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ABROGATION OF ONCOGENIC K-RAS FUNCTION BY ANDROGRAPHOLIDE DERIVATIVES VIA IN SILICO, IN VITRO, AND IN VIVO APPROACHES

QUAH SHUN YING

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By

QUAH SHUN YING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chair : Prof. Johnson Stanslas, PhD
Faculty : Medicine and Health Sciences

The rat sarcoma (Ras) proteins are small guanosine triphosphatases (GTPases) that act as molecular switches in major signalling pathways involved in cell proliferation, differentiation, and survival, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades. Ras exists in three isoforms – K-Ras, H-Ras, and N-Ras. Approximately 30% of all human cancers harbour Ras mutations, with the most frequently mutated isoform being K-Ras, which exclusively appears in pancreatic ductal adenocarcinoma (PDAC). Mutated K-Ras proteins are constitutively active with the GTPase activity being compromised. Oncogenic K-Ras is currently a valuable oncology target and its inhibition represents an important therapeutic strategy. Recent *in silico* study has revealed a direct binding of andrographolide (AGP) and its benzylidene derivatives, SRJ09 and SRJ23, to K-Ras oncoprotein, which abrogated its function and downstream MAPK signalling. The present study aims to investigate the potential of AGP derivatives as anti-Ras therapeutics through *in silico*, *in vitro*, and *in vivo* approaches. The anticancer potential of SRJ09 and SRJ23 has been well-demonstrated in the human colon (HCT-116) and prostate (PC-3) cancer cells, respectively. These two cell lines have been made resistant to the compounds previously and were used in the present study to examine the altered gene profile in relation to the expression of regulatory genes involved in the compounds' anticancer activity using microarray analysis. Regulatory genes associated with autophagy and apoptotic processes, such as *ATG12* and *HMOX1*, as well as MAPK and PI3K pathways, such as *FGF19* and *SPRY2* that play major roles in promoting cell growth and survival, were found to be altered. New benzylidene derivatives have been previously synthesised using SRJ09 and SRJ23 as parent compounds, yielding SRS compounds. In the present investigation, the most druggable binding pocket on K-Ras mutants namely p2 was revealed through *in silico* simulations. SRJ23 and SRS157 were found to bind *via* intermolecular hydrogen bonding to this pocket. The

anti-PDAC activity of selected AGP derivatives (SRJ23, SRJ09, SRS07, and SRS157) and their mechanisms of action were elucidated *in vitro*. SRJ23 and SRS157 were shown to perform differently particularly in terms of activity on Erk, a crucial signalling protein in the K-Ras-associated MAPK cascade. Its activation was unanticipatedly enhanced by SRJ23 and significantly suppressed by SRS157 upon 24-h treatment of the compounds. SRS07 presented as a superior anti-PDAC agent by promoting oxidative stress, possibly through enhancement of Akt activation in the K-Ras-mediated PI3K pathway. A simple pharmacokinetic study performed in BALB/c mice at a single dose of 100 mg/kg SRJ23 revealed that the compound achieved a maximum plasma concentration of 18.8 μM after 30 min of administration, with long half-life (4.28 h) and mean residence time (6.30 h). Subsequent *in vivo* antitumour study reported that 100 mg/kg SRS157 delayed the doubling of tumour growth in the PDAC-xenograft nude mouse model more effectively than SRJ23 at the same dose. In conclusion, the outcomes of the present study provide a strong indication of the potential of AGP derivatives, which specifically target the oncogenic K-Ras and abrogate its function, as promising clinical anti-pancreatic cancer candidates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMANSUHAN FUNGSI K-RAS ONKOGENIK OLEH DERIVATIF ANDROGRAPHOLIDE MELALUI KAEDAH-KAEDAH *IN SILICO*, *IN VITRO*, DAN *IN VIVO*

