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

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14. ABSTRACT
In the second year of this three-year project, we have completed most of the tasks following the timeline of our Statement of Work, although the COVID-19 pandemic has severely affected progress 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. These successes greatly help the research project focus on the effectiveness of HMCD-SIM as an effective anti-tumor agent in the third year.

15. SUBJECT TERMS

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1. Introduction

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 (SIM), 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, cytochrome C release 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 and KPC transgenic pancreatic cancer models will be used to validate therapeutic efficacy of the HMCD-SIM.

2. Key words

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

3. Accomplishments

What were the major goals of the project?

Aim 1: To validate HMCD-SIM as a promising new drug for PDAC targeting and therapeutic sensitization.

1.1. Determining the therapeutic efficacy of HMCD-SIM on cultured PDAC cells, human PDAC xenograft tumors, and spontaneous PDAC tumors in immune intact transgenic mice. **Partially Completed.**

1.2. Confirming the ability of HMCD-SIM to re-sensitize therapeutically resistant PDAC cell lines to chemotherapy, by obtaining reduced half maximal inhibitory concentrations (IC50) of gemcitabine (GEM), paclitaxel (PTX), csplatin (CDDP) and tyrosine kinase inhibitors (TKIs) in the presence of the sensitizing HMCD-SIM in representative PDAC cell lines. **Partially Completed.**

1.3. Examining sera levels of the G protein coupled receptor-associated sorting protein 1 (GASP-1) peptide in correlation with therapeutic responses of PDAC tumors and using GASP-1 peptide levels in sera to differentiate the aggressiveness of PDAC tumors in patients treated with chemotherapy, either with or without therapeutic relapse. **Completed.**

Aim 2: To determine the mechanisms of HMCD-SIM-mediated cancer cell kill and therapeutic sensitization.

2.1. Examining the toxic effect of HMCD-SIM conjugate on mitochondrial and lysosomal structure and function, and the corresponding loss of vitality in PDAC cell lines. **Partially Completed.**

2.2. Assessing the mechanism of HMCD-SIM-mediated sensitization. The effect of HMCD-SIM on the uptake and retention functions of other anti-tumor drugs will be investigated. **Partially Completed.**

2.3. Identifying the mechanism by which HMCD-SIM-induced cholesterol loss down-regulates the Shh signaling axis to inhibit PDAC cell interaction with cells in the tumor microenvironment to reduce PDAC progression and prevent the occurrence of therapeutic resistance. **Partially Completed.**

What was accomplished under these goals?

Major task 1: Determining the therapeutic efficacy of HMCD-SIM.

Chung
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). 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.

Pandol
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 many of them in the present study. We are now in the process of generating enough breeding pairs to generate KPC mice to use for testing HMCD-SIM. We will have enough KPC mice in the next 2-4 months to start the treatment.

Chung
We have now successfully established new pancreatic cancer cell lines from surgical tumor specimens of two patients (months 7 – 18). Eight cell lines have been established from a single tumor of the first patient. Though these cell lines are mutually divergent in terms of their growth patter and cellular morphology, all have been found to have tumorigenicity as assayed with xenograft tumor formation. Cell lines from the second patient are currently been cultured continuously to passage 41. We will continue to culture these cells to 60 passages to demonstrate immortality of the cell line.

We have so far determined the therapeutic efficacy of HMCD-SIM on all the six PDAC cell lines, including the two commonly used human PDAC cell lines MiaPaCa II and BXPC-3, the two established from spontaneous PDAC tumors of the KPC transgenic mice (UN-KPC-960 and UN-KPC-961), and the two newly established human PDAC cell line from this research project (CS-P-2 and CS-P-124). After postponing in the COVID-19 pandemic season, HMCD-SIM has been tested on human PDAC xenograft tumors in athymic mice, on PDAC tumors in immune intact and syngeneic mice (129 mice), and on spontaneous PDAC tumors in KPC transgenic mice. Additional animal studies are needed to complete the proposed study (months 19 – 24).

Major task 2: Confirming the ability of HMCD-SIM to sensitize chemotherapies.

Chung
We have determined IC50 of GEM and CDDP on BXPC-3 and MIA PaCa-2 cells, individually and in combination with HMCD-SIM (months 3 – 15). Experiments are currently underway for reduced IC50 of GEM, PTX, CDDP, and mTOR inhibitors in combinatory treatment with HMCD-SIM (7 – 18). Control xenograft tumor formation with CS-P-2 cell lines has been completed.

As a representative study, we have tested the combinatory use of HMCD-SIM with gemcitabine. HMCD-SIM acted fast in cancer cell kill, causing complete cancer cell death within 24 hours. In contrast, the effect of gemcitabine was slow and mild, taking more than 3 days to display growth inhibition. Consequently, no additive or synergism was observed between HMCD-SIM and gemcitabine in the first 24 hours of combinatory treatment. Next, the dose of HMCD-SIM will be further reduced while the time of treatment will be extended to 96 hours in order to observe tumor cell sensitization (months 13 – 24).

Major task 3: Examining sera GASP-1 peptide levels as a PDAC companion biomarker.

