UNIVERSITI PUTRA MALAYSIA

EFFECTS OF GAMMA-SECRETASE INHIBITOR GSI-34 ON BLADDER CANCER CELL VIABILITY, MIGRATION AND NOTCH-ASSOCIATED GENE EXPRESSION LEVELS

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By

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May 2017

Chairman : Chau De Ming, PhD
Faculty : Medicine and Health Sciences

Bladder cancer is a prevalent cancer that is associated with many risk factors including aging and exposure to tobacco. On a molecular level, activating mutations of oncogenes such as Fibroblast Growth Factor Receptor, *FGFR3* and the loss-of-function of tumour-suppressor genes such as *p53* and *pRb* have been associated with bladder cancer progression. Previous studies have shown that aberrant Notch signalling has been linked with many cancers including breast cancer, head and neck cancer as well as bladder cancer. Recent genetic studies have shown that Notch receptor mutations are associated with bladder cancer but the mechanism of action of these Notch receptor mutations remains unclear. One of the key enzymes that activates the Notch signalling pathway is gamma-secretase. Thus, gamma-secretase is an attractive drug target. The aim of this study was to determine the effect of a gamma-secretase inhibitor, GSI-34, on bladder cancer cell viability, cell migration and expression levels of Notch receptor genes and Notch downstream targets genes. Using MTT assay, the cytotoxic effect of GSI-34 on the viability of bladder cancer cells was tested. GSI-34 showed a cytotoxic effect against RT112 cells but did not affect the cellular viability of EJ28, SW780 and 5637. Another gamma-secretase inhibitor, DAPT also reduced the RT112 cell viability and minimally supressed 5637 cell viability. The effect of GSI-34 on bladder cancer cell migration was also evaluated using an *in vitro* migration assay. GSI-34 inhibited SW780 and 5637 cell migrations but enhanced EJ28 cell migration. However, GSI-34 had no effect on RT112 cell migration. Further examination on the effect of GSI-34 on the gene expression levels of Notch receptor genes and Notch downstream target genes was performed using RT-qPCR. Based on the analysis of gene expression, *NOTCH2* genes was consistently upregulated in all bladder cancer cells examined. In contrast, *HES1* was downregulated in EJ28, SW780 and 5637 cells upon GSI-34 treatment. The *DUSP1* gene was also consistently upregulated in all bladder cancer cells. This study identified that RT112 cells are sensitive towards GSI-34 while other cells; EJ28, SW780 and 5637 cells are more resistant towards GSI-34. This study also showed that bladder cancer cells are

potentially resistant towards GSI-34 through the downregulation of *HES1* and the upregulation of *NOTCH2*. In addition, higher levels of *DUSP1* expression is associated with greater sensitivity towards GSI-34 treatment. The findings from this study identifies GSI-34 as a potential therapeutic target for a subset of bladder cancers. However, the role of NOTCH genes as well as associated genes need to be further understood to ensure effective translation of potential gamma-secretase inhibitors such as GSI-34 as effective bladder cancer therapeutics in the future.

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

KESAN PERENCAT GAMMA-SECRETASE, GSI-34 KEATAS PERTUMBUHAN SEL-SEL KANSER PUNDI KENCING, MIGRASI DAN TAHAP EXPRESI GEN BERKAITAN NOTCH

Oleh

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Kanser pundi kencing adalah kanser yang lazim dikaitkan dengan pelbagai faktor risiko termasuk penuaan dan pendedahan kepada karsinogen tembakau. Pada peringkat molekul, pengaktifan mutasi onkogen seperti Fibroblast Growth Factor Receptor, *FGFR3* dan ketidaksempurnaan gen pembantut-tumor seperti *p53* dan *pRB* telah dikaitkan dengan perkembangan kanser pundi kencing. Kajian terdahulu telah menunjukkan bahawa ketidaksempurnaan isyarat Notch telah dikaitkan dengan pelbagai jenis kanser termasuk kanser payudara, kanser kepala dan leher serta kanser pundi kencing. Kajian genetik terkini telah menunjukkan bahawa mutasi pada Notch reseptor berkait dengan pundi kencing akan tetapi mekanisma mutasi Notch reseptor adalah masih tidak jelas. Salah satu enzim utama yang mengaktifkan isyarat Notch ialah gamma-secretase lalu menjadikan sasaran gamma-secretase sebagai target yang menarik. Tujuan kajian ini adalah untuk menentukan kesan merencat gamma-secretase dengan menggunakan perencat gamma-secretase, GSI-34, pada pertumbuhan sel-sel kanser pundi kencing, migrasi sel dan tahap ekspresi gen reseptor Notch dan Notch sasaran hiliran gen. Dengan menggunakan MTT assay, kesan sitotoksik GSI-34 ke atas pertumbuhan sel-sel kanser pundi kencing telah diuji. GSI-34 menunjukkan kesan toksik terhadap sel-sel RT112 dan tidak menjejaskan kehidupan sel EJ28, SW780 dan 5637. DAPT juga merencat pertumbuhan sel-sel RT112 dan merencat sel-sel 5637 secara minimum. Kesan GSI-34 ke atas kadar migrasi sel-sel kanser pundi kecing juga telah dinilai dengan menggunakan ujian migrasi *in vitro*. GSI-34 menunjukkan perencatan dalam migrasi sel-sel SW780 dan 5637 dan merangsang migarsi sel-sel EJ28. Walaubagaimanapun, GSI-34 tidak mempunyai kesan terhadap migrasi sel-sel RT112. Pemeriksaan lanjut mengenai kesan GSI-34 ke atas kadar expresi gen Notch reseptor dan Notch gen sasaran hiliran dilakukan dengan menggunakan kaedah RT-qPCR. Berdasarkan analisis gen, gen *NOTCH2* meningkat secara konsisten dalam semua sel-sel kanser pundi kencing yang diperiksa manakala expresi *HES1* gen berkurangan dalam sel-sel EJ28, SW780 dan 5637 dengan rawatan GSI-34. Gen *DUSP1* juga konsisten dinaikkan dalam kesemua sel kanser pundi kencing. Kajian kini