Oleh

QUAH SHUN YING

April 2021

Pengerusi : Prof. Johnson Stanslas, PhD
Fakulti : Perubatan dan Sains Kesihatan

Protein sarkoma tikus (Ras) adalah trifosfatase guanosin kecil (GTPases) yang bertindak sebagai pertukaran molekul dalam laluan isyarat utama yang terlibat dalam percambahan, perbezaan, dan kelangsungan hidup sel-sel, contohnya laluan isyarat mitogen-activated protein kinase (MAPK) dan phosphatidylinositol 3-kinase (PI3K). Ras wujud dalam tiga isoform – K-Ras, H-Ras dan N-Ras. Anggaran 30% daripada semua kanser manusia mempunyai mutasi Ras dengan isoform yang paling kerap bermutasi adalah K-Ras yang dijumpai khasnya dalam adenokarsinoma saluran pankreas (PDAC). Protein K-Ras yang bermutasi aktif secara konstitutif disertai oleh aktiviti GTPase yang dikompromikan. Kini, K-Ras onkogenik merupakan sasaran onkologi yang berharga dan perencatanya merupakan satu strategi terapi yang penting. Kajian *in silico* terkini telah mendedahkan pengikatan langsung andrographolide (AGP) dan derivatif benzylidene (SRJ09 dan SRJ23) pada protein mutan K-Ras dimana ia dapat memansuhkan fungsi dan isyarat MAPK. Kajian ini bertujuan untuk menyelidik potensi derivatif AGP sebagai terapeutik anti-Ras melalui kaedah-kaedah *in silico*, *in vitro*, dan *in vivo*. Potensi anti-kanser SRJ09 dan SRJ23 terhadap sel-sel kanser usus besar manusia (HCT-116) dan prostat (PC-3) telah didedahkan. Kedua-dua sel ini dicipta untuk menahan rintangan terhadap sebatian tersebut sebelumnya dan ia digunakan dalam kajian ini untuk memeriksa profil gen yang diubah berkait rapat dengan ekspresi gen pengawalselian melibatkan sebatian aktiviti anti-kanser dengan menggunakan analisis mikroarray. Gen pengawalseliaan yang berhubungan dengan proses autophagy dan apoptosis, seperti *ATG12* dan *HMOX1*, serta laluan isyarat MAPK dan PI3K, seperti *FGF19* dan *SPRY2* yang memainkan peranan utama dalam menggalakkan pertumbuhan sel dan kelangsungan hidup, telah didapati terjejas. Derivatif benzylidene yang baru disintesis sebelumnya dengan menggunakan SRJ09 dan SRJ23 sebagai sebatian induk telah menghasilkan sebatian SRS. Dalam penyiasatan ini, poket pengikat yang diramalkan paling sesuai digunakan pada protein mutan K-Ras iaitu p2 telah

didedahkan melalui simulasi *in silico*. SRJ23 dan SRS157 didapati mengikat pada poket ini melalui ikatan hidrogen antara molekul. Kegiatan anti-PDAC sesetengah derivatif AGP yang terpilih (SRJ23, SRJ09, SRS07, dan SRS157) dan mekanisme tindakan mereka telah didedahkan secara *in vitro*. SRJ23 dan SRS157 bertindak secara berbeza terutamanya dari segi aktiviti pada Erk (satu protein isyarat yang penting dalam laluan isyarat MAPK berhubungan dengan K-Ras. Pengaktifan ditingkatkan tanpa disangka-sangka oleh SRJ23 tetapi ditindas dengan ketara oleh SRS157 selepas rawatan sebatian selama 24 jam. SRS07 telah ditunjukkan kemampuannya sebagai agen kanser anti-PDAC yang unggul dengan menggalakkan tekanan oksidatif dan meningkatkan pengaktifan Akt dalam laluan isyarat PI3K yang dimediasi oleh K-Ras. Kajian farmakokinetik yang dilakukan pada tikus BALB/c dengan menggunakan dos tunggal 100 mg/kg SRJ23 telah menunjukkan bahawa sebatian tersebut mencapai kepekatan plasma maksimum iaitu 18.8 μM selepas setengah jam pemberian dengan jangka hayat (4.28 jam) dan purata masa ketinggalan (6.30 jam) yang panjang. Kajian anti-kanser *in vivo* selanjutnya melaporkan bahawa 100 mg/kg SRS157 memperlambat penggadaan pertumbuhan tumor pada model tikus PDAC-xenograft dengan lebih berkesan berbanding dengan SRJ23 pada dos yang sama. Kesimpulannya, hasil kajian ini memberi petunjuk yang lebih kuat mengenai potensi derivatif AGP sebagai calon-calon klinikal untuk anti-kanser pankreas yang menjanjikan, di mana secara khususnya mereka boleh menyasarkan dan memansuhkan fungsi K-Ras onkogenik.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Johnson Stanslas, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Ho Kok Lian, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Nizar bin Abd Manan, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Pran Kishore Deb, PhD
Associate Professor
Faculty of Pharmacy
Philadelphia University
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 9 September 2021

