RGC Ref. No.:
UGC/FDS25/M02/17
(please insert ref. above)

RESEARCH GRANTS COUNCIL
COMPETITIVE RESEARCH FUNDING SCHEMES FOR
THE LOCAL SELF-FINANCING DEGREE SECTOR

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report
(for completed projects only)

Submission Deadlines: *1. Auditor's report with unspent balance, if any: within **six** months of the approved project completion date.*
*2. Completion report: within **12** months of the approved project completion date.*

Part A: The Project and Investigator(s)

1. Project Title

A mechanistic study on the combination uses of doxorubicin with *Salvia Miltiorriza* Bunge (Danshen) in treating drug-resistant hepatocellular carcinoma *in vitro* and *in vivo*

2. Investigator(s) and Academic Department(s) / Unit(s) Involved

Research Team	Name / Post	Unit / Department / Institution
Principal Investigator	Dr. CHAN Shun Wan / Associate Professor	Faculty of Science & Technology, Technological and Higher Education Institute of Hong Kong.
Co-Investigator(s)	Prof. KWAN Yiu Wa / Professor	School of Biomedical Sciences, The Chinese University of Hong Kong
Co-Investigator(s)	Dr. MOK Daniel Kam-wah / Associate Professor	Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University

3. Project Duration

	Original	Revised	Date of RGC / Institution Approval *(must be quoted)*
Project Start Date	1st Jan 2018	NA	NA
Project Completion Date	31st Dec 2020	30th Jun 2021	11th Dec 2020 by THEi

		31st Dec 2021	24th June 2021 by RGC
Duration *(in month)*	36	42	11th Dec 2020 by THEi
		48	24th June 2021 by RGC
Deadline for Submission of Completion Report	31st Dec 2021	30th Jun 2022	11th Dec 2020 by THEi
		31st Dec 2022	24th June 2021 by RGC

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. *To investigate the in vitro cytotoxic effects of doxorubicin with or without Danshen extract and their underlying mechanisms*
2. *To evaluate the effectiveness of the combination uses of doxorubicin with Danshen extract on drug-resistant HCC in nude mice*
3. *To use metabolomics approach for the comparison of serum metabolic changes in normal nude mice, nude mice with xenograft and nude mice with xenograft treated with doxorubicin in combination with Danshen extract*
4. *To investigate whether the anti-tumor effect of using doxorubicin in combination with Danshen extract is via the expressions of sodium-hydrogen exchanger isoform-1 (NHE-1), P-glycoprotein (P-gp) and multidrug resistance (MDR)-associated protein 1 (MRP-1) as well as the potential systemic pathways identified by metabolomics study*

5.2 Revised objectives

Date of approval from the RGC: N/A

Reasons for the change: _____

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_____

5.3 Realisation of the objectives

(Maximum 1 page; please state how and to what extent the project objectives have been achieved; give reasons for under-achievements and outline attempts to overcome problems, if any)

Objective 1:

To achieve this objective, doxorubicin (Dox)-resistant HepG2 cell line (R-HepG2) was developed. The *in vitro* cytotoxic effects of Dox with or without Danshen extract were investigated. It was found that the synergistic effect of Danshen extract on the cytotoxic effects of Dox in HepG2 and R-HepG2 is time-dependent, suggesting that long-term treatment of Danshen extract may help increase the cytotoxicity of Dox in both HepG2 and R-HepG2. Additionally, LO2 cells (normal liver cells line) were applied as the indicator of liver damage or protection caused by Dox. Danshen extract was found to induce a significant cytotoxicity in LO2 cells, suggesting Danshen extract may not be suitable for Hepatocellular carcinoma (HCC). Four major active ingredients of Danshen extract were selected to determine their *in vitro* cytotoxic effects in R-HepG2 and LO2. Tanshinone IIA was found synergistically enhanced the cytotoxicity of Dox in R-HepG2 cells but had minimum cytotoxic effect to LO2. The underlying mechanism of Danshen extract and Tanshinone IIA was investigated using Western blot. Various pathways such as the expressions of N-cadherin, vitmentin, and MMP-13 and caspases, nuclear factor kappa-B were measured and compared.

