EVALUATION OF PANCREATIC CANCER CELL LINES AS A SOURCE OF CANCER STEM CELLS (CSC) AND THEIR UTILISATION FOR ASSESSING ANTI-CSC ACTIVITIES OF KRAS BINDERS

By

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October 2020

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Background: High incidence of recurrence has been recorded at 80% of pancreatic ductal adenocarcinoma (PDAC) cases, despite tumour resection and an adjuvant chemotherapy with gemcitabine are prescribed. Pancreatic cancer stem cells (CSCs) are believed to have initiated recurrent PDAC. Growing evidence indicates that these cells resist gemcitabine as a result of aberrant activation of KRAS, a molecular switch that regulates cancer-associated signalling pathways, including MAPK and PI3K-AKT. Vismodegib is the earliest known anti-pancreatic CSC agent, but its anti-CSC activity has been proven to be not translatable from bench to clinical settings. It is crucial to seek for an alternative to vismodegib. Patient-derived CSC culture is prevalently used for assessing potential anti-CSC agents *in vitro*, but patients' biopsies may not be accessible to some researchers. **Objectives:** In the present study, PDAC cell lines were evaluated as a source of CSCs and they were utilised for assessing the anti-CSC potential of KRAS binders (DCAI and SRJ23) with their mechanisms of action being elucidated. **Methods:** Cell viability assays were performed to evaluate the cytotoxicity of gemcitabine, vismodegib, and KRAS binders in PDAC cell lines (BxPC-3, PANC-1, Capan-2, and MIA PaCa-2). Flow cytometry was employed to analyse the expression of pancreatic CSC surface markers (CD44, CD24, and CD133) on a single-cell basis. While tumoursphere-forming ability was investigated by culturing cells in serum-free and non-adherent conditions *in vitro*, the tumourigenicity of PDAC cells was assessed in immunocompromised mice. Molecular events were delineated by Western blotting and the presence of SOX2-expressing cells in tumour xenografts was evaluated by immunohistochemistry. **Results:** PANC-1 and Capan-2 cell lines showed exceptional resistance to gemcitabine with Capan-2 contains a notable number of putative CSCs co-expressing CD44, CD24, and CD133 (3.7% of the total population). These cells resisted the cytotoxicity of gemcitabine,

but responded to vismodegib. Tumourspheres were found arising from BxPC-3 and Capan-2 cell lines only, but the latter had closer resemblance to CSCs based on their responsiveness to the inhibition induced by foetal bovine serum. The presence of tumoursphere-forming cells is consistent with BxPC-3 and Capan-2 cell lines being highly tumourigenic in immunocompromised mice. DCAI and SRJ23 abrogated the self-renewal of Capan-2 tumourspheres with the effect of SRJ23 being the greatest. DCAI and SRJ23 diminished SOX2 expression and signalling pathways that were essentially activated in Capan-2 tumourspheres. SOX2-expressing cells were found correspondingly present in xenografts of highly tumourigenic BxPC-3 and Capan-2 cell lines, except for PANC-1 xenografts, of which SOX2-expressing cells were present despite poorly tumourigenic. **Conclusion:** Capan-2 cell line contains a small subset of putative CSCs, suggesting that cancer cell line is a valid source of CSCs in the discovery of novel anti-CSC agents. This study provides first evidence of anti-CSC activity in DCAI and SRJ23, which is attained through inhibiting KRAS signalling. It is postulated that SRJ23 induces an additional inhibitory circuitry that involves protein phosphatase 2A, a dephosphorylating enzyme that inactivates NF-κB and WNT pathways, such that it emerges as a more promising candidate than DCAI in the future development of anti-CSC drug.

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

PENILAIAN TITISAN SEL KANSER PANKREAS SEBAGAI SUMBER SEL KANSER STEM DAN PENGGUNAANNYA UNTUK MENGUJI SIFAT AKTIVITI ANTI-SEL KANSER STEM PERENCAT KRAS

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Pengenalan: Walaupun tumor dapat dibedah dan gemcitabine dipreskrib dalam kemoterapi, adenokarsinoma duktus pankreas (PDAC) berulang kerap berlaku. Sel kanser stem pankreas telah dikenal pasti sebagai dalang kanser berulang. Pelbagai bukti mengatakan bahawa pengaktifan KRAS, molekul pengawal kepada laluan isyarat yang berhubung kait dengan kanser, yang tidak terkawal menyebabkan sel kanser stem pankreas menentang kesan antikanser gemcitabine. Vismodegib merupakan ejen anti-sel kanser stem yang paling awal ditemui di dalam makmal, namun ia dibuktikan tidak berkesan dalam membasmi kanser berulang. Oleh itu, usaha pencarian gantian vismodegib adalah penting, akan tetapi, tiada hasil yang berjaya setakat ini. **Objektif:** Dalam kajian ini, kehadiran sel kanser stem dalam titisan sel PDAC telah disahkan bahawa ia mampu dijadikan subjek ujikaji yang melibatkan perencat KRAS (DCAI dan SRJ23). Potensi DCAI dan SRJ23 dalam membasmi sel kanser stem dinilai dan mekanismenya turut dijelaskan. **Kaedah:** Aktiviti sitotoksik gemcitabine, vismodegib dan perencat KRAS dalam titisan sel PDAC (BxPC-3, PANC-1, Capan-2, and MIA PaCa-2) dinilai melalui ujian daya maju sel. Sitometri aliran dijalankan untuk menganalisis kehadiran penanda (CD44, CD24 dan CD133) pada permukaan sel kanser stem pancreas tunggal. Kebolehan titisan sel PDAC untuk membentuk sfera tumor dikaji dalam vitro melalui keadaan kultur yang bebas serum dan tidak melekat. Kemampuan ketumbuhan xenograf sel PDAC diuji pada mencit imunokompromi. Perubahan sel pada peringkat molekul dikesan dan digambarkan melalui ujian serap Western dan kehadiran sel xenograf tumor yang mengekspresikan SOX2 dikesan melalui ujian imunohistokimia. **Hasil Ujikaji:** Sel PANC-1 dan Capan-2 menunjukkan kesan rintangan yang luar biasa. Namun, hanya Capan-2 mengandungi sejuzuk sel (3.7% daripada seluruh populasi) yang mengekspresi CD44, CD24 dan CD133. Sel-sel ini menentang sitotoksik gemcitabine, tetapi mereka adalah sensitif

terhadap kesan vismodegib. Walaupun kedua-dua BxPC-3 dan Capan-2 mengandungi sel yang mampu membentuk sfera tumor, sel sfera tumor Capan-2 lebih menyerupai sel kanser stem berdasarkan respons mereka terhadap perencatan pertumbuhan yang disebabkan oleh serum anak lembu. Kehadiran sel-sel yang membentuk sfera tumor selaras dengan keupayaan BxPC-3 dan Capan-2 dalam mencetuskan pertumbuhan tumorigenik pada mencit imunokompromi. DCAI dan SRJ23 merencat kemampuan sel sfera tumor Capan-2 untuk mengswabaharui dan kesan SRJ23 adalah lebih menonjol. Kedua-duanya mengurangkan ekspresi SOX2 dan aktiviti laluan isyarat yang berperanan penting dalam sel sfera tumor Capan-2. Namun begitu, aktiviti SRJ23 dalam merencatkan isyarat NF-κB dan WNT adalah lebih kuat daripada DCAI. Kehadiran sel yang mengekspresikan SOX2 dalam xenograf tumor BxPC-3 dan Capan-2 adalah selaras dengan keupayaan mereka dalam mencetuskan pertumbuhan tumor. Walau keupayaan tumorigenik berkurang dalam mencetuskan pertumbuhan tumor, xenograf tumor PANC-1 turut mengandungi sel yang mengekspresikan SOX2. **Kesimpulan:** Titisan sel Capan-2 mengandungi sejumlah kecil sel seiras dengan sel kanser stem yang mengekspresikan CD44, CD24 dan CD133 serta melawan kesan antikanser kemoterapi. Sel-sel ini mungkin terlibat dalam pembentukan sfera tumor dalam vitro dan mencetuskan pertumbuhan xenograf tumor dalam vivo. Sifat anti-sel kanser stem yang dimiliki DCAI dan SRJ23 adalah dilaporkan buat kali pertama dalam kajian ini. Sebagai perencat KRAS, kedua-duanya membantut pertumbuhan sel kanser stem melalui sekatan isyarat KRAS dan laluan isyarat di bawah kawalannya, iaitu MAPK dan AKT, diikuti penyahaktifan isyarat NF-κB and WNT, dan diakhiri pengurangan ekspresi SOX2. Meskipun ada persamaan antara DCAI dan SRJ23 dalam mekanisme anti-sel kanser stem, adalah dicadangkan bahawa kebolehan SRJ23 melakukan sekatan tambahan terhadap isyarat penting dalam sel kanser stem melalui protein phosphatase 2A merupakan kelebihan yang ada pada SRJ23 apabila dibanding dengan DCAI. SRJ23 berpotensi untuk diusahakan sebagai anti-sel kanser stem.

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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:

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7-AAD	7-amino-actinomycin D
ABC	ATP-binding Cassette
ADP	Adenosine Diphosphate
AGP	Andrographolide
ALDH	Aldehyde Dehydrogenase
AML	Acute Myeloid Leukaemia
APC	Allophycocyanin
APL	Acute Promyelocytic Leukaemia
APS	Ammonium Persulfate
ATCC	American Type Culture Collection
αTOS	α-tocopheryl Succinate
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblastic Growth Factor
BSA	Bovine Serum Albumin
BZIM	Benzimidazole
CAI	Carboxyamidotriazole
CI	Confidence Interval
CMYC	Cellular Myelocytomatosis Oncogene
CNS	Central Nervous System
CO	Carbon Monoxide
CSC	Cancer Stem Cell
DAB	3,3'-diaminobenzidine
DCAI	4,6-dichloro-2-methyl-3-aminoethyl-indole
DMEM	Dulbecco's Modified Eagle's Medium

DMEM/F12	Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELDA	Extreme Limiting Dilution Analysis
ERK	Extracellular Signal-regulated Kinase
ESA	Epithelial-specific Antigen
ESC	Embryonic Stem Cell
FACS	Fluorescence-activated Cell Sorting
FBS	Foetal Bovine Serum
FGFR1	Fibroblast Growth Factor Receptor 1
FITC	Fluoroscein Isothiocyanate
FMO	Fluorescence Minus One
GAP	GTPase-activating Protein
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
GRB2	Growth Factor Receptor-bound Protein 2
GSK3β	Glycogen Synthase Kinase-3β
GTPase	Guanosine Triphosphatase
hESCs	Human Embryonic Stem Cells
HSP40	Heatshock Protein 40

HVR	Hypervariable Region
IHC	Immunohistochemistry
IκBα	IkappaB Alpha
IκBs	Inhibitors of NF-κB
IKKs	IκB Kinases
IL1β	Interleukin-1β
IL1R	Interleukin-1 Receptor
iPSC	Induced Pluripotent Stem Cell
KLF4	Kruppel-like Factor 4
Kobe0065	N-(3-chloro-4-methylphenyl)-2-[2,6-dinitro-4-(trifluoromethyl)phenyl]-hydrazinecarbothioamide
KRAS	Kirsten rat sarcoma
LDA	Limiting Dilution Assay
MACS	Magnetic-activated Cell Sorting
MAPK	Mitogen-activated Protein Kinase
MEK	Mitogen-activated Protein Kinase Kinase
mIBG	Meta-iodobenzylguanidine
MPTP	1-methyl 4-phenyl 1,2,3,6 Tetrahydropyridine
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor kappa B
NO	Nitric Oxide
NSCLC	Non-small Cell Lung Cancer
OCT4	Octamer-binding Protein 4
OXPHOS	Oxidative Phosphorylation

PanIN	Pancreatic Intraductal Neoplasia
PBS	Phosphate-buffered Saline
PDAC	Pancreatic Ductal Adenocarcinoma
PDX1	Duodenal Homeobox Protein 1
PE	R-phycoerythrin
PH	Pleckstrin-homology
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PP2A	Protein Phosphatase 2A
PTCH	Patched
RalGDS	Ral Guanine Nucleotide Dissociation Stimulator
RBD	Ras-binding Domain
RSK-1	Ribosomal S6 Kinase 1
RSK-2	Ribosomal S6 Kinase 2
RTK	Receptor Tyrosine Kinase
SCID	Severe Combined Immune-deficient
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SFN	Sulforaphane
SOX2	SRY-box Transcription Factor 2
SP	Side Population
SRJ09	3,19-(2-bromobenzylidene)andrographolide
SRJ23	3,19-(3-chloro-4-fluorobenzylidene)andrographolide
SHH	Sonic Hedgehog

SMO	Smoothened
TBST	Tris-buffered saline/Tween-20
TEMED	Tetramethylethylenediamine
TIC	Tumour-initiating Cell

CHAPTER 1

INTRODUCTION

Background of the Study

With a global incidence of 458,918 cases in 2018, pancreatic ductal adenocarcinoma (PDAC) is ranked 14th in the list of the commonest cancers worldwide (Bray et al., 2018). It is the seventh most lethal cancer with 432,232 deaths were recorded worldwide in 2018 (Bray et al., 2018). It is believed that PDAC arises from the epithelial cells that constitute the lining of pancreatic ducts, through which digestive enzymes produced by acinar cells are channelled into the duodenum (Stark & Eibl, 2015). Even though tumour masses can be removed by resection alone, the prognosis of PDAC patients remains poor (Gbolahan et al., 2019).

PDAC begins with a neoplastic precursor lesion, known as pancreatic intraepithelial neoplasia (PanIN) and its development is described as a progressive accumulation of multiple genetic mutations (Hruban et al., 2000). Among the key proto-oncogenes, Kirsten rat sarcoma (*KRAS*) gene is mutated at the early stage of PDAC development and oncogenic KRAS protein has been found in more than 90% of the PDAC cases (Eser et al., 2014), suggesting that it is a promising therapeutic target in the prevention and treatment of PDAC. The pivotal role of KRAS in promoting stem cell-like characteristics has also been confirmed in pancreatic cancer cells (Seguin et al., 2014). This finding is consistent with previous studies suggesting that acinar cells (Wei et al., 2016) and β-cells (Pour & Schmied, 1999; Pour et al., 2003) can be transdifferentiated into duct-like cells and function as the precursor of PanIN and PDAC by expressing oncogenic KRAS mutant proteins.

Cancer stem cells (CSCs) are identified as the fraction of cells, which show remarkable resistance to the cytotoxic effect of chemotherapeutic drugs, found within the heterogenous population of the bulk tumour. In contrast to their non-CSC counterparts, CSCs express drug-metabolising enzymes and drug efflux transporters (Thomas et al., 2015) as well as demonstrate enhanced ability of DNA repair (Liu et al., 2006). Contemporary chemotherapeutics eliminate fast-dividing non-CSCs effectively, leaving slow-cycling or quiescent CSCs unaffected during chemotherapy (Viale et al., 2009). The residual CSCs give rise to recurrent tumour in few years after the chemotherapy is terminated as a result of undetectable tumour volume.

In a landmark study by Hocker et al. (2013), it was reported that andrographolide (AGP) and its benzylidene derivatives, 3,19-(2-bromobenzylidene)andrographolide (SRJ09) and 3,19-(3-chloro-4-fluorobenzylidene)andrographolide (SRJ23) inhibit both wild-type and oncogenic KRAS proteins. This previous study has paved the way for initiating the present study with the aim of drugging KRAS, which has been known to be undruggable for many decades, in the context of targeting CSCs to eradicate pancreatic cancer recurrence. None of the known KRAS binders, which were found to have antagonistic activity against aberrant KRAS signalling preclinically, have been tested for their anti-pancreatic CSC activities.

Effort of drugging KRAS is still relevant in seeking potential treatment for PDAC patients, despite being challenging. It is believed that KRAS is a prominent therapeutic target in view of its pivotal role in the carcinogenesis and progression of PDAC. In the present study, previously known KRAS binders, namely benzimidazole (BZIM; Maurer et al., 2012), 4,6-dichloro-2-methyl-3-aminoethyl-indole (DCAI; Maurer et al., 2012), N-(3-chloro-4-methylphenyl)-2-[2,6-dinitro-4-(trifluoromethyl)phenyl]-hydrazinecarbothioamide (Kobe0065; Shima et al., 2013), andrographolide (AGP) and its derivatives (SRJ09 and SRJ23), were tested for their anti-pancreatic CSC property in PDAC cell lines with differential KRAS mutational statuses. These cell lines harbour wild-type KRAS (BxPC-3) and mutant KRAS: $KRAS^{G12D}$ (PANC-1), $KRAS^{G12V}$ (Capan-2), $KRAS^{G12C}$ (MIA PaCa-2).

Problem Statement

Treatment options of PDAC remain scarce and they are limited to tumour resection that precedes an adjuvant chemotherapy with the use of either gemcitabine or 5-fluorouracil (Gbolahan et al., 2019). Although the adjuvant gemcitabine therapy was reported to have significantly improved the overall survival and disease-free survival of patients with resectable PDAC (Oettle et al., 2013), 80% of these patients would experience a recurrence that will eventually lead to fatality within two years after diagnosis (Gbolahan et al., 2019).

Pancreatic CSCs are known to have initiated recurrent and metastatic PDAC. Following the isolation and characterisation of pancreatic CSCs based on surface markers (CD44, CD24, and CD133), expression of embryonic SOX2 transcription factor, and an association of these cells with chemoresistance (Hermann et al., 2007; Herreros-Villanueva et al., 2013; Li et at., 2007), a few anti-pancreatic CSC agents were discovered. Vismodegib is the earliest known anti-pancreatic CSC agent that targets Hedgehog signalling (Singh et al., 2011), of which activity against pancreatic CSCs was proven to be promising preclinically. Nevertheless, recent clinical study revealed that combining vismodegib and gemcitabine

did not reduce the CSC content of tumour biopsies and the prognosis of patients with metastatic PDAC was not significantly improved as compared with gemcitabine alone (De Jesus-Acosta et al., 2019). This finding suggested that the anti-pancreatic CSC activity of vismodegib is not translatable from bench to clinical settings. Hence, it is crucial to seek for an alternative to vismodegib.