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of Chairman of Supervisory Committee: Prof. Dr. Johnson Stanslas

Signature: _____
Name of Member of Supervisory Committee: Associate Prof. Dr. Ho Kok Lian

Signature: _____
Name of Member of Supervisory Committee: Dr. Nizar bin Abd Manan

Signature: _____
Name of Member of Supervisory Committee: Associate Prof. Dr. Pran Kishore Deb

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0QR	N-(6-aminopyridin-2-yl)-4-fluorobenzenesulfonamide
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, and excretion
AGP	Andrographolide
ANOVA	Analysis of variance
APS	Ammonium persulphate
APT1	Acyl protein thioesterase 1
aRNA	Anti-sense RNA
ATG12	Autophagy-related gene 12
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-xL	Bcl-2-extra large
BH3	Bcl-2 homology 3
BSA	Bovine serum albumin
BWC	Body weight change
Cα	Alpha carbon
CADD	Computer-aided drug design
Cdc25	Cell division cycle 25
cDNA	Complementary DNA
CGM	Complete growth medium
CO	Carbon monoxide
CO_2	Carbon dioxide
COSMIC	The Catalog of Somatic Mutations in Cancer

CPU	Central processing unit
CRD	Cysteine-rich domain
cRNA	Complementary RNA
CSC	Cancer stem cell
CV	Coefficients of variation
CYP	Cytochrome P450s
DCFDA	2',7'-dichlorofluorescein diacetate
DDAG	14-deoxy-11,12-didehydroandrographolide
DEGs	Differentially expressed genes
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	EGF receptor
EMT	Epithelial-mesenchymal transition
ERBB4	Erb-B2 receptor tyrosine kinase 4
Erk	Extracellular signal-regulated kinase
EtBr	Ethidium bromide
FBS	Foetal bovine serum
FDR	False discovery rate
FGF19	Fibroblast growth factor 19
FGFR4	Fibroblast growth factor receptor 4
GLOBOCAN	Global Cancer Statistics
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GI_{50}	Drug concentration that inhibits cell growth by 50%
Glide	Grid-based Ligand Docking with Energetics
GO	Gene Ontology
Grb2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
H-bond	Hydrogen-bond
H&E	Haematoxylin-eosin
HKP	Housekeeping proteins
HMOX1	Haem oxygenase 1
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HVR	Hypervariable region
IC_{50}	Inhibitory concentration of a drug that reduces cell population to half of the control value
IC_{80}	Drug concentration that reduces cell population by 80%, relative to vehicle control
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICMT	Isoprenylcysteine carboxymethyl transferase
IgG	Immunoglobulin
i.p.	Intraperitoneal
IVC	Individually ventilated cage

INHAND	International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice
KITLG	KIT ligand
LBDD	Ligand-based drug discovery
LC_{50}	Drug concentration that kills cells by 50%
LC3	Microtubule-associated protein 1A/1B-light chain 3
LOD	Limit of detection
LOQ	Limit of quantitation
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
MD	Molecular dynamics
ME	Matrix effect
Mek	MAPK/ERK kinase
MM-GBSA	Molecular mechanics-generalised Born surface area
MOMP	Mitochondrial outer membrane permeabilisation
MOPS	3-(N-morpholino)propane sulfonic acid
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
mTORC	Mechanistic target of rapamycin complex
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NCI	National Cancer Institute
NCI-60	NCI's panel of 60 cancer cell lines
NF-kB	Nuclear factor kappa B
Nrf-2	Nuclear factor erythroid-2-related factor 2
OECD	The Organisation for Economic Co-operation and Development
OH	Hydroxyl

OPLS	Optimised Potentials for Liquid Simulations
p-loop	Phosphate-binding loop
p70S6K	p70 ribosomal protein S6 kinase
PAGE	Polyacrylamide Gel Electrophoresis
PanIN	Pancreatic intraepithelial neoplasia
PAT	Protein acyltransferase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PDB	Protein Data Bank
PDE6δ	Phosphodiesterase 6 delta
PDK1	3-phosphoinositide-dependent protein kinase 1
PE	Process efficiency
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
PK	Pharmacokinetics
PKB	Protein kinase B
PPI	Protein-protein interaction
PTEN	Phosphatase and tensin homolog
R^2	Correlation coefficient
RAD	Ras-association domain
Ras	Rat sarcoma

