensitizing effect of HMCD-SIM on GEM will be confirmed in 30 months.
3. The ability of HMCD-SIM to overcome GEM-resistance will be confirmed in 30 months.
4. HMCD-SIM inhibition on PDAC tumors will be histopathologically confirmed at the end of the study.
5. HMCD-SIM inhibition on PDAC tumor will be statistically proven at the end of the proposed study.</td></tr>
<tr><td colspan="5">Major Task 3: Examining sera GASP-1 peptide levels as a PDAC companion biomarker.</td></tr>
<tr><td>Subtask 1: Conduct GASP-1 ELISA assays on the 685 patient sera samples in Biobank and statistical correlation with patient disease history.</td><td>7 - 24</td><td>10%
(This study was aborted because of the lack of cancer-specificity, as inflammatory samples were found with GASP-1 elevation)</td><td>10%</td><td>10%</td></tr>
<tr><td>Subtask 2: Conduct GASP-1 IHC on 28 tumor specimens and statistical correlation with PDAC progression and metastasis. Specimens to be tested include 16 “normal” and pair-matched tumors from clinical trials of Project #0003 and RTOG 0848 (Table 1), and 12 from Major Task 1, Subtask 3. Additional “normal” specimens will be obtained from clinical trial of Panc-Bank (Table 1), in case that some tumor specimens are not accompanied with matched “normal” pairs.</td><td>7 - 33</td><td>50%
(This study was done on 20 PDAC specimens and was then aborted because of the lack of cancer-specificity)</td><td>N/A</td><td>50%</td></tr>
</table>

Subtask 3: Conduct GASP-1 ELISA on mouse blood and cell culture medium, and statistical correlation to PDAC progression and metastasis.	7 - 36	60% (This work is done with PDAC cell culture medium. Not statistically significant correlation was found)	100%	60%
Milestone(s) Achieved: 1. The 685 patient sera samples in Biobank will be subjected GASP-1 ELISA in the first 24 months. 2. Sera from xenograft tumor-bearing mice and cell culture medium will be subjected to GASP-1 ELISA. 3. PDAC specimens in Biobank together with newly acquired specimens will be subjected to GASP-1 IHC. 4. Blood GASP-1 peptide level as a PDAC companion biomarker will be defined at the end of the study.				
Specific Aim 2: To determine the mechanisms of HMCD-SIM for cancer cell kill and sensitization.				
Major Task 4: Examining the toxic effect of HMCD-SIM conjugate.				
Subtask 1: Examining the six HMCD-SIM treated human PDAC cell lines, as listed in Major Task 1, Subtask 2, for impaired mitochondrial function.	7 - 18	100%	N/A	N/A
Subtask 2: Examining the six HMCD-SIM treated human PDAC cell lines, as listed in Major Task 1, Subtask 2, for impaired lysosomal function.	13 - 18	33% (This work has been done only with BxPC-3 and MiaPaCa II cells)	N/A	N/A
Milestone(s) Achieved: 1. The direct toxicity of HMCD-SIM on PDAC cell mitochondria will be confirmed in 1.5 year. 2. The direct toxicity of HMCD-SIM on PDAC cell lysosome will be confirmed in 1.5 years.				
Major task 5: Assessing the mechanism of HMCD-SIM-mediated sensitization.				
Subtask 1: Confirming HMCD-SIM-facilitated cancer cell entry of gemcitabine-^{13}C-$^{15}N2$ in human BXPC-3 and MiaPaCa II PDAC cells.	7 - 12	0%	N/A	0%?
Subtask 2: Using site directed mutagenesis to replace the two charged amino acid residues with neutral residues in the OATP protein.	13 - 18	0% (This work is not done because we realized that a single PDAC cell line may express several protein members of the OATP family)	N/A	N/A
Subtask 3: Studying the mechanism of HMCD-SIM for promoting cancer cell entry of the conventional chemotherapeutic drugs, using the six HMCD-SIM treated human PDAC cell lines, as listed in Major Task 1, Subtask 2.	19 - 24	0%	N/A	N/A