Objective 2:

To achieve this objective, two *in vivo* studies were performed. In the first *in vivo* study, it was found that administrations of 2 mg/kg bw and 5 mg/kg bw Dox increased the tumor size. ED_{50} of Dox could not be determined. 1.5 mg/kg bw Dox was selected. Further reduction of the dosage of Dox was found to be not effective to reduce the tumor size. For Tanshinone IIA 30 mg/kg bw was found to be close to the ED_{50}. The second *in vivo* studies were performed using the following experimental grouping.

- Group 1: Control group (nude mice without R-HepG2 xenograft)
- Group 2: Model group (nude mice with R-HepG2 xenograft)
- Group 3-4: Dox (1.5 mg/kg bw) & Tanshinone IIA (30 mg/kg bw) groups (nude mice with R-HepG2 xenograft plus either selected dosage of Dox or ED_{50} of Tanshinone IIA)
- Group 5-7: Dox+Tanshinone IIA-A, Dox+Tanshinone IIA-B & Dox+Tanshinone IIA-C groups (nude mice with R-HepG2 xenograft plus selected dosage of Dox in combination with Tanshinone IIA, ¼ of the ED_{50}, ½ of the ED_{50} and the ED_{50}, respectively)

Objective 3:
To achieve this objective, serum metabolites in various experimental groups will be extracted and isolated for ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) analysis. The serum metabolite changes in various treatment groups were investigated.

Objective 4:
To achieve this objective, the expressions of sodium-hydrogen exchanger isoform-1 (NHE-1), P-glycoprotein (P-gp) and multidrug resistance (MDR)-associated protein 1 (MRP-1) in R-HepG2 tumors xenograft in different were compared using qPCR.

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. To investigate the *in vitro* cytotoxic effects of doxorubicin with or without Danshen extract and their underlying mechanisms	✓	100%
2. To evaluate the effectiveness of the combination uses of doxorubicin with Danshen extract on drug-resistant HCC in nude mice	✓	100%
3. To use metabolomics approach for the comparison of serum metabolic changes in normal nude mice, nude mice with xenograft and nude mice with xenograft treated with doxorubicin in combination with Danshen extract	✓	100%
4. To investigate whether the anti-tumor effect of using doxorubicin in combination with Danshen extract is via the expressions of sodium-hydrogen exchanger isoform-1 (NHE-1), P-glycoprotein (P-gp) and multidrug resistance (MDR)-associated protein 1 (MRP-1) as well as the potential systemic pathways identified by metabolomics study	✓	100%

6. Research Outcome

6.1 Major findings and research outcome
(Maximum 1 page; please make reference to Part C where necessary)

The *in vitro* cytotoxic effects of Dox with or without Danshen extract were investigated in doxorubicin (Dox)-resistant HepG2 cell line (R-HepG2). Results showed that the synergistic effect of Danshen extract on the cytotoxic effects of Dox in HepG2 and R-HepG2 is time-dependent, suggesting that long-term treatment of Danshen extract may help increase the cytotoxicity of Dox in both HepG2 and R-HepG2. Dox in combination with Danshen extract would result in a greater magnitude of cytotoxicity especially in Dox-R-HepG2 cells compared with Dox is given alone (Figure 1). However, Danshen extract demonstrated higher cytotoxic effect on LO2 cells (normal liver cells line) (Figure 2). Screening of major active ingredients from Danshen extract has performed and identify Tanshinone IIA can synergistically enhanced the cytotoxicity of Dox in R-HepG2 cells and has no effect on cell viability of LO2 (Figure 3). The cytotoxic effect was found to be related to the upregulation of FASL and downregulations of BAX and CCND1 mRNA expression but not related to the MDR1 mRNA expression. The combination treatment of Dox and tanshinone IIA also modulate the protein expression of the metastasis-associated proteins in R-HepG2 cells (Figure 4). To evaluate drug interactions between Dox and tanshinone IIA, combination index (CI) values were calculated, tanshinone IIA was found to synergistically enhance the cytotoxicity of Dox in R-HepG2 cells (Table 1, with CI > 1). (Addressing objective 1 of the current study).