RasGRF	Ras guanylnucleotide releasing factors
RBD	Ras binding domain
Rce-1	Ras-converting enzyme type 1
RCSB	Research Collaboratory for Structural Bioinformatics
RE	Recovery
Rheb	Ras homolog enriched in brain
RT	Retention time
RIN	RNA integrity number
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RNase	Ribonucleases
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RTK	Receptor tyrosine kinase
RTV	Relative tumour volume
SAR	Structure-activity relationship
SBDD	Structure-based drug discovery
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SH2	Src homology 2
SHC	Src homology 2 domain-containing
SID	Simulation Interaction Diagram
SOS	Son of Sevenless
SPRY2	Sprouty homolog 2

SPSS	Statistical Package for Social Sciences
SRJ09	3,19-(2-bromobenzylidene)andrographolide
SRJ23	3,19-(3-Chloro-4-fluorobenzylidene)-andrographolide
STD-NMR	Saturation transfer difference nuclear magnetic resonance
STRING	Search Tool for the Retrieval of Interacting Genes/ Proteins
T/C	Optimal treatment-to-control ratio
TAE	Tris-acetate-ethylenediaminetetraacetic acid
T_d	Tumour growth delay
TGI	Drug concentration that totally inhibits cell growth
TSC	Tuberous sclerosis complex
TuGI	Tumour growth inhibition
vdW	van der Waals

CHAPTER 1

INTRODUCTION

1.1 Overview of the Study

Cancer is one of the most common chronic diseases, affecting both men and women globally, with increasing prevalence over the past three decades (Tu et al., 2017). In 2018, more than 18 million new cancer cases have been diagnosed and the number has been increasing annually (Bray et al., 2018). The major risk factors of cancer are often associated with aging and growth of the world population (Weir, Thompson, Soman, Møller, & Leadbetter, 2015). Cancer has been recognised as the second leading cause of death worldwide (Bray et al., 2018). Considering population growth and increased urbanisation, the number of cancer patients is expected to rise to 29.5 million by the year 2040, with an estimated 73% increase in the number of cancer-related deaths worldwide (NCI, 2020).

Fundamentally, human cancers involve a transformation of normal cells into tumorous cells at both the genetic and molecular levels of the organism (Bukhtoyarov & Samarin, 2015). One of the initial phases of cancer cell transformation is the activation and/or expression of oncogenes, which drive the tumour initiation and cancer progression (Vicente-Dueñas, Romero-Camarero, Cobaleda, & Sánchez-García, 2013). *RAS* oncogenes, which possess a central role in regulating cell growth and survival, are among the first to be discovered and often found to harbour point mutations in a wide spectrum of human cancers. Three functional *RAS* oncogenes (*HRAS*, *NRAS*, and *KRAS*) have been identified since the 1980s, and they encode highly similar proteins (H-Ras, N-Ras, K-Ras) with inherent guanosine triphosphatase (GTPase) activity (Wennerberg, Rossman, & Der, 2005). It is estimated now that over 30% of all human tumours have activating mutations in one of the *RAS* genes (Kodaz et al., 2017). Mutations on *KRAS* constitute more than 80% of all *RAS* mutations (Arrington et al., 2012; Hobbs, Der, & Rossman, 2016). Analysis of a significant number of human tumours has demonstrated a variation in mutant *KRAS* incidence among different tumour types examined (Bos, 1989). In particular, the highest incidence of mutated *KRAS* genes is found in tumours from the exocrine pancreas (> 90%), typically pancreatic ductal adenocarcinoma (PDAC), followed by colorectal cancers (30% – 50%) and lung cancers (> 30%).

Being a key node in cellular signalling responsible for growth and survival, oncogenic K-Ras activates several downstream effectors, including canonical mitogen-activated protein kinase (MAPK) and phosphoinositide

3-kinases (PI3K) (Eser, Schnieke, Schneider, & Saur, 2014), resulting in constitutive activation of these effector signalling pathways. Tumours with activating *KRAS* mutations were found to be resistant to standard therapies (Lu, Jang, Gu, Zhang, & Nussinov, 2016). To date, the existing cancer drugs available in the market are not catered to cancer patients with mutations in *KRAS*, such as PDAC, which is an aggressive disease characterised by poor prognosis and therapeutic resistance. Therefore, the search for better treatment, using K-Ras oncoprotein as the central anticancer therapeutic target, is especially urgent.