Milestone(s) Achieved: 1. HMCD-SIM-promoted cancer cell entry of chemotherapeutics will be confirmed in the first year. 2. The mechanism of OATP-facilitated HMCD-SIM entry to cancer cells will be confirmed in 1.5 years. 3. The mechanism of HMCD-SIM-facilitated cancer cell entry of conventional chemotherapeutic drugs will be elucidated in the first 24 months.				
Major Task 6: Investigating HMCD-SIM-induced cholesterol loss and Shh signaling inhibition.				
Subtask 1: Confirming HMCD-SIM inhibition of endogenous cholesterol level in the six HMCD-SIM treated human PDAC cell lines, as listed in Major Task 1, Subtask 2, by HPLC cellular cholesterol quantitation.	7 - 12	100%	100%	100%
Subtask 2: Confirming that HMCD-SIM inhibits Smo activity with promoter-reporter assays, using the six HMCD-SIM treated human PDAC cell lines, as listed in Major Task 1, Subtask 2,	13 - 18	33% (This work is done with BxPC-3 and MiaPaCa II cells only)	33%	33%
Subtask 3: Examining for altered cancer-stromal interaction in HMCD-SIM-treated human MiaPaCa II and BxPC-3 PDAC cells under 2-D and 3-D co-culture conditions.	19 - 24	25% (This work is done under 2-D co-culture conditions only)	50%	25%
Subtask 4: Examining for altered cancer-stromal interaction in HMCD-SIM treated human CS-P-2 xenograft PDAC tumors by analyzing the ratio of human and mouse cells.	25 - 36	25% (We have prepared CS-P-2 xenograft tumor specimens but have not checked them for cancer-stromal interaction)	N/A	25%
Milestone(s) Achieved: 1. HMCD-SIM inhibition on PDAC endogenous cholesterol synthesis will be confirmed in the first year. 2. The inhibitory effect of HMCD-SIM on Smo activity and Shh signaling will be confirmed in 1.5 years. 3. HMCD-SIM-induced alterations in cancer-stromal interaction in culture will be determined in 2 years. 4. HMCD-SIM-induced alterations in cancer-stromal interaction in human PDAC xenograft tumors will be determined at the end of the study.				
Biostatistics, Bioinformatics, Manuscript and Progress Report: Study design and experimental data analyses will be consulted with CSMC Biostatistics and Bioinformatics Core.				
Biostatistical and computational support Manuscript and progress report preparation	7 - 36	75% (One manuscript has been published)	100%	75%

What was accomplished under these goals?

1. The results from these studies so far demonstrated that HMCD-SIM, by itself, could kill PDAC cells. HMCD-SIM could also sensitize PDAC cells to the treatment of GEM or CDDP.

2. The results from these studies so far demonstrated that HMCS-SIM kills PDAC cells by attacking mitochondria to cause cytochrome C release, mitochondrial membrane leakage and transmembrane potential reduction.

3. The results from these studies so far demonstrated that HMCD-SIM could also affect PDAC cell metabolism, inhibiting cholesterol biosynthesis.

4. We have established new pancreatic cancer cell lines CS-P-2 and CS-P-124, which show xenograft tumorigenicity. These cell lines will be used in the proposed studies.

5. We have initiated a phase 1 clinical trial with a phase 1b extension in pancreatic cancer and glioblastoma.

Major task 1: Determining the therapeutic efficacy of HMCD-SIM. We have synthesized and purified enough amount of HMCD-SIM conjugate for the entire study in this proposal (months 1 – 2), and we have determined efficacy of the HMCD-SIM on BXPC-3, MIA PaCa-2, UN-KPC-960 and UN-KPC-961 cell lines (months 3 – 6). We now established two new human pancreatic cancer cell lines: CS-P-2 and CS-P-124. All the 6 cell lines have been tested for HMCD-SIM treatment (Figure 1). The results were in good agreement with our previous finding. HMCD-SIM effectively killed these pancreatic cancer cells. IC50 values were around 5 µM after 24 hours of treatment. Mice bearing BXPC-3 and MIA PaCa-2 xenograft tumors have been tested for efficacy on inhibiting tumor formation by HMCD-SIM alone. Preliminary analysis indicated significant inhibition of tumor growth.

