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CYTOTOXICITY OF SRJ23 AND ITS DERIVATIVES, EXPRESSION AND SRJ23 BINDING OF KRAS G12V ONCOPROTEIN

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By

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CYTOTOXICITY OF SRJ23 AND ITS DERIVATIVES, EXPRESSION AND SRJ23 BINDING OF KRAS G12V ONCOPROTEIN

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December 2017

Chair: Professor Johnson Stanslas, PhD
Faculty: Medicine and Health Sciences

Oncogenic KRas signaling is often associated with a poor prognosis of pancreatic cancer. Effort to target oncogenic KRas signaling persists for years but without much success due to its 'undruggable' property. In recent years, several small-molecule KRas inhibitors such as SRJ23 were developed to inhibit oncogenic KRas signaling at the guanine nucleotide exchange level. MTT cell viability assay was used to evaluate the cytotoxicity of SRJ23 and its derivatives towards breast, colon, prostate and pancreatic cancer cell lines. SRJ23 is a semi-synthetic derivative of andrographolide (AGP) which lacks a distinct selectivity towards specific cancer type by demonstrating an equally good cytotoxicity in breast, colon, prostate, and pancreatic cancer cell lines. A few novel derivatives of SRJ23 were synthesised to improve its target specificity. One of the derivatives (SRS 151) shows selective total growth inhibition on pancreatic cancer cells harbouring oncogenic KRas (MIA PaCa-2 and Capan-2). SRS157 (GI_{50} = 2.4 μM), although is as equally potent as SRJ23 (GI_{50} = 2.1 μM) in pancreatic cancer cell lines, was found selectively targeting pancreatic cancer. Previous study has revealed a direct binding of SRJ23 to KRas *in silico*. To validate a physical interaction between oncogenic KRas G12V and SRJ23 *in vitro*, KRas G12V was expressed by using Champion pET SUMO protein expression system and was used for X-ray crystallography to study the physical interaction between KRas G12V and SRJ23. The hanging drop vapour diffusion method in X-ray crystallography did not yield diffractable protein crystals, therefore saturation transfer difference-nuclear magnetic resonance (STD-NMR) was applied to study KRas G12V-SRJ23 interaction. The STD-NMR suggested a potential physical interaction between KRas G12V and SRJ23 that involves mainly the three-membered ring and the hydroxyl group on the lactone ring of SRJ23. In conclusion, the findings of this study showed that SRJ23 could be a promising anti-Ras drug.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Sarjana Sains

SITOTOKSIKSITI SRJ23 DAN DERIVATIF, EKSPRESI DAN INTERAKSI SRJ23 DENGAN KRAS G12V ONKOPROTEIN

Oleh

MICHELLE TAN SIYING

Disember 2017

Pengerusi: Profesor Johnson Stanslas, PhD
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Isyarat sel berunsurkan KRas onkogenik sering dihubungkait dengan ramalan perkembangan kanser pancreas yang tidak menyenangkan hati. Usaha membanteras isyarat Ras onkogenik telah dijalankan bertahun-tahun tetapi tidak berjaya kerana sifat Ras yang sukar disasarkan oleh perencat. Sejak kebelakangan ini, beberapa molekul kecil seperti SRJ23 yang berpotensi menjadi perencat isyarat Ras onkogenik pada tahap 'guanine nucleotide exchange' telah ditemui. Ujian sitotoksik MTT digunakan untuk menentu aktiviti sitotoksik SRJ23 dan terbitannya terhadap payudara, kolon, prostat dan pankreas. SRJ23 ialah kompaun sintetik yang diterbit daripada andrographolide (AGP). Kompaun ini tidak mempunyai sasaran kanser yang nyata dengan menunjukkan aktiviti sitotoksik yang setara terhadap sel-sel kanser payudara, kolon, prostat dan pankreas. Beberapa terbitan SRJ23 telah disintesis agar terbitannya menyasarkan jenis kanser tertentu. Salah satu terbitannya (SRS151) didapati hanya bertindak ke atas pertumbuhan sel-sel kanser pankreas yang bergantung kepada ekspresi KRas onkogenik (MIA PaCa-2 and Capan-2). SRS157(GI_{50} = 2.4 μM) menunjukkan aktiviti sitotoksitik yang setanding SRJ23 (GI_{50} = 2.1 μM) ke atas sel-sel kanser pankreas, tetapi aktiviti sitotoksiknya adalah selektif ke atas kanser pankreas jika dibandingkan SRJ23. Kajian terdahulu membuktikan kewujudan ikatan langsung antara KRas dan SRJ23 *in silico*. Untuk mengesahkan interaksi fizikal antara protein onkogenik Kras G12V dan SRJ23 secara *in vitro*, KRas G12V dihasilkan melalui sistem ekspresi protein yang dikenali sebagai Champion pET SUMO dan digunakan dalam X-ray kristalografi untuk mengkaji kewujudan interaksi fizikal antara protein onkogenik KRas G12V dan SRJ23. Disebabkan oleh X-ray kristalografi menggunakan teknik 'hanging drop vapour diffusion' tidak menghasilkan kristal protein yang sesuai untuk diffraksi, teknik ini telah diganti dengan 'saturation transfer difference-nuclear magnetic resonance' (STD-NMR) untuk mengkaji interaksi KRas G12V-SRJ23. Kajian STD-NMR