The bioactive compound from the well-known herb *Andrographis paniculata*, andrographolide (AGP), has been studied for its anticancer properties for nearly two decades by the Stanslas' group (Stanslas et al., 2001; Jada et al., 2006; Jada et al., 2007). Structural modifications of AGP led to the generation of benzylidene derivatives, namely 3,19-(2-bromobenzylidene)andrographolide (SRJ09) and 3,19-(3-chloro-4-fluorobenzylidene)-andrographolide (SRJ23), with improved potency and selectivity of anticancer activity (Jada et al., 2008). Further modifications on SRJ09 and SRJ23 yielded SRS07 and eight derivatives (SRS150 – SRS157), respectively. Using *in silico* molecular simulation study of protein-ligand interactions, a previous study revealed that these benzylidene derivatives were capable of binding oncogenic K-Ras directly (Hocker et al., 2013). With this direct binding, these compounds demonstrated remarkable inhibition on the activation of K-Ras and its downstream signalling molecules of the MAPK pathway, thereby jeopardising the viability of the cancer cells harbouring either wild-type or oncogenic K-Ras.

The present investigation is conceptualised based on the possible attenuation of the aberrant mutant K-Ras-mediated signalling pathways by benzylidene derivatives of AGP *via* binding to a druggable allosteric site in the protein. The study further explored the preclinical antitumour potential of these AGP derivatives in PDAC tumour xenograft nude mice model.

1.2 Hypothesis

The semisynthetic AGP derivatives bind oncogenic K-Ras proteins, abrogating their functions and exerting anticancer activity through their inhibitory effects on K-Ras-mediated signalling pathways, such as MAPK and PI3K cascades, as well as reduce the tumour growth in preclinical xenograft models.

1.3 Objectives

1.3.1 General Objective

The main objective of this study was to investigate the potential of AGP derivatives as anti-Ras therapeutics through *in silico*, *in vitro*, and *in vivo* approaches.

1.3.2 Specific Objectives

The specific objectives as follows:

i. To decipher the mechanisms of action and mechanisms of acquired resistance of SRJ09 and SRJ23 in colorectal and prostate cancer cells.

ii. To identify the binding modes and molecular interactions of the AGP derivatives with K-Ras proteins *via in silico* approach.

iii. To determine the *in vitro* growth inhibitory effects of AGP derivatives on a panel of PDAC cell lines harbouring either wild-type or oncogenic K-Ras, as well as their effects on K-Ras-mediated MAPK and PI3K pathways.

iv. To determine the pharmacokinetics of SRJ23 in mice.

v. To evaluate the preclinical antitumour activity of SRJ23 and SRS157 against PDAC tumour xenografts in nude mice.

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BIODATA OF STUDENT

Quah Shun Ying was born in Penang, Malaysia on 12th November, 1991 as the eldest daughter in the family. She graduated from Penang Chinese Girls High School, Penang, Malaysia in 2008, and achieved her degree in Bachelor of Science (Chemistry and Biology) with Magna cum Laude in 2013 from Campbell University, Buies Creek, North Carolina, United States of America. During her degree study, she obtained a few awards including Book Prize Award, The Matthew Baillie Prize for Chemistry and The Samuel Martin Brown Prize for Biology.

Same year after graduation, she started work as a research analyst in AcuBiz Consulting Sdn. Bhd., Kuala Lumpur, Malaysia, mainly involved in collecting data needed for consulting projects through extensive secondary and primary research, analysing data collected and preparing project reports under the guidance of project supervisor. She then worked as a research assistant in Pharmacotherapeutics Lab, Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Malaysia.

In 2015, Shun Ying pursued her study further with a degree of Ph.D. in Pharmacology and Toxicology, under the supervision of Prof. Dr. Johnson Stanslas from the same faculty. Her research studies mainly involved cancer and molecular pharmacology and toxicology. During her course of Ph.D. study, she participated in several scientific conference events, including the 1st MACR Scientific Conference organised in MAHSA University, Selangor, Malaysia, of which she was awarded with MACR Student Award (3rd Prize). She also became the Assistant Secretary of The International Conference on Drug Discovery and Translational Medicine 2018 (ICDDTM '18) organized by the Malaysian Association for Cancer Research.

LIST OF PUBLICATIONS

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