We have recently published a paper showing a study using the same KPC pancreatic cancer mouse model we plan to use in our project. The measurements performed in this study include fibrosis, inflammation, epithelial to mesenchymal transition and metastasis, cancer stemness and drug resistance, glucose metabolism and cytokine secretions (Edderkaoui M, et al., Gastroenterology; 2018 Dec;155(6):1985-1998). All these measurements represent key mediators of tumor growth and promotion and we plan to perform most of them in the present study. We have generated enough KPC mice to treat them with HMCD-SIM. KPC mice are now ongoing the treatment for a survival study.

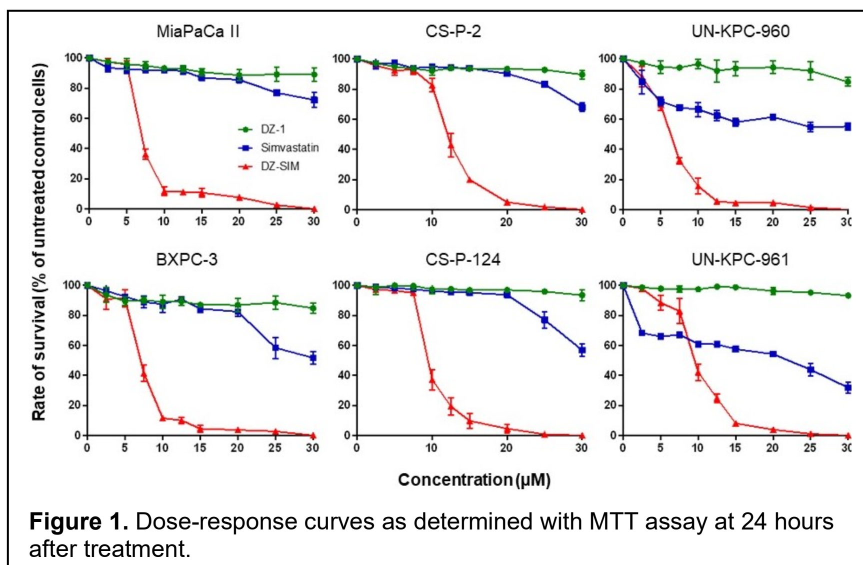

Figure 1. Dose-response curves as determined with MTT assay at 24 hours after treatment.

We have now successfully established new pancreatic cancer cell lines from surgical tumor specimens of two patients (months 7 – 18). Eight cell lines (clones CS-P-2-1 to CS-P-2-8) have been established from a single tumor of the first patient. Though these cell lines are mutually divergent in terms of their growth pattern and cellular morphology, all have been found to have tumorigenicity as assayed with xenograft tumor formation. A single cell line from the second patient (CS-P-124) has now been cultured continuously to passage 60. Immortality of the cell line is thus demonstrated. We have determined that like the CS-P-2 cell line, CS-P-124 can also form xenograft tumors. These cell lines have now been tested for short tandem repeat (STR) analysis, which revealed unique DNA fingerprint, conforming that these cells are indeed newly established cell lines of human origin. These two new cell lines have now been tested with HMCD-SIM treatment and therapeutic efficacy assessment has been completed. We are using commercial contract service now for karyotyping analysis to detect aneuploidy.

We have obtained HRPO approval for acquiring fresh patient surgical specimens for ex vivo culture to establish and characterize two new human PDAC cell lines. Surgical tumor specimens will be acquired for tumor tissue culture starting next month (assuming a rate of 1 cell line successfully established from every 6 specimens) (months 7 – 18).