mencadangkan kewujudan interaksi antara KRas G12V dan SRJ23. Interaksi ini melibatkan bahagian-bahagian pada struktur SRJ23 iaitu ‘three-membered ring’ dan kumpulan hidroksil yang terikat pada ‘lactone ring’. Pada kesimpulannya, hasil kajian ini menunjukkan SRJ23 berpotensi menjadi ubat anti-Ras.

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I certify that a Thesis Examination Committee has met on 29 December 2017 to conduct the final examination of Michelle Tan Siying on her thesis entitled "CYTOTOXICITY OF SRJ23 AND ITS DERIVATIVES, EXPRESSION AND SRJ23 BINDING OF KRAS G12V ONCOPROTEIN" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.
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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 Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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Name of Member of Supervisory Committee : Dr. Sreenivasa Rao Sagineedu

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PDAC	Pancreatic ductal adenocarcinoma
NSCLC	Non-small cell lung cancer
Ras-MAPK	Ras-mitogen activated protein kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K	Phosphoinositide 3-kinase
GEF	guanine nucleotide exchange factors
GDP	Guanine diphosphate
GTP	Guanine triphosphate
GAP	GTPase activating proteins
HVR	Hypervariable C-terminal region
Q61	Glutamine
AGP	Andrographolide
NCI	National Cancer Institute
MTT	Microculture tetrazolium
EGFR	Epidermal growth factor receptor
RalGDS	Ral guanine nucleotide dissociation stimulator
PLC_{ε}	Phospholipase C_{ε}
FTIs	Farnesyltransferase inhibitors
Ub	Ubiquitin
E. coli	*Escherichia coli*
AP	Ammonium persulfate
PCR	Polymerase chain reaction
NMR	Nuclear magnetic resonance
STD-NMR	Saturation transfer difference-nuclear magnetic resonance
PDB	Protein Data Bank
FDA	Food and Drug Administration
HTS	High-throughput screening
NADH	Nicotinamide adenine dinucleotide
DMSO	Dimethyl sulfoxide
SD	Standard deviation
SAR	Structure-activity relationship
SI	Selective index
IMAC	Ion metal affinity chromatography
SOS	Son of sevenless
NOE	Nuclear overhauser effect
Ppm	Parts per million
MD	Molecular dynamic

CHAPTER 1

INTRODUCTION

1.1 Overview

Cancer is the leading cause of death worldwide caused by loss of normal regulatory mechanisms which inhibit cell cycle progression. Breast, prostate, and lung cancers are among the top cancers being diagnosed currently with lung cancer remains as the top cancer killer (Torre et al., 2015). Thyroid cancer is expected to replace colorectal cancer as the fourth leading cancer being diagnosed by year 2030, while melanoma and uterine cancer stand at the fifth and sixth position of the most common cancer, respectively (Rahib et al., 2014). Pancreatic cancer is the third leading cause of cancer-related death in the United States surpassing breast cancer and expected to surpass prostate and colorectal cancer by year 2030 to become the second leading cause of cancer-related death in the United States (Rahib et al., 2014). Pancreatic cancer is considered deadly as it has the highest mortality rate of all major cancers and surgical resection is a possible curative treatment for only a minority of pancreatic cancer patients (10 to 15%) in the early stages (Jemal et al., 2005). Pancreatic cancer patients have low survival rates with a mortality rate of 97 to 98% within five years of diagnosis (Ghaneh et al. 2007). Most pancreatic cancer patients often die from a cancer-related death due to the late diagnosis of neoplasm which often occurs at the later stages of the disease, after local spread and distant metastases (Li et al., 2004). In an exacerbating way, the symptoms mostly appeared to be unspecific in nature (Ghaneh et al. 2007). Approximately 10% of pancreatic cancer cases are familial. Pancreatic ductal adenocarcinoma (PDAC) which accounts for more than 80% of the malignant neoplasms of pancreas is the most common epithelial, exocrine pancreatic malignancy (Alexakis et al., 2004). PDAC harbours genetic abnormalities which affect the mechanisms controlling G_1 to S cell cycle progression and cellular proliferation. Mutation in *p16*, *p53*, *Smad4* tumour suppressors gene and *Ras* proto-oncogene are examples of genetic abnormalities often found in more than 80% of pancreatic cancer (Rozenblum et al., 1997, Naumann et al., 1996; Huang et al., 1996; Chen et al., 1996; Redston et al., 1994).The *Ras* gene was known as cancer-related genes with *KRas* being the most frequently altered gene in pancreatic cancer. Besides pancreatic cancer harbouring approximately 95% of *KRas* mutation, approximately 30% to 40% of colon cancers harbour *KRas* mutation (Arrington et al., 2012). *KRas* mutation is also found mainly in 16% to 40% of non-small cell lung cancer (NSCLC) (Nelson et al., 1996).