Chung
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 GEM 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 compared to 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 ($p=0.0036$).The nuclear membrane level of GASP-1 was also higher in metastatic and GEM-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 from patients with confirmed pancreatitis.

Pandol/Tomlinson
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.

Elevated GASP-1 levels in the serum of PDAC patients was observed compared to those of the healthy donors. On the other hand, when spectrum of patient serum sample enlarged, many pancreatitis patient serum samples were found with similarly elevated GASP-1 levels. These results suggested that GASP-1 might not be an ideal tumor cell biomarker, as it was also elevated under inflammation conditions (months 7 – 24).

Major task 4: Examining the toxic effect of HMCD-SIM conjugate.

Chung
We have documented impaired mitochondrial function using three 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 mitochondria were pre-loaded with either rhodamine 123 or JC1 (months 1 – 6).

After PDAC cells were treated with HMCD-SIM, a significant loss of mitochondrial transmembrane potential was observed with JC-1 staining method. Oxidative phosphorylation was suppressed by HMCD-SIM in Seahorse assays. Reactive oxygen species (ROS) level was elevated as determined by MitoSOX stain. On the other hand, mitochondrial fission inhibitor was found to be able to counter HMCD-SIM-induced PDAC cell death (months 7 – 18).

Major task 5: Assessing the mechanism of HMCD-SIM-mediated sensitization.

Chung

We are currently locating an isolated cell culture incubator for culture cells with radioactive gemcitabine-13C-15N2 (months 1 – 6). For mutagenesis studies, we decided to obtain a human full length OATP1B3 cDNA clone from commercial sources (months 13 - 18).

A series of combinatory HMCD-SIM and gemcitabine treatment was used with MiaPaCa II cells in order to identify the optimal experimental condition for the study of HMCS-SIM affecting uptake and retention of other anti-tumor drugs (months 19 – 24).

Major task 6: Investigating HMCD-SIM-induced cholesterol loss and Shh signaling inhibition.

Chung

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). HMCS-SIM inhibited Smo activity with promoter-reporter assays (months 13 – 18). When HMCD-SIM was used to treat BXPC-3 cells in 3-dimensional cancer-stromal co-culture, the BXPC-3 cells were found to be more sensitive to the treatment than the stromal cells (months 19 – 24).

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

The project provided training for postdoctoral fellows Lijuan Yin, Liyuan Yin, and Ji Lyu, in a novel area in pancreatic research with Dr. Chung, an expert in cancer biology and metastasis. These fellows also worked with Drs. Pandol and Tomlinson who are experts in clinical pancreatic cancer research and treatment.

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

In the next reporting period, we will keep close observation to the timeline of our Statement of Work. With our HRPO and ACURO protocols approved, we are using xenograft tumor formation to assess the efficacy of HMCD-SIM on inhibiting pancreatic cancer growth and metastasis.

4. IMPACT

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

In the first year of this three-year project, we have completed tasks following the timeline of our Statement of Work. These works laid a solid foundation for second year mechanistic investigation of the molecular mechanism of HMCD-SIM mediated pancreatic cancer cell killing.

In the second year of this project, we have validated the efficacy of HMCD-SIM on all the six representative pancreatic cancer cell lines. Studies on the role of HMCD-SIM in sensitizing cancer cells to conventional therapy are under way both *in vitro* with cancer cell lines and *in vivo* with xenograft tumors and transgenic mouse spontaneous pancreatic cancer models.

What was the impact on other disciplines?

In our first-year studies, HMCD-SIM is determined to be able to kill pancreatic cancer cells. These results will encourage us to test HMCD-SIM to kill other human cancer cells. At the same time when this project is going on, our colleagues have tested HMCD-SIM on prostate and lung cancer cell lines. As anticipated, HMCD-SIM was found to induce rapid death of these cancer cells.

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

Nothing to report.

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

During the high COVID-19 pandemic season, vivarium in our institution was shut down for almost three months. Laboratory research of the project was also delayed because of the pandemic. We will ask for extension of the project.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications

Nothing to report

Books or other non-periodical, one-time publications

Nothing to report

Other publications, conference papers and presentations

Development of agents targeting membrane cholesterol and mitochondria to accelerate pancreatic cancer cell kill. Accelerating the pipeline for improving pancreatic cancer, Translational Symposium, Digestive Disease Week (DDW) 2019, San Diego Convention Center, 33ABC, May 18. 2019

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and /or licenses

Nothing to report.

Other products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Leland W. K. Chung (Initiating PI)	Experimental design	6 months
Yi Zhang	HMCD-SIM synthesis and purification	2 months
Ruoxiang Wang	Culture of patient samples	4 months
Ji Lyu	Small animal studies	6 months
Stephen Pandol (Partnering PI)	Patient recruitment and protocol preparation	2 months
Edderkaoui Mouad	Animal breeding	3 months
Nicholas Nissen	Patient recruitment	6 months
Andrew Hendifar	Patient recruitment	2 months
James Tomlinson	Patient recruitment and protocol preparation	2 months
Michael Lewis	Biomarker evaluation	2 months

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes, Gina Chia-Yi Chu has left for another job opportunity.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

COLLABORATIVE AWARDS

Nothing to report.

QUAD CHARTS

Nothing to report.

9. APPENDICES

Nothing to report.