We have obtained ACURO approval for determining anti-tumor efficacy of HMCD-SIM by treating human BXPC-3, MIA PaCa-2, CS-P-2, CS-P-26 and mouse UN-KPC-960 and UN-KPC-961 xenograft tumors, and spontaneous PDAC

tumors in KPC mice (months 7 – 18). We are now testing whether the CS-P-26 cell line is tumorigenic and metastatic with xenograft tumor formation in athymic nude mice.

To establish UN-KPC-960 and UN-KPC-961 tumor models, we found that these cells could not form tumors in C57BL/6 mice after subcutaneous inoculation. We are now doing two experiments: 1) inoculating to nude mice to make sure that these cell lines can form tumors at all after subcutaneous inoculation or pancreatic orthotopic inoculation; and 2) inoculating to 129 mice to make sure that these cell lines can form tumors at all after subcutaneous inoculation or pancreatic orthotopic inoculation. Due to COVID-19 pandemic, we were not able to purchase nude mice for about 2.5 months. We only recently obtained permission from Vivarium for starting to purchase mice for this study.

We have established image-guided approaches to evaluate the cancer specificity of HMCD-SIM and to monitor therapeutic efficacy with near infrared fluorescence (NIRF) and bioluminescence (BL) imaging. We are developing a LC-MS/MS protocol for quantitative analysis of HMCD-SIM for blood clearance and biodistribution studies.

For performing lymphocyte depletion in tumor-bearing mice to investigate the role of host immunity in HMCD-SIM-induced inhibition (months 19 - 24), the proposed study has not been started due to COVID-19 pandemic. KPC mice are being bred for this study.

We now conducted an alternative study, in which syngeneic 129 mice were used as host for intra-splenic inoculation of UN-KPC-960 mouse pancreatic cancer cells (5×10^5 cells/injection). In this study, tumor-bearing mice treated with HMCD-SIM (n = 6) were found with prolonged survival.

In the current reporting quarter, we are performing the third study. Each B6 129 momice are inoculated intrasplenically with 5×10^5 cells (n=6). Mice are being treated with HMCD-SIM (10 mg/kg, intraperitoneally, twice a week). After 2 months, all 6 mice in the control group have died of cancer metastasis, whereas, all 6 mice treated with HCMD-SIM were still alive (Fig. 2). These results strongly indicate effectiveness of the HMCD-SIM in inhibiting pancreatic tumor growth and metastasis. This study is now completed. Histopathologic staining is being carried out to confirm tumor metastasis in various mouse host organs.

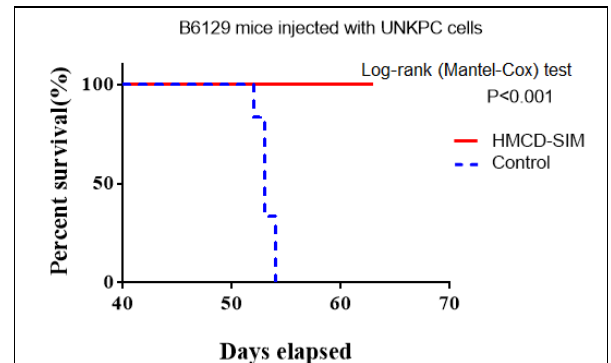

Figure 2. HMCD-SIM significantly improve survival in syngeneic mice with pancreatic cancer cells.

To further determine anti-tumor efficacy of HMCD-SIM (months 7 – 18), KPC mice were bred. The first study using low dose of HMCD-Sim (5mg/Kg) did not show any improvement in KPC mice survival. We then increased the dose to 25mg/kg and we have now a significant increase in KPC mice survival in the HMCD-SIM treatment group. The median survival time increased significantly (*p=0.03*) from 115 days in control group to 145 days in DZ-SIM group (Fig. 3).

