GLYCOGEN SYNTHASE KINASE-3β (GSK-3β) INHIBITORS FROM SOIL ACTINOMYCETES OF SABAH RAINFORESTS: SCREENING, PURIFICATION AND IDENTIFICATION

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2015

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

31 August 2014

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ABSTRACT

Glycogen Synthase Kinase-3 (GSK-3) is a multitasking enzyme involved in various processes cell. It is expressed in two isoforms in mammalian cells; GSK-3α and GSK-3β. Dysregulation of GSK-3 is a causal factor of diseases such as cancer and diabetes. In drug discovery, GSK-3β is usually being targeted as its activity is more understood compared with GSK-3α. Small molecule originated from nature is regarded as the best inhibitor for GSK-3β that be used for treatment due to their novel structural features and potent activity. Actinomycetes are recognized as prolific producers of active compounds, including potent GSK-3β inhibitor such as staurosporine and manzamine A. External factors such as nutrient availability and pH may influence the production of secondary metabolites by actinomycetes. GSK-3β inhibitors were previously identified in *Streptomyces* sp. isolated from primary rainforests of Sabah. Due to unexplored potential of actinomycetes from Sabah rainforests, the possibility of finding more inhibitors from actinomycetes of Sabah is promising. In this study, 640 strains of actinomycetes were isolated from 156 soil sample of different forest types in Sabah. Kruskal-Wallis analysis revealed that a large number of isolated strains from secondary forest in which significantly related to the soil pH. Slightly acidic soils were found to yield more strains compared with alkaline soils. Based on the preliminary screening using a yeast-based assay of 505 strains, 14 positive strains were identified. Three strains (FA013, FH025 and H11809) were chosen to be partitioned using LLE. Of the three, H11809 was chosen to be further fractionated using column chromatography due to consistent and potential inhibitory activity. Fractionation of H11809 chloroform extract yielded two active fractions; F4 and F8, in which F8 showed no toxic activity against yeast. Further analysis of F8 using FTIR revealed that carbonyl ester as the major functional group. It was supported by GCMS in which carbonyl ester is the functional group of major compound; dibutyl phthalates (SI >90 %). Carbonyl group was reported to facilitate the binding of numerous inhibitors with GSK-3β. Minor compound; cyclo-leu-pro (SI >80 %), was also chosen to be further studied due to the presence of carbonyl group as well (carbonyl amide). Identification was supported by spiking using commercially-purchased pure compounds. Both compounds were shown to inhibit the activity of GSK-3β based on a yeast-based and kinase assays. Michaelis-Menten and Lineweaver-Burke plots showed that dibutyl phthalates inhibited GSK-3β with mixed inhibition and cyclo-leu-pro uncompetitive inhibition. IC_{50} values of dibutyl phthalates indicated active (IC_{50} =3.1 μM) inhibitory activity against GSK-3β while cyclo-leu-pro exhibited moderate inhibition (IC_{50} =12.94 μM). In conclusion, GSK-3β inhibitors were successfully identified from actinomycetes strain H11809, and may serve as a good lead to be further developed since non-ATP competitive inhibitors are the main interests in drug discovery.

ABSTRAK

PERENCAT GLIKOGEN SINTASE KINASE-3β (GSK-3β) DARIPADA AKTINOMISET TANAH HUTAN HUJAN SABAH: PENYARINGAN, PENULENAN DAN PENGENALPASTIAN

Glikogen sintase kinase-3 (GSK-3β) merupakan enzim pelbagai fungsi yang terlibat dalam banyak proses sel. Ia diekspresikan dalam dua isoforms di dalam sel mamalia; GSK-3α and GSK-3β. Gangguan pada regulasi GSK-3 merupakan punca kepada kanser dan diabetes. Dalam proses penemuan ubat-ubatan, GSK-3β sering disasarkan kerana aktivitinya lebih difahami berbanding GSK-3α. Molekul kecil daripada sumber semulajadi dianggap sebagai perencat yang baik kerana sturktur kimianya yang unik dan aktiviti yang kuat. Aktinomiset merupkan pengeluar sebatian kimia yang banyak, termasuklah perencat GSK-3β seperti staurosporine dan manzamine A. faktor luaran seperti nutrisi dan pH boleh mempengaruhi metabolit sekunder yng dihasilkan oleh aktinomiset. Sebelum ini, perencat GSK-3β telah dikenal pasti daripada Streptomyces sp. yang diisolasi daripada hutan hujan primer Sabah. Memandangkan aktinomiset di hutan hujan Sabah kurang dikaji, kemungkinan untuk menjumpai lebih banyak perencat adalah tinggi. Dalam kajian ini, 640 strain aktinomiset telah diisolasi daripada 156 tanah daripada pelbagai jenis hutan di Sabah. Analisa Kruskal-Wallis menunjukkan bahawa, bilangan strain yang kebanyakannya diisolasi daripada hutan sekunder dipengaruhi oleh pH tanah. Tanah yang sedikit berasid didapati menghasilakn lebih banyak strain berbanding tanah alkali. Saringan awal terhadap 505 strain menggunakan esei berasaskan yis, dan 14 strain aktif telah dikenal pasti. Tiga strain (FA013, FH025 dan H11809) telah dipilih untuk pemisahan menggunakan teknik LLE. H11809 telah dipilih daripada tiga strain untuk proses fraksinasi menggunakan kromatografi turus kerana aktiviti perencat yang konsistent dan berpotensi. Fraksinasi ekstrak kloroform H11809 menghasilkan dua fraksi aktif; F4 dan F8, dimana F8 tidak menunjukkan aktiviti toksik kepada yeast. Analisa selanjutnya keatas F8 menggunakan FTIR menunjukkan bahawa karbonil ester merupakan kumpulan berfungsi utama. Ianya disokong melalui GCMS dimana karbonil ester adalah kumpulan berfungsi dibutil phthalate (SI >90 %). Kumupulan karbonil dilaporkan membantu perlekatan pelbagai perencat kepada GSK-3β. Sebation minor (cyclo-leu-pro (SI >80 %)) juga dipilih untuk kajian selanjutnya kerana kewujudan kumpulan karbonil (karbonil amid). Pengenalpastian disokong dengan spiking menggunakan sebatian komersial tulen yang dibeli. Kedua-dua sebatian merencat aktiviti GSK-3β berdasarkan esei berasaskan yis dan kinase. Plot Michaelis-Menten and Lineweaver-Burke menunjukkan dibutil phthalate merencat GSK-3β dengan rencatan campuran dan cyclo-leu-pro rencatan tidak kompetitif. Nilai IC_{50} