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The Research Grants Council of Hong Kong
NSFC/RGC Joint Research Scheme
Joint Completion Report

(Please attach a copy of the completion report submitted to the NSFC by the Mainland researcher)

Part A: The Project and Investigator(s)

1. Project Title

Reversal of P-gp-mediated Paclitaxel Resistance: Identification of Modulator-binding Site on P-gp and Rational Design of Next Generation P-gp Modulators

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

	Hong Kong Team	Mainland Team
Name of Principal Investigator *(with title)*	Prof. Ming-cheung Larry CHOW	Prof. Yan SUN
Post	Professor	Professor & Cheung Kong Scholar
Unit / Department / Institution	Department of Applied Biology and Chemical Technology / The Hong Kong Polytechnic University	School of Chemical Engineering and Technology / Tianjin University
Contact Information	larry.chow@polyu.edu.hk	ysun@tju.edu.cn
Co-investigator(s) *(with title and institution)*	N.A.	Dr. Fufeng LIU / Tianjin University

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted)*
Project Start date	1 January 2016	N.A.	
Project Completion date	31 December 2019		
Duration *(in month)*	48		
Deadline for Submission of Completion Report	31 December 2020		

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1) Identification of flavonoid-binding sites on P-gp by photocrosslinking and mass spectrometry;
2) Molecular simulation of interaction between flavonoid compounds, PTX and P-gp;
3) Design, synthesis and characterization of next generation flavonoid compounds to improve paclitaxel bioavailability.

5.2 Revised Objectives
N.A.

6. Research Outcome

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

Please see next page.

(1) Identification of flavonoid-binding sites on P-gp by photocrosslinking and mass spectrometry

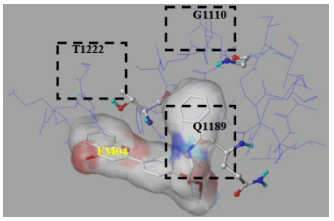
Based on the parent compound FM04, seven FM04 derivatives with photo-crosslinking (phenyl azide or diazirine) and click chemistry functional groups (azide or alkyne) were synthesized. FM04X1, FM04H1, FM04X4 were active in reversing the P-gp-mediated resistance with EC_{50} from 66 to 110 nM compared to parent compound FM04 (90 nM).

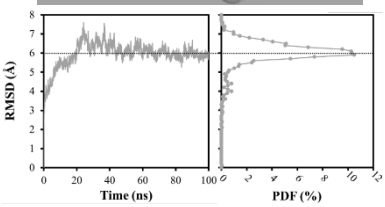
FM04 90 ± 15.0 nM	FM04X1 110 ± 10.1 nM	FM04X2 733 ± 32.2 nM	FM04X3 602 ± 20.4 nM
FM04X4 66 ± 8.5 nM	FM04X2-2 Inactive	FM04H1 107 ± 15.7 nM	FM04H2 3010 ± 100.8 nM

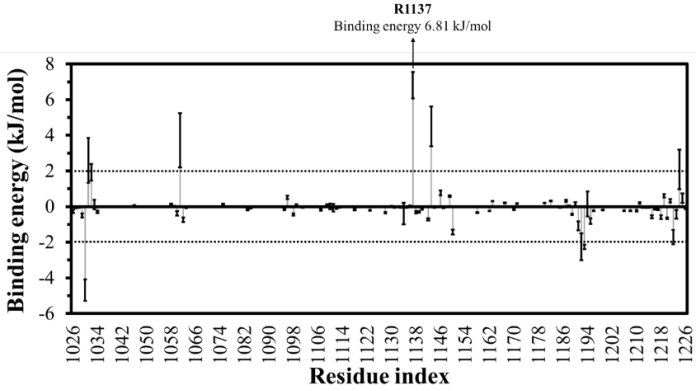
Mouse MDR3 overexpressed in *P. pastoris* was pulled down by FM04X4 or FM04H1 after photo-crosslinking and clicked with biotin-PEG_4-N_3. Purified P-gp was subjected to protease digestion and analyzed by LC-MS/MS to identify the peptides with an expected increase in mass. Sequence coverage was 80.7%. Together, FM04X4 and FM04H1 can crosslink to peptides Q1189-K1216 (identified 4 times), Q1189-K1208 (identified once), T1222-K1246 (identified once) and G1110-1119 (identified twice). MSMS analysis further pinpointed photocrosslinked residues to Q1189, T1222 or C1223, G1110 or I1111.

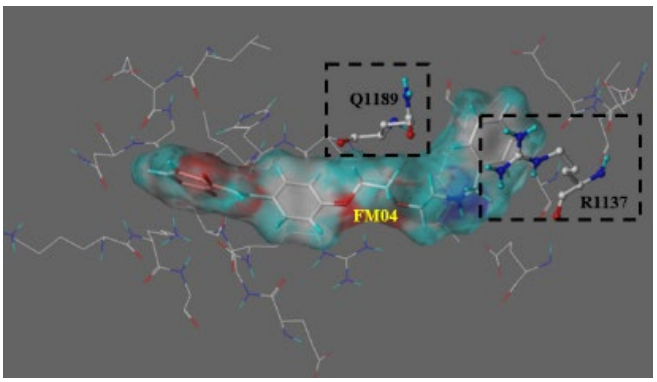
(2) Molecular simulation of interaction between flavonoid compounds, PTX and P-gp

FM04 was docked into the ligand binding site of the P-gp (PDB code: 4M1M) with Q1189 as the center of grid box. Topology of FM04 was constructed by Automated Topology Builder (ATB) and analyzed by molecular dynamics (MD) with P-gp using Gromacs. The root means square deviation (RMSD) and probability distribution function (PDF) results showed that the system stabilized after 50 ns. From the time of 90-100 ns, the average binding energy and standard deviation between each amino acid and FM04 was calculated and it was found that R1137 has largest clash with FM04. The charged side chain of R1137 was spatially close to the hydrophobic benzene ring of FM04, further supporting the MD results.