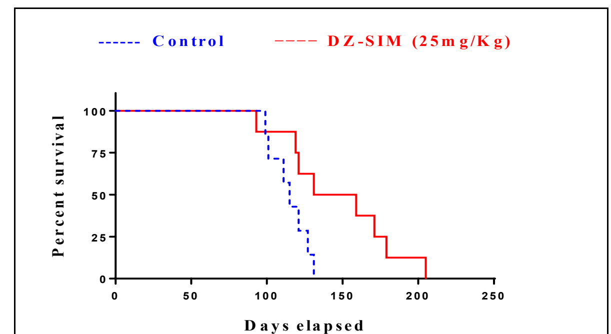

Figure 3. HMCD-SIM significantly improve survival in transgenic KPC pancreatic cancer mice.

Major task 2: Confirming the ability of HMCD-SIM to sensitize chemotherapies. We have determined IC50 of gemcitabine (GEM), cisplatin (CDDP) and paclitaxel (PTX) on BXPC-3 and MIA PaCa-2 cells, individually and in combination with HMCD-SIM (months 7 – 18). Experiments are currently underway for reduced IC50 of GEM, PTX, CDDP, and mTOR inhibitors in combinatory treatment with HMCD-SIM (months 7 – 18). Control xenograft tumor formation with CS-P-2 cell lines has been completed. Control xenograft tumor formation with CS-P-26 cells will be started in the next month, followed by treating mice bearing BXPC-3, MIA PaCa-2, CS-P-2 and CS-P-26 tumors with GEM in combination with a low dose HMCD-SIM to improve animal survival (months 13 – 24). The study of treating GEM-resistant human MIA PaCa-2 xenograft tumors with HMCD-SIM to exhibit xenograft tumor growth and metastasis to improve animal survival (months 19 – 30) has just been started. Histopathologic analysis and GASP-1 detection on HMCD-SIM-treated xenograft tumors (months 13 – 36) is halted at the moment till the cross-reactivity issue of GASP-1 ELISA in pancreatitis specimens is resolved.

We conducted a series of analyses to assess whether a low dose HMCD-SIM treatment could make pancreatic cancer cells become sensitive to conventional chemotherapeutic drugs (months 13 – 24). HMCD-SIM at 1 μM, 3 μM and 5 μM did not significantly make pancreatic cancer cells more sensitive to gemcitabine treatment. This study will be repeated to confirm that HMCD-SIM does not sensitize pancreatic cancer cells to other anti-tumor treatments. Through repeated experiments, we noticed a conspicuous issue on time difference between HMCD-SIM-induced cancer death and the death by other anti-tumor treatments. While HMCD-SIM kills cancer cells within 24 hours, the effect of other treatments was much slower. We will repeat the combinatory treatment in the next with extended time (96 hours) and to assess the effect of sensitization.

It turned out difficult to study HMCD-SIM sensitization with cultured cancer cells. The main hurdle is probably the difference in the time of action between this conjugate and chemotherapeutics. Time of action by HMCD-SIM is within 24 hours, while chemotherapeutics such as GEM, CDDP and PTX inhibits cancer cell growth in a much slower pace, beyond 48 hours. We decided to use xenograft tumor models to assess the effect of HMCD-SIM sensitization.

To treat GEM-resistant human MiaPaCa II xenograft tumors with HMCD-SIM to inhibit xenograft tumor growth and metastasis to improve animal survival (months 19 – 30), we conducted two preliminary and pioneer animal studies during the last reporting quarter to assess *in vivo* experimental conditions. In the first study, nude mice (n=3) were inoculated with human pancreatic cancer MiaPaCa II cells (2×10^6/site, subcutaneously) and treated with HMCD-SIM (5 mg/kg, intraperitoneally and twice a week) for 4 weeks. Compared to the controls, tumor size in the treatment group was reduced, although the reduction did not reach statistical significance, most likely due to the small group size. Through this study, we determined that a 10 mg/kg dose was to be used to a group of 10 mice (n=10) in the next study in order to reach statistically significant difference between control and treatment groups.