In the animal studies, Dox (1.5 mg/kg bw) and tanshinone IIA (30 mg/kg bw) alone could not provide a statistically significance in reducing the weight of the tumor induced by R-HepG2 xenograft in nude mice (Figure 5, $p > 0.05$). It is interesting to note that increasing the dose of Dox to 2 and 4 mg/kg bw could significant increase the tumor size (data shown in pervious progress report). Combining Dox (1.5 mg/kg bw) and tanshinone IIA (30 mg/kg bw) was found to significant reduce the weight of the tumor (Figure 5, $p < 0.05$). The serum metabolites in various experimental groups were analyzed and compared. It was found that the metabolomics profile of Dox and tanshinone IIA combination drug therapy group was significantly different from that of the model group but it was getting closer to that of the control group (Figure 6 and 7). The distribution of 19 metabolites in various treatment groups acquired in UPLC-Orbitrap-MS were shown in a heatmap (Figure 6 and 7). Our results also It was found that the effect of the combination therapy used in the current study is related to the alteration of mRNA expression of sodium-hydrogen exchanger isoform-1, NHE-1 (Slc9a1), but not multidrug resistance (MDR)-associated protein 1, MDR-1 (Abcb1a, Abcb1b), in the tumors (Figure 8). Further investigations on the mechanism(s) of the combination therapy on the expression of Slc9a1 and related pathways can help identify drug target site(s) to handle multidrug resistance in HCC. Data may provide a new direction on the therapy of HCC. Thus, further studies on comparing different NHE-1 agonists in combination with doxorubicin to handle multidrug resistance in HCC are proposed. It can help confirm the approach of using combination therapy in treating HCC. (Addressing objective 2-4 of the current study).

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6.2 Potential for further development of the research and the proposed course of action *(Maximum half a page)*

We will continue to investigate the possibility of using combination uses of doxorubicin with tanshinone IIA to treat multidrug resistance in HCC. Metabolomics study will also be continued to determine different serum biomarkers of HCC so as to clarify the pathogenesis of HCC and identify potential therapeutic targets and/or diagnostic markers for HCC. In this study we confirmed that using doxorubicin in combination with tanshinone IIA is an effective approach to handle multidrug resistance in HCC. In the metabolomics study, 19 metabolites was found to alter significantly in HCC animal treated with doxorubicin in combination with tanshinone IIA. It was found that the effect of the combination therapy used in the current study is related to the alteration of mRNA expressions of sodium-hydrogen exchanger isoform-1, NHE-1 (Slc9a1), but not multidrug resistance (MDR)-associated protein 1, MDR-1 (Abcb1a, Abcb1b), in the tumors. Further investigations on the mechanism(s) of the combination therapy on the expression of Slc9a1 and related pathways can help identify drug target site(s) to handle multidrug resistance in HCC. Data may provide a new direction on the therapy of HCC. Thus, further studies on comparing different NHE-1 agonists in combination with doxorubicin to handle multidrug resistance in HCC are proposed. It can help confirm the approach of using combination therapy in treating HCC.

