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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

Development of Beclin1-specific Autophagy Modulators to Inhibit Lung Cancer Cell Proliferation

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

	Hong Kong Team	Mainland Team
Name of Principal Investigator *(with title)*	Prof. Yanxiang ZHAO	Prof. Renxiao WANG
Post	Professor	Professor
Unit / Department / Institution	Dept. of Applied Biology and Chemical Technology / The Hong Kong Polytechnic University	State Key Laboratory of Bioorganic and Natural Products Chemistry / Shanghai Institute of Organic Chemistry / Chinese Academy of Sciences
Contact Information	yanxiang.zhao@polyu.edu.hk	wangrx@mail.sioc.ac.cn
Co-investigator(s) *(with title and institution)*	Dr. Vincent KENG, The Hong Kong Polytechnic University	Dr. Yongwen JIANG, Chinese Academy of Sciences

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted)*
Project Start date	1 Jan 2017	N.A.	
Project Completion date	31 Dec 2020	N.A.	
Duration *(in month)*	48	N.A.	
Deadline for Submission of Completion Report	31 Dec 2021	N.A.	

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1. Structure-based design of stapled peptides that target the Beclin1 coiled coil domain (joint effort by the Hong Kong team and the Mainland team);

2. Chemical synthesis of stapled peptides with designed sequence and hydrocarbon links (the Mainland team);

3. Biochemical characterization of the stapled peptides and structure-based optimization (joint effort by the Hong Kong team and the Mainland team);

4. Cell- and animal-based functional study of the stapled peptides in promoting autophagy and suppression lung cancer cell proliferation (the Hong Kong team).

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)

During this project period, we determined the crystal structure of the Beclin1-UVRAG coiled coil complex and showed that the Beclin1- UVRAG heterodimer interface was strengthened by extra hydrophobic pairings and electrostatically complementary interactions. Guided by this structure, we developed Beclin1-targeting stapled peptides by mimicking a short segment within its coiled coil domain. A hydrocarbon staple of 13-carbon length was added to each designed peptide to stabilize its α-helical structure. Computational screening and biochemical assays identified several lead candidates that specifically interacted with Beclin1 coiled coil domain, reduced its homodimerization and promoted Beclin1-Atg14L/UVRAG interaction. Further cell-based studies confirmed that one lead candidate Tat-SP4 induced autophagy in HEK293T cells in dose-dependent manner. This peptide also promoted the endolysosomal degradation of cell surface oncogene receptors like EGFR in multiple NSCLC cell lines. A manuscript reporting these findings was published in ***PNAS*** in 2018 (Part C, item 8[1]).

To further enhance the potency of the designed peptides, we conducted optimization by staple scanning and sequence permutation. Using Tat-SP4 as the initial template, we positioned the hydrocarbon staple on six different positions on the peptide but maintained the (*i, i*+*7*) linkage. Our results show that placing the hydrocarbon staple closer to the Beclin 1-peptide interface enhanced the binding affinity by ∼10- to 30-fold. One such peptide i7-01s showed potent anti-proliferative efficacy in cancer cells that overexpressed EGFR and HER2 by enhancing their endolysosomal degradation and inducing necrotic cell death. A manuscript reporting these findings was published in ***J. Med. Chem*** in 2021 (Part C, item 8[6]).

Besides these structure-based design efforts, the research team also carried out mechanistic studies to delineate the molecular pathways that drive tumorigenesis and metastasis. The HBx gene found in predominantly Asian genotype B of chronic hepatitis B virus (HBV) showed stronger tumorigenic effect than its common variants (Part C, item 8[2]). Schwann cell-specific Pten inactivation in mice led to enlarged inguinal white adipose tissue, thus suggesting a novel role of the sympathetic nervous system in adipose tissue development (Part C, item 8[3]). Additionally, the ZBTB20 gene was shown to be an oncogenic driver for liver cancer by downregulating PPARG and activating the Wnt/β-catenin pathway (Part C, item 8[4]). Lastly, a forward genetic Sleeping Beauty (SB) insertional mutagenesis screen identified CNYP2 and ACTN2 as two potential oncogenic drivers for cancer metastasis (Part C, item 8[4]).