Despite current available diagnostic means, medical practitioners have met with challenges in early detection and differential diagnosis of pancreatic cancer. As a result, many academic and pharmaceutical researchers have paid

greater attention to understanding molecular events which lead to the development of this cancer in order to provide a basis for development of effective strategies for prevention, diagnosis and treatment. Recently, the focus has switched to targeting the Ras signaling pathway as cancer is often associated with the deregulation of the Ras signaling pathway caused by mutation of *Ras* gene in more than 30% of all cancers (Bos, 1989; Prior et al., 2012). *Ras* has a central role in regulating growth and cell survival in a wide spectrum of human tumours, the Ras-mitogen activated protein kinase (Ras-MAPK) signaling pathway is a well-validated oncogenic cascade. As the earliest and most common genetic mutation, *Ras* mutation drives transformation and tumour progression in the pancreas as evidenced by sequence analysis (Lemoine et al., 1992).

Ras protein, an essential component in Ras signaling is a small molecular weight GTPase that functions as a molecular switch in regulating pathways involved in fundamental cellular processes such as proliferation, differentiation, motility, transcription and survival. This protein couples extracellular signal to intracellular effectors such as the MEK kinase pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) andphosphoinositide 3-kinase (PI3K) pathway. The molecular switch cycles between "off" and "on" conformations and the cycling is controlled by two key regulators; guanine nucleotide exchange factors (GEF) which promote GDP dissociation and GTP binding, and GTPase activating proteins (GAP) which stimulate intrinsic GTPase activity in Ras. Ras bound to GDP is inactive and will transit to the active state when bound to GTP and this process is controlled by GEF. The active state of Ras bound to GTP will hydrolyse to its inactive state by GAP in which it turns off the Ras signaling.

The Ras protein is commonly referred to as $p21^{ras}$ with a molecular weight of approximately 21kDa (Barbacid, 1987) and is encoded by three ubiquitously expressed genes known as *NRas,HRas*and *KRas*. The four different Ras proteins (HRas, NRas, and the KRas 4b and the less abundant KRas 4a splice variants) encoded by these three genes have a highly conserved primary amino acid sequencehowever differ in their hypervariable C-terminal region (HVR) as a result of lipid posttranslational modifications (Dekker and Hedberg, 2011). According to the COSMIC dataset, KRas is confirmed as the most frequently mutated isoform, being found in 22% of all tumours analysed, followed by 8% for NRas and 3% for HRas. Mutation in *Ras* genes render Ras oncoprotein to constitutively bound to GTP and results in activation of downstream effector pathways even in the absence of extracellular stimuli (Karnoub and Weinberg, 2008). Each Ras isoform has a distinctive codon mutation. Point mutations in codons 12, 13 and 61 of *KRas*with codon 12 being the most common site of *Ras* activation in pancreatic adenocarcinoma results in expression of constitutively active mutated Ras proteins which transform cells into a malignant phenotype (Santos and Nebreda, 1989). This research focuses on targeting KRas especially on the mutation of codon 12 due to their

higher occurrence in 75% to 100% of pancreatic cancers (Mu et al., 2004; Cox and Der, 2010).