Major task 3: Examining serum GASP-1 peptide levels as a PDAC companion biomarker. In preparation for the current study, we have used legacy samples to optimize an ELISA method for measuring GASP-1 peptide in patient serum samples (months 1 – 12). We have recently tested the hypothesis that GASP-1, a G protein coupled receptors (GPCRs)-associated sorting protein-1, a 156 kDa cytosolic protein, as a serum marker for human bladder cancer. This approach allows us to standardize our technology and protocol. When overexpressed in cancer cells, DASP-1 directs ligand-bound GPCRs to plasma membrane as a signal enhancer promoting cell proliferation. We collected serum samples from 13 healthy donors as control and 30 bladder cancer patients with IRB approval. Serum GASP-1 was assessed by ELISA (Proplex Technologies, Dresher, PA). Data were analyzed with Graph-pad prism 6.0 for statistical clinical correlation. GASP-1 protein was also measured in 8 cultured bladder cancer cell lines with or without Gemcitabine treatment by western blot and 12 archived cancerous and 5 normal bladder tissues by immunohistochemistry. Our results reveal that higher serum GASP-1 expression was found in bladder cancer patients than controls ($p<0.001$). The area under the ROC curve (AUC) for GASP-1 to discriminate bladder cancer from normal was 0.8096 (95% confidence interval [CI], 0.7202 to 0.899; $P<0.0001$). GASP1 expression in stage-Ta, -T1 and -TIS and PUNLMP, low- and high-grade were all higher than controls statistically (all $p<0.05$). GASP-1 was detected in clinical bladder cancer tissue specimens and cultured bladder cancer cell lines. At tissue level, semi-quantitative IHC expression of cytoplasmic GASP-1 was comparable in normal and cancerous bladder epithelial cells but nuclear membrane GASP-1 expression in the cancer group was significantly higher than control ($p=0.0036$) also higher in metastatic and Gemcitabine-treated bladder cancer cells. This method will be used for this DoD study using serum samples collected from pancreatic cancer patients, in comparison to specimens collected from normal controls and also from patients with confirmed pancreatitis.

We have now obtained the first 40 plasma samples of pancreatic cancer patients. GASP-1 ELISA of these samples will be performed in the coming month. We are also collecting more patient plasma from clinical pancreatic cancer patients. After testing the plasma samples, however, it was found that only serum samples were suitable for the ELISA platform. Additional patient serum samples have now to be prepared. Alternatively, we are testing whether commercial GASP-1 ELISA kit could be used to detect plasma samples. We have now obtained the first 40 serum samples of pancreatic cancer patients. GASP-1 ELISA of these samples has been completed and the data indicate a significant two-fold increase in the GASP-1 level in the serum of patients with pancreatic cancer (stages I and II) and a 3-fold increase in patients with stages II and IV pancreatic cancer (Figure 2). However, there was no significant difference between pancreatitis and cancer patients. This lack of specificity may prevent development for clinical application.

We have obtained HRPO approval for conducting GASP-1 IHC on 28 tumor specimens and statistical correlation with PDAC progression and metastasis (months 7 – 33). In primary tests, tumor sections from 4 PDAC cases were tested for GASP-1 level, indicating that the detection protocol was appropriate. Interpretation of the results is halted now until the cross-reactivity issue of GASP-1 ELISA in pancreatitis specimens is resolved.