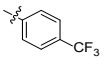
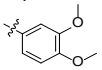
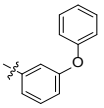
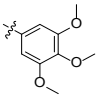
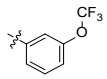
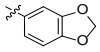






3) Design, synthesis and characterization of next generation flavonoid compounds to improve paclitaxel bioavailability.

Based on MD data, ten new FM04 derivatives were synthesized and their P-gp reversing activity was measured. It was found that FM04i has the highest potency (EC_{50} = 53.7 ± 4.7nM) which is significantly lower than the parent compound FM04 (EC_{50} = 90 ± 15.0 nM).

Compound	R=	EC_{50} (nM)	Compound	R=	EC_{50} (nM)
FM04		90 ± 15.0			
FM04a		146.8 ± 35.6	FM04f		253.0 ± 76.6
FM04b		69.3 ± 9.4	FM04g		315.2 ± 72.2
FM04c		63.5 ± 7.6	FM04h		141.7 ± 42.5
FM04d		66.0 ± 15.9	FM04i		53.7 ± 4.7
FM04e		117.5 ± 31.0	FM04k		99.3 ± 18.6

Summary

FM04 a potent P-gp modulator but with unknown mechanism of action. This project has successfully used photocrosslinking plus mass spectrometry to identify the target of FM04 on P-gp. Three residues (Q1189, G1110 and T1222) were photocrosslinked to FM04 photo-crosslinking derivatives. MD suggested that FM04 is in close contact with R1137. Second generation FM04 derivatives were synthesized with improved potency (FM04i with EC_{50}= 53.7nM). This suggests that the FM04 is modulating P-gp via the ATP-binding domain on P-gp. Problems encountered: social unrest in 2019 at our campus (PolyU) has a significant impact on the research progress.

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

1) This project has resulted in the rational design of a new series of FM04 modulator, including the improved FM04i (EC_{50}=53.7 nM) with almost double the activity of parent compound FM04 (EC_{50}=90 nM)).

2) We will verify the FM04-target on P-gp (Q1189, G1110 and T1222) by site directed mutagenesis. It is anticipated that these residues are important in the regulation of the P-gp activity.

7. The Layman's Summary

(describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Multidrug resistance in cancer is a serious problem in effective chemotherapy. We have previously developed a series of novel compounds, based on natural product flavonoids, to reverse multidrug resistance in cancer by inhibiting P-glycoprotein which would otherwise pump the cancer drugs out of the cancer cells. This project has identified the detailed target amino acids on P-glycoprotein. The significance of this project is that we now have better understanding of how flavonoids interact with P-gp and be able to design and improve these P-gp modulators with higher activity.

Part C: Research Output

8. Peer-reviewed journal publication(s) arising directly from this research project

(Please attach a copy of each 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) *(**bold** the authors belonging to the project teams and 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 this Joint Research Scheme *(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)*						
2018	2018			**Wong ILK, Zhu X, Chan K-F, Law MC, Lo AMY, Hu X, Chow LMC * and Chan TH***	"Discovery of Novel Flavonoid Dimers To Reverse Multidrug Resistance Protein 1 (MRP1, ABCC1) Mediated Drug Resistance in Cancers Using a High Throughput Platform with "Click Chemistry" J Med Chem 2018 61(22):9931-9951	Yes (2020 completion report)	Yes	Yes	Yes
2021	2020		2020	**Liu Z, FF Liu, Cui, JH, Wong ILK, Chan Y-W, Sun G, Yan S*, Chan THC* and Chow L***	Reversal of P-gp mediated drug resistance: identification of modulator-binding sites on P-gp and rational design of next generation P-gp modulators	No	No	Yes	Yes

9. Recognized international conference(s) in which paper(s) related to this research project was/were delivered *(Please attach a copy of each delivered paper. All listed papers must acknowledge RGC's funding support by quoting the specific grant reference.)*

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 this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
2019	Identification of Flavonoid Monomer Binding Site on P-gp using Photo-crosslinking and LC-MS	Gordon Research Conference: Mechanisms of Membrane Transport Folding and Evolution in the Membrane Environment: New Ways of Understanding Transport Mechanisms	Yes (2020 Dec completion report)	Yes	Yes	Yes

10. 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
Liu, Zhen	Ph.D.	04-Jul-2016	Intended to submit in April 2020

11. Other impact *(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)*

1. Technology Transfer Award (PolyU) given to the highest licensing income received for the financial year at PolyU. 2016

2. Patent "Alkyne-, azide- and triazole-containing flavonoids as modulators for multidrug resistance in cancers" (IP-721). China patent application has been granted on 1-March-2017 (CN Patent Application No. 201380012019.1).

3. Patent application for "Triazole linked flavonoid dimers and method to treat glioblastoma" was filed on 28-6-2018 US 62/690,340 with LMC Chow, T-H William Chan, X Hu, ILK Wong and K-F Chan as inventors.

12. Statistics on Research Outputs *(Please ensure the summary statistics below are consistent with the information presented in other parts of this report.)*

	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 [or conference]	2 including 1 in preparation	1	0	1	N.A.