The cellular signaling networks of a living cell are quite complex. It is not the simple rule that molecular level outcome will be determined byenvironmental signal.Ras proteins have been coined “undruggable” as their activation mechanism is poorly understood. Activating Ras mutation was found to be resistant to standard therapies (Lu et al., 2016). There is no clinically useful drug available as an anti-Ras therapy due to the “undruggable” properties of KRas in which the protein lacks pocket for small molecule to bind with high affinity. The resemblance of mutant protein and normal KRas protein from a structural point of view and similar GDP/GTP-binding domain with other members of Ras superfamily of small GTPases such as Ral (Nicely et al., 2004), results in specific therapies even more challenging (McCormick, 2016). Despite being “undruggable”, there are a few interesting exceptions which were discovered over the past few years that lead to new insights on KRas structure and function. Structural biology and *in silico* drug design had discovered a few promising inhibitors using small molecules which reveal opportunities for interventions and developing KRas therapies. In order to understand the molecular details of Ras signaling cascades, there are five main approaches in targeting oncogenic Ras signaling using small molecules which are:

1) Inhibition of nucleotide exchange factor which abrogated the Ras-GTP formation
2) Interference with the Ras membrane association
3) Inhibition of Ras-effector interactions
4) Downregulation of Ras proteins
5) Enhancement of intrinsic Ras GTPase activity

Using ensemble docking and innovative cell-based assays, small-molecule ligands known as andrographolide (AGP) and its benzylidene derivatives such as SRJ23 are found to directly bind to transient pocket on KRas and inhibit the nucleotide exchange factor binding of both its wild type KRas and oncogenic mutant KRas G12V (Hocker et al., 2013). AGP has been shown to exhibit great *in vitro* anticancer activities in various cancer cells particularly breast and colon cancers by inducing G1 cell-cycle arrest and apoptosis. The benzylidene derivatives of AGP exhibit inhibitory potency at nanomolar to micromolar concentrations (Jada et al., 2008). One of the andrographolide derivatives known as SRJ23 showed enhanced cytotoxicity and selectivity than AGP in the National Cancer Institute (NCI) screenings (Jada et al., 2008). SRJ23 displayed growth inhibition at low micromolar concentration and selectivity towards PC-3 prostate cancer cell lines among the various prostate cancer cell lines tested Wong et al., 2009).

1.2 Hypothesis

Despite SRJ23 having good cytotoxicity in prostate cancer cell lines (Wong et al., 2014), this compound is yet to be tested in pancreatic cancer which commonly harbours KRas mutation. Therefore, this situation had led to testing the cytotoxicity of SRJ23 using microculture tetrazolium (MTT) cell proliferation assay in various pancreatic cancer cell lines such as MIA PaCa 2, Capan-2, and BxPC-3. The *in vitro* anticancer activity of SRJ23 on pancreatic cancer cell line was hypothesised to involve direct inhibition of SRJ23 to Ras protein. The derivatives of SRJ23 were synthesised in order to discover compound with better potency and selectivity than SRJ23. Derivatives of SRJ23 were hypothesised to have better cytotoxicity activity than SRJ23. Although one of the derivatives (SRS157) displayed greaterselectivity in Capan-2 pancreatic cancer cell line as compared to HCT116 colorectal cancer cell line but overall, SRJ23 still showed better potency as compared to its derivatives in various cancer cell lines such as MCF7 breast, BxPC-3 pancreatic, MIA PaCa 2 pancreatic, HCT116 colorectal cancer cell line and PC-3 prostate cancer line.

Furthermore, the*in silico* docking using 75 unique KRas conformers showed SRJ23 is able to bind to three distinct pocketsknown as p1, p2, and p3 of oncogenic mutant KRas Q61H which involved switch 1 and 2 (Hocker et al., 2013). Ras has reduced affinity for effectors and harbours open pockets in state 1 and it was hypothesised that small molecule inhibitor such as SRJ23 which can stabilise the state 1 conformation has the potential to inhibit Ras signalling through interference with either effector or nucleotide exchange factor. Using molecular dynamics simulations, KRas-SRJ23 complex was found to be stable with SRJ23 favoured binding at p1 and state 1-like conformers with an open switch 1.SRJ23 was hypothesised to form complex with KRas G12V while STD NMR signals were hypothesised to show protons of SRJ23 are in close contact with the surface of the binding pocket ofKRas G12Vprotein, indicating interaction between SRJ23 and KRas G12V protein. With promising *in vitro* and *in silico* findings, this has prompted the research to focus on expression of KRas G12V using bacterial system and purification of protein to determine their potential interaction with SRJ23 through X-ray crystallography and saturation transfer difference-nuclear magnetic resonance (STD-NMR).

1.3 Objectives

The general objective of this study was to determine the potentiality of SRJ23 compound and its derivatives to be developed as an anti-Ras therapy.

1.4 Specific Objectives

The general objective could be achieved through the specific objectives as followed:

i) To determine the *in vitro* cytotoxicity of SRJ23 and its derivatives among a panel of cancer cell linesand to discover compound with better potency and selectivity than SRJ23.
ii) To express and purify KRas G12V.
iii) To determine potential interaction between KRas G12V protein and SRJ23 compound through X-ray crystallography and saturation transfer difference-nuclear magnetic resonance.

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