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

With close inter-disciplinary collaboration between the Hong Kong and mainland labs, our research team successfully developed Beclin1-targeting stapled peptides and showed that these designed peptides interacted with Beclin1 with high binding affinity, promoted cellular autophagic flux and enhanced endolysosomal degradation of cell surface oncogenic receptors like EGFR and HER2. These peptides also showed potent anti-proliferative efficacy in cell- and animal-based models for lung cancer. Building upon these results, we plan to the following two directions in the future.

First of all, we plan to carry out mechanistic studies to understand how our designed peptides inhibit cancer cell proliferation. Our data so far suggests that these peptides induced necrotic cell death but not apoptosis. Although excessive autophagy has been linked to a form of necrotic cell death termed autosis, it is not clear whether our peptides would function in similar manner. We plan to investigate

this issue using a pharmacological approach, i.e. testing whether cell death induced by our designed peptides can be rescued by inhibitors of known cell death programs such as apoptosis, necroptosis, and pyroptosis etc. If one or a few cell death programs are confirmed to be involved, we will then proceed to knockdown key plays such as Bcl-2 in apoptosis and see if it would affect the anti-proliferative efficacy of our peptides. Overall, we hope to understand how our Beclin1-targeting peptides engage one or a few cell death programs to inhibit cancer cell proliferation.

Secondly, we also plan to expand the scope of our research from lung cancer to other cancer types. Autophagy is known to have an intimate and complex relationship with cancer, acting like a double-edged sword in context-dependent manner. We initially proposed to investigate the impact of our designed peptides on lung cancer because Beclin1 was reported to play a major role in regulating the endolysosomal degradation of EGFR, a major driver oncogene in lung cancer. In fact, Beclin1 is monoallelically deleted in 40-75% cases of human sporadic breast, ovarian and prostate cancer. Thus we hope to test the anti-tumor efficacy of our designed peptides in these cancer types too. We plan to conduct further rounds of optimization to enhance the potency and specificity of our peptides and then assess their potency in cell- and animal-based models of these cancer types. Overall, we hope our work will validate the autophagy process and Beclin1 as suitable therapeutic targets for cancer drug discovery.

7. The 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)

Autophagy is an evolutionarily conserved process that degrades and recycles cytosolic content to maintain cellular homeostasis. Beclin1 is an essential autophagy gene and also a tumor suppressor with frequent monoallelic deletion in human cancer cases. There is intense interest to develop Beclin1-targeting candidates to modulate autophagy and serve as potential cancer therapeutics.

Our research team determined the crystal structure of the Beclin1 coiled coil domain and its complex with UVRAG, a major autophagy modulator. Guided by these structures, we used computational modeling and virtual screening to develop stapled peptides that would bind to Beclin1 coiled coil domain. A hydrocarbon stapling was added to each peptide to maintain its α-helical structure, a structural feature essential for Beclin1 binding. Lead candidates identified by computational screening were then chemically synthesized and functionally characterized. One of our designed peptides was shown to bind to Beclin1 with high affinity and promoted autophagic flux in multiple cell lines. Additionally, this peptide enhanced the endolysosomal degradation of oncogenic receptor EGFR in multiple lung cancer cell lines and inhibited their proliferation.

In summary, our study has demonstrated that Beclin 1-targeting stapled peptides may serve as effective autophagy modulators to inhibit cancer cell proliferation.

<u>Part C: Research Output</u>

8. Peer-reviewed journal publication(s) arising <u>directly</u> 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				Wu S, He Y, Qiu X, Yang W, Liu W, Li X, Li Y, Shen HM, **Wang R**, Yue Z, **Zhao Y***	"Targeting the potent Beclin1–UVRAG coiled-coil interaction with designed peptides enhances autophagy and endo lysosomal trafficking" **Proc Natl Acad Sci USA.** 115 (25):E5669-E5678.	Yes (Dec. 2018)	Yes	Yes	Yes
2019				Chiu AP, Tschida BR, Sham T, Lo LH, Branden SM, Li X, Lo RC, Hinton DE, Rowlands DK, Chan C, Mok DM, Largaespada DA, Warner N, **Keng VW***	"HBx-K130M/V131I Promotes Liver Cancer in Transgenic Mice via AKT/FOXO1 Signaling Pathway and Arachidonic Acid Metabolism" **Mol Cancer Res.** 17(7):158201593	No	Yes	Yes	Yes