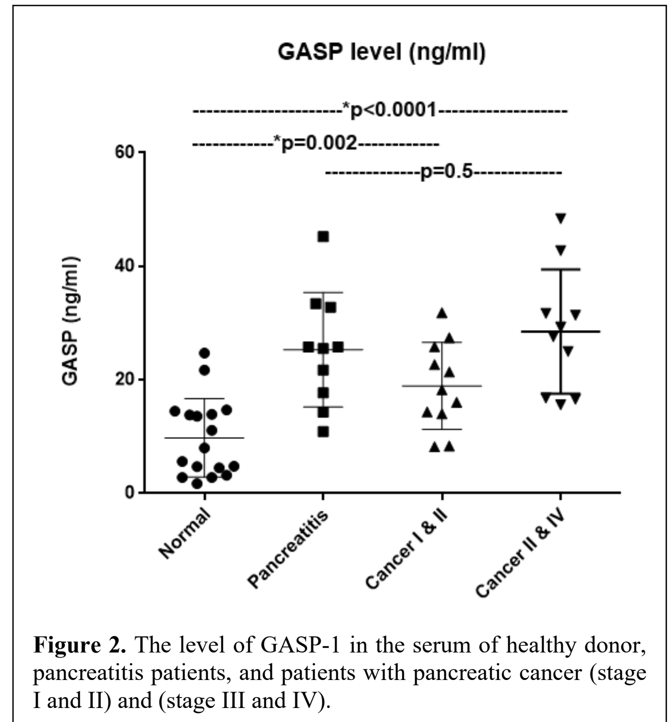

Figure 2. The level of GASP-1 in the serum of healthy donor, pancreatitis patients, and patients with pancreatic cancer (stage I and II) and (stage III and IV).

Major task 4: Examining the toxic effect of HMCD-SIM conjugate. We have documented impaired mitochondrial function using 3 methods: 1) immunohistochemical staining for cytochrome C release from mitochondria to cytosol following HMCD-SIM treatment; 2) Quantitative flow cytometry for reduced mitochondrial membrane potential with HMCD-SIM-treated cancer cells whose mitochondrial were pre-loaded with either rhodamine 123 of JC1 (months 7 – 18). The same methods will be used to examine toxic effect on UN-KPC-960, UN-KPC-961 and the two newly established PDAC cell lines, which will be analyzed in the next month (months 7 – 18).

We recently determined that the cancer cell death caused by HMCD-SIM could be alleviated by Mdivi-1, an inhibitor for mitochondrial division (mitochondrial fission). In the presence of Mdivi-1, MiaPaCa II and BXPC-3 cells became resistant to HMCD-SIM-induced death. We will generalize this finding with CS-P-2, CS-P-124, UN-KPC-960 and UN-KPC-961 cells to affirm that mitochondrial dynamics of fusion and fission play a critical role in cancer cell survival.

Major task 5: Assessing the mechanism of HMCD-SIM-mediated sensitization. We are currently locating an isolated cell culture incubator for culture cells with radioactive gemcitabine-^{13}C-$^{15}N2$ (months 7 – 18). For mutagenesis studies, we determined to obtain a human full length OATP1B3 cDNA clone from commercial sources. The mutagenesis work will be started as soon as COVID-19 pandemic recedes (months 13 – 18). The study of the mechanism of HMCD-SIM for promoting cancer cell entry of the conventional chemotherapeutic drugs, using the 6 HMCD-SIM treated human PDAC cell lines (19 – 24) is under way.

We noticed in the literature reporting that HMCD forms adduct with albumin (Osama *et al*., 2018, 2020), suggesting that OATP may not be the only thoroughfare for tumor cell uptake. We initiated a project assessing the role of albumin in HMCD-SIM uptake, by comparing adduct protein formation between HMCD and HMCD-SIM groups. Our results thus far suggest that HMCD binds with certain proteins in cell culture (Figure 3). When whole cell lysates were analyzed on SDS-PAGE, a protein adduct could be seen with a molecular weight around 60 kD. Interestingly, this analysis revealed that HMCD-SIM may affect additional proteins (Figure 3), as several additional bands became visible. These results suggest that, different from its precursor HMCD, the conjugate of HMCD-SIM may form adducts with other cellular proteins to compromise their biological function. Protein adducts formation may be an explanation for cytotoxicity of the HMCD-SIM. Identification of these proteins may elucidate the mechanism of HMCD-SIM-induced cancer cell death.