7. Layman's Summary

(Describe <u>in layman's language</u> the nature, significance and value of the research project, in no more than 200 words)

Hepatocellular carcinoma (HCC) is responsible for a large proportion of cancer deaths in the world. China has one of the highest population of patients with liver cancer (every year with 350,000 new cases) which accounts for ~50% of the global liver cancer diagnosed, and liver cancer is one of the top killers in the Southeast China, including Hong Kong and Guangdong province. HCC classically arises and grows in asymptomatic fashion, making most HCC cases are only diagnosed in an advanced stage. When life expectancy falls to 4 to 6 months on average, chemotherapy is the only recommended treatment. For the non-specific cytotoxic effects of most anticancer drugs, they may cause drug resistance, toxicities and side effects that result in treatment withdrawal. Therefore, improving treatment with less toxicity is an essential component of cancer therapy. Our study showed that using doxorubicin in combination with tanshinone IIA, an active ingredient from Danshen, provided synergistic effect in treating nude mice with HCC xenograft. It can establish a foundation for the future pre-clinically and clinical studies using combination drug therapy, which help to improve existing strategies for HCC prevention and treatment. The development of better HCC treatment can reduce the prevalence of HCC.

Part C: Research Output

8. Peer-Reviewed Journal Publication(s) Arising Directly From This Research Project

(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(denote the corresponding author with an asterisk*)*	Title and Journal / Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the Institutional Repository *(Yes or No)*
Year of Publication	**Year of Acceptance** *(For paper accepted but not yet published)*	**Under Review**	**Under Preparation** *(optional)*						
N/A	**N/A**	**N/A**	**Under Preparation**	**Zhang H, Ng YF, Kwan YW, Mok DKW, Chan SW***	**Cytotoxic effect of Salvia Miltiorriza Bunge (Danshen) extract in doxorubicin-resistant HepG2 cells. Evidence-Based Complementary and Alternative Medicine.**	**No**	**No**	**Yes**	**N/A**
N/A	**N/A**	**N/A**	**Under Preparation**	**Ng YF, Zhang H, Man KY, Kwan YW, Mok DKW, Chan SW***	**The synergistic effect of using doxorubicin in combination with tanshinone IIA in treating drug-resistant hepatocellular carcinoma xenograft in vivo. Frontiers in Pharmacology.**	**No**	**No**	**Yes**	**N/A**

9. Recognized International Conference(s) In Which Paper(s) Related To This Research Project Was / Were Delivered

(Please attach a copy of each conference abstract)

Month / Year / Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the Institutional Repository *(Yes or No)*
May / 2023 / Osaka, Japan	The synergistic effect of using doxorubicin in combination with tanshinone IIA in treating drug-resistant hepatocellular carcinoma xenograft in vivo	9th Annual World Congress of Food and Nutrition 2023	No	No	Yes	No

10. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning

(Please elaborate)

The research experience and new knowledge generated from the current project has been contributed to the development of final year projects of BSc (Hons.) in Testing and Certification and BSc (Hons.) in Food Science and Safety. The established research technique has been used in the practical sessions and developing teaching and learning materials of some BSc (Hons.) in Testing and Certification teaching modules, such as Biochemical & DNA Technologies and Advanced Instrumentation Analysis.

11. Student(s) Trained

(Please attach a copy of the title page of the thesis)

Name	Degree Registered for	Date of Registration	Date of Thesis Submission / Graduation
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

12. Other Impact

(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, teaching enhancement, etc.)

This project further consolidates the collaboration between the Hong Kong Polytechnic University and the Technological and Higher Education Institute of Hong Kong (THEi). Additionally, new research collaborations on related area have been established between other higher education institutions, such as Tung Wah College. Recently, our team have secured another Faculty Development Scheme (FDS) project entitled, “A mechanistic study on the

combined use of esculetin and probiotics in preventing Parkinson's disease in mice" (Ref. No.: UGC/FDS25/M03/21). It is another project about using combination therapy to treat chronic diseases.

13. Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (please specify)	
No. of outputs arising directly from this research project	2 (under preparation)	1	0	0	Type	No.
					Undergraduate final year projects	4

14. Public Access Of Completion Report

(Please specify the information, if any, that cannot be provided for public access and give the reasons.)

Information that Cannot Be Provided for Public Access	Reasons
Nil	