menunjukkan bahawa sel-sel RT112 sensitif terhadap GSI-34 manakala sel-sel lain; EJ28, SW780 dan 5637 sel-sel lebih tahan terhadap GSI-34. Kajian ini menunjukkan bahawa sel-sel kanser pundi kencing lebih tahan terhadap GSI-34 melalui penurunan gen *HES1* dan peningkatan gen *NOTCH2*. Di samping itu, peningkatan gen *DUSP1* dikaitkan dengan sensitiviti terhadap rawatan GSI-34. Penemuan daripada kajian ini mengenal pasti GSI-34 sebagai sasaran terapeutik potensi untuk subset kanser pundi kencing. Walau bagaimanapun, peranan gen Notch serta gen yang berkaitan perlu difahami selanjutnya untuk memastikan keberkesanan translasi perencat gamma-secretase seperti GSI-34 sebagai terapeutik kanser pundi kencing pada masa hadapan.

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I certify that a Thesis Examination Committee has met on 17 May 2017 to conduct the final examination of Nadzatul Nabila binti Roslan on her thesis entitled "Effects of Gamma-Secretase Inhibitor GSI-34 on Bladder Cancer Cell Viability, Migration and Notch-Associated Gene Expression Levels" 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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The 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:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

˚C	degrees celsius
±	plus-minus
μM	micromolar
μg/mL	microgram per mililitre
APP	amyloid precursor protein
cDNA	complementary deoxyribonucleic acid
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transitions
FAM	fluorescein dye
FBS	foetal bovine serum
FGFR	fibroblast growth factor receptor
g	g force (relative centrifugal force)
gm	gram
HKG	housekeeping gene
GSI	gamma-secretase inhibitor
GOI	gene of interest
HCl	hydrochloric acid
LNA	Locked Nucleic Acid
M	molar

MMC	Mitomycin C
MAPK	mitogen-activated protein kinase
mg/mL	milligram per millilitre
$MgCl_2$	magnesium chloride
n.d.	not detectable
NaCl	sodium chloride
NaOH	sodium hydroxide
NICD	notch intracellular domain
nM	nanomolar
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
RT-qPCR	real-time qpcr
SEM	standard error of mean
v/v	volume to volume
TAE	tris-acetate-EDTA
UPL	universal probe library
VEGF	vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

Bladder cancer is a type of malignancy that arises from the epithelial lining of the urinary bladder. It is the ninth most common cancer worldwide and affects more than 350,000 new patients every year and causes more than 100,000 deaths annually worldwide (Ferlay et al., 2010; Jemal et al., 2011). The majority of the people who are diagnosed with bladder cancer are elderly patients and the disease is more common in men as compared to women (Matalka et al., 2008; Gupta et al., 2009; Bajbuk et al., 2011; Burger et al., 2013). More than 90 % of bladder cancers are transitional-cell carcinomas (TCC). Less than 10 % of bladder cancers comprise the other rare forms of bladder cancers including squamous cell carcinoma (SCC), adenocarcinoma, small cell carcinoma and rare neoplasms as well as sarcomas (Sharma et al., 2009; Kaufman et al., 2009).

Approximately 70 % of patients are diagnosed with non-muscle invasive (NMIBC) cancer that is morphologically well differentiated with constant nuclei. Non-muscle invasive cancers are often successfully treated with endoscopic resection. However, there is a high tendency of cancer recurrences within 5 years. About 15 % of NMIBC cases progress to high grade with invasive properties. Since there is a high rate of recurrence and potential of progression, patients with this type of cancer require close follow-up for life, with periodic cystoscopic examination (Ather and Nazim, 2015; Babjuk et al, 2011). This surveillance is invasive, uncomfortable, time-consuming and expensive (Mowatt et al, 2010); thus making bladder cancer as one of the most expensive cancers to manage (Rhijn et al., 2009; Babjuk et al., 2011; Bosch et al., 2011). The other 30 % of bladder cancer patients are diagnosed with muscle invasive bladder cancer (MIBC) that has a very poor prognosis and exhibits invasive properties at the time of diagnosis. This type of cancers is often associated with a high propensity to metastasise. Muscle invasive bladder cancer requires more radical treatment and have low survival rates (Kaufman et al., 2009; Stenzl et al, 2011).