2020				Li X, Zhang SJ, Man KY, Chiu AP, Lo LH, To JC, Chiu CH, Chan CO, Mok DKW, Rowlands DK, **Keng VW***	"Schwann cell-specific Pten inactivation reveals essential role of the sympathetic nervous system activity in adipose tissue development" **BBRC** 531(2):118-124	No	Yes	Yes	Yes
2021				To JC, Chiu AP, Tschida BR, Lo LH, Chiu CH, Li X, Kuka TP, Chan WC, Bell JB, Moriarity BS, Largaespada DA*, **Keng VW***	"ZBTB20 regulates WNT/CTNNB1 signaling pathway by suppressing PPARG during hepatocellular carcinoma tumorigenesis" **JHEP Reports** 3(2): 100223	No	Yes	Yes	Yes
2021				Lo LH, Lam CY, To JC, Chiu CH, **Keng VW***	"Sleeping Beauty insertional mutagenesis screen identifies the pro-metastatic roles of CNPY2 and ACTN2 in hepatocellular carcinoma tumor progression" **BBRC** 541: 70-77.	No	Yes	Yes	Yes

2021				Yang Q, Qiu X, Zhang X, Wei X, Yu Y, Feng G, Li Y, **Zhao Y*** and **Wang R***	"Optimization of Beclin 1-Targeting Stapled Peptides by Staple Scanning Leads to Enhanced Antiproliferative Potency in Cancer Cells" **J. Med. Chem.** 64(18)13475-13486	No	Yes	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)*
3/2018/ Lucca (Barga), Italy	Structural study of the mammalian Beclin 2 to delineate its distinct roles in regulating autophagy and GPCR trafficking	2018 Gordon Research Conference Autophagy in Stress, Development and Disease	Yes (Dec. 2018)	Yes	Yes	Abstract attached for reference
7/2019/ Tianjin, P.R.China	Targeting the Beclin1 coiled coil domain for autophagy regulation: molecular mechanism and design of novel modulators	17th Chinese Biophysics Congress	No	Yes	Yes	Abstract attached for reference

8/2019/ Cold Spring Harbor, U.S.A.	A Beclin1-targeting stapled peptide induces cell death with mixed features and inhibits cancer cell proliferation	2019 Cold Spring Harbor Laboratory Cell Death Meeting	No	Yes	Yes	Abstract attached for reference

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
CHEN Jingyi	Ph.D.	September 2018	August 2022
ZHANG Shuqi	Ph.D.	September 2018	August 2022

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

- Patents:
(1) US patent application titled "Autophagy-inducing Peptide Analogs" Serial No. 62/355,883 granted on April 14th 2020
(2) China patent application titled "Beclin 1-targeting stapled peptides and their use as pharmaceutical agents" Serial No. 202110518586.0 filed on May 12th 2021

- Collaboration:
The research team has developed an extensive network of collaboration not only within Hong Kong and China but also internationally. Some examples of the research institutes we have collaborated with are listed below and grouped by the team member involved:

(Prof. YX Zhao)
School of Pharmacy, Fudan University, Shanghai, P.R.China
No. 2 Affiliated Hospital, Xi'an Jiaotong University, Xi'an, P.R.China
Department of Molecular and Cell Biology and Department of Chemistry, University of California at Berkeley, Berkeley CA, U.S.A.
Boston Children's Hospital, Harvard Medical School, Boston MA, U.S.A.
Ernest Mario School of Pharmacy, Rutgers University, Piscataway NJ, U.S.A.

(Dr. Vincent Keng)
Medical School, University of Minnesota, Twin Cities MN, U.S.A.

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]	6	3	N/A	1	N/A