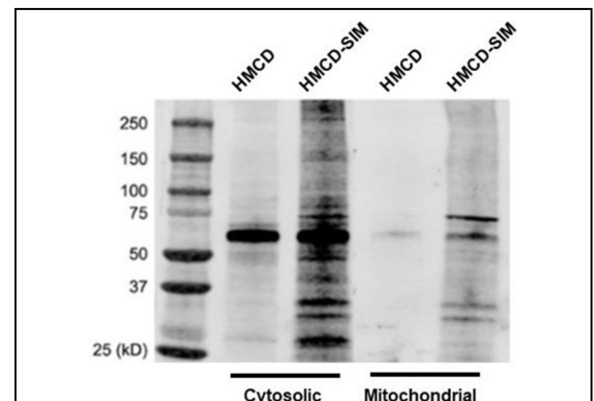

Figure 3. Differential protein adduct formation between HMCD and HMCD-SIM. In this study, cancer cells were treated with HMCD or HMCD-SIM. Whole cell lysates and mitochondrial lysates were fractionated on SDS-PAGE and subjected to near infrared detection of adduct proteins.

In this reporting quarter, we started to isolate the bands revealed in the SDS-PAGE by cutting out the gel pieces for proteomic identification of the adduct protein by mass spectroscopy.

Major task 6: Investigating HMCD-SIM-induced cholesterol loss and shh signaling inhibition. We have confirmed that HMCD-SIM inhibits cholesterol level significantly in treated cancer cells, with more prominent effect on cholesterol level of the mitochondria (months 7 – 12). A luc reporter driven by a Smo promoter will be

transfected to BXPC-3 and MiaPaC2 cells starting in the next month. This work is currently under way (months 13 – 18). The study for altered cancer-stromal interaction in HMCD-SIM-treated human MIA PaCa-2 and BxPC-3 PDAC cells under 2-D and 3-D con-culture conditions is under way (19 – 24).

We have confirmed that the use of HMCD-SIM indeed causes cholesterol reduction in the cell culture medium. However, when we used high cholesterol to treat pancreatic cancer cells, the treated cells did not become resistant to HMCD-SIM treatment. On the other hand, cholesterol-free cell culture medium did not cause death of the cancer cells. Decrease cholesterol level, therefore, may not be the reason for HMCD-SIM-induced cancer cell kill. With the HMCD-SIM-treated xenograft tumor specimens, we will next to examine whether HMCD-SIM kills cancer cells but leaves mesenchymal stromal cells unhurt in the tumor microenvironment.

We have obtained CS-P-2 xenograft tumor specimens by inoculating the cells to SCID mice for 2 months. Following inoculation with our protocol, CS-P-2 cells yielded 100% tumor formation. These tumors are fixed in formalin. Part of the tumor tissue was embedded and sectioned for histopathology assessment.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal

disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have initiated a phase 1 clinical trial with a phase 1b extension in pancreatic cancer and glioblastoma. Thus, the results of this study have led to clinical application of a novel therapeutic.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

This work will have an impact on multiple disciplines involved in pancreatic cancer treatment including oncologists, surgeons, pharmacologists, radiologists.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

The results led to licensing of the technology to industry followed by pre-clinical toxicology studies with plans for initiating a phase 1 clinical trial with a phase 1b extension in pancreatic cancer and glioblastoma. Thus, the results of this study have led to clinical application of a novel therapeutic.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*

- *improving social, economic, civic, or environmental conditions.*

Treatment innovations for pancreatic cancer with potential for improved survival. The outcome will also affect knowledge for both practitioners and patients indicating that there are potential novel and improved treatments for pancreatic cancer.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Although there were delays due to the COVID pandemic, key milestones have been met. Some SOW items were not completed for reasons described in the table at the beginning of this report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

No such changes.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

None.

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Ou Y, Wang R, Chu GCY, Elmadbouh OHM, Lim A, Chung LWK, Edderkaoui M*, Zhang Y*, Pandol SJ*. (*Co-Senior Authorship). "Novel DZ-SIM Conjugate Targets Cancer Mitochondria and Prolongs Survival in Pancreatic Ductal Adenocarcinoma", Advanced Therapeutics. https://doi.org/10.1002/adtp.202200021. (Jul. 2022).

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

N/A

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

N/A

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

N/A

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

N/A

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

N/A

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

N/A

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc.,*

available to project staff);

- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

No

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*