There are many factors that are linked with bladder cancer incidence including environmental factors such as smoking and occupational exposure to carcinogens. Spontaneous or carcinogen-induced genetic mutations involving the activation of oncogenes such as *FGFR3* and *HRAS* or loss-of-function of tumour-suppressor genes such as *pRB* and *p53* (Dinney et al., 2004; Rhijn et al., 2004; Saito et al., 2006) are common molecular events in bladder cancer. One of the many genes that has been found to be mutated in bladder cancer is Notch. Recently, evidence of inactivating mutation of *NOTCH1* and *NOTCH2* genes was identified in patient bladder cancer samples through the large-scale genetic studies (Rampias et al., 2014).

Notch is a membrane-bound receptor that plays a central role in the Notch signalling pathway. Growing evidence have shown that Notch signalling plays multiple roles in

cellular processes including cell differentiation, proliferation, apoptosis, angiogenesis, as well as maintaining the stem cells (Leong and Karsan, 2006). Many studies have shown that aberrant Notch signalling is associated with human cancers including breast cancer, lung cancer, leukemia, and many other cancers (Lobry et al., 2011; Ranganathan et al., 2011). An overexpression of a constitutively active form of Notch was initially found to be associated with T-Cell Acute Lymphoblastic Leukemia (T-ALL) (Ellisen et al., 1991). Subsequently, Weng et al. (2004) identified activating *NOTCH1* mutations in more than 50 % of T-ALL cases and demonstrated *NOTCH1* mutation as the oncogene in T-ALL cases. With this evidence, many studies have been conducted to probe the association between Notch signalling and other cancers.

Recent studies by Greife et al. (2014) demonstrated that Notch signalling is suppressed through the downregulation of *NOTCH1* in bladder cancer. Rampias and colleagues revealed a potential tumour-suppressive role of Notch in bladder cancer; whereby inactivating mutations of Notch components were found in more than 40 % of bladder cancers that were examined (Rampias et al., 2014). Although studies have shown that Notch act as tumour-suppressor in bladder cancer, another recent study by Hayashi et al. (2016) revealed that *NOTCH2* activation correlates with a poor prognosis of bladder cancer. These findings suggest that *NOTCH2* acts as an oncogene and is involved in the progression of bladder cancer. Thus, Notch can potentially play a dual role as an oncogene or tumour-suppressor. However, the exact mechanism of Notch signalling during bladder cancer progression and the effect on cellular growth, migration and gene expression have not been fully explored.

Numerous studies of Notch inhibition have been performed to better understand the effects of targeting Notch pathway either using small molecule drugs or antibodies (Purow, 2012). One of the current strategies is to inhibit Notch signalling by inhibiting the activity of the gamma-secretase enzyme. Gamma-secretase inhibitors (GSIs) were first developed as potential therapies for Alzheimer's disease and are currently being adapted for cancer therapy. One of the potent GSIs that are being studied is GSI-34 and it has been shown to have low IC_{50} in A20 mouse lymphoma of about 1.0 nM Shelton et al. (2009). Although Notch signalling is implicated in bladder cancer, the gamma-secretase and Notch signalling roles in bladder cancer growth and progression are still not fully understood. Therefore, there is a need to study and characterise the role of gamma-secretase and Notch signalling in bladder cancer development. To date there is still lack of evidence on targeting Notch signalling through inhibiting gamma-secretase by using GSI in bladder cancer. Thus, there is a need to study the effects of GSI-34 on bladder cancer progression as it could be a novel drug as one of the Notch signalling therapeutic agents to treat bladder cancer more effectively.

Problem statement

The effects of inhibiting gamma-secretase by using GSI-34 on bladder cancer cells have not been fully investigated and explored. The phenotypic effects of GSI-34 on bladder cancer cell viability, migration and Notch gene expression is unknown to date.

Hypothesis

It was hypothesised that gamma-secretase inhibitor, GSI-34 represses bladder cancer cell growth and migration. It was also hypothesised that the inhibition of gamma-secretase results in the down-regulation of the expression of Notch genes and Notch downstream targets.

General objective and specific objectives

The main objective of this study was to identify the effect of a gamma-secretase inhibitor, GSI-34 on bladder cancer cell viability and migration as well as the expression levels of Notch genes and Notch downstream targets in the treated cells.

The specific objectives of this study include:

1. to determine the effect of GSI-34 on bladder cancer cell viability;
2. to evaluate the effect of GSI-34 on bladder cancer cell migration;
3. to evaluate the effect of GSI-34 on the expression levels of selected Notch genes and Notch downstream targets in bladder cancer cells.

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