The existence of CSCs in continuous culture of cancer cell lines remains controversial. Many have questioned the authenticity of these microenvironment-sensitive cells after prolonged culturing in the presence of foetal bovine serum *in vitro*. Although patient-derived CSC culture is preferred for experimentations to study CSC biology and discover novel anti-CSC agents, the inaccessibility of patients' biopsies poses a great hurdle to research activities. Therefore, investigating the presence of CSCs in PDAC cell lines and characterising them molecularly in the context of their self-renewal mechanism are of value to researchers with difficulty in establishing patient-derived CSC culture.

Research Hypothesis

CSCs exist in PDAC cell lines as a rare subset of cells, of which hallmark features include showing resistance to gemcitabine and sensitivity to vismodegib, co-expressing pancreatic CSC surface markers, forming tumourspheres *in vitro*, and being highly tumourigenic *in vivo*. Based on the notion that KRAS signalling plays a determining role in the self-renewal of breast, colorectal, and pancreatic CSCs. It is hypothesised that KRAS binders inhibit the self-renewal of CSCs in PDAC cell lines.

Objectives of the Study

Main Objective

The general objective of the present study was to identify binders of KRAS with anti-pancreatic CSC activity.

Specific Objectives

The specific objectives of the present study were:

1. To characterise CSCs in PDAC cell lines based on resistance to gemcitabine and sensitivity to vismodegib, co-expression of pancreatic CSC surface markers, tumoursphere-forming ability *in vitro*, and tumourigenicity *in vivo*.
2. To establish a test model for assessing the inhibitory effects of KRAS binders on the self-renewal of CSCs in PDAC cell lines.

3. To assess the inhibitory effects of KRAS binders on the self-renewal of CSCs in PDAC cell lines.
4. To elucidate the molecular mechanism of the self-renewal of CSCs in PDAC cell lines and the mechanism of action in abrogating it by KRAS binders.
5. To evaluate the relevance of correlating the presence of SOX2-expressing cells in PDAC tumour xenografts with varying degrees of tumourigenicity *in vivo*.

Thesis Outline

The present study has two main aims:

1. Identifying and characterising pancreatic CSCs to establish a test model for evaluating the anti-pancreatic CSC property of KRAS binders.
2. Evaluating the anti-pancreatic CSC property of KRAS binders.

It is presented in a total of 7 chapters. A brief preview of each chapter is as follows:

Chapter 1
An introductory chapter includes the background, significance, problem statements, hypotheses, and objectives of the present study to help the readers in appreciating the rationale behind the initiation of the present study.

Chapter 2
This chapter notes fundamental knowledge for the conception, design, and execution of the present study. In addition, the knowledge is helpful to the readers in apprehending and appreciating the work done in the present study and the content of this thesis.

Chapter 3
This chapter explores the existence of CSCs in PDAC cell lines based on the chemoresistant nature of CSCs and co-expression of pancreatic CSC surface markers (CD44, CD24, and CD133). The $CD44^{+}CD24^{+}CD133^{+}$ cells of Capan-2 cell line were found to be resistant to gemcitabine, a chemotherapeutic drug, and sensitive to vismodegib, an anti-pancreatic CSC agent.

Chapter 4
This chapter addresses the question of whether CSCs exist in PDAC cell lines based on two hallmark characteristics of CSCs, namely tumoursphere formation *in vitro* and tumourigenicity *in vivo*.

Chapter 5
This chapter reports the cytotoxic activities of KRAS binders, namely benzimidazole (BZIM), DCAI, Kobe0065, AGP, SRJ09, and SRJ23 in PDAC cell lines. DCAI and SRJ23 were selected to be further tested for their activities in abrogating the self-renewal of Capan-2 tumourspheres, which is pertaining to anti-pancreatic CSC property.

Chapter 6
This chapter delineates the molecular mechanism of the formation of Capan-2 tumourspheres in serum-free and non-adherent culture conditions. In addition, the modulating effects of gemcitabine, vismodegib, DCAI, and SRJ23 on the biology of Capan-2 tumourspheres were revealed. The relevance of relating the presence of SOX2-expressing cells, which are known to be pancreatic CSCs, in PDAC tumour xenografts to varying degrees of their tumourigenicity *in vivo* was investigated and reported.

Chapter 7
This chapter summarises the discussion and conclusion of each chapter. Future direction of the present study is suggested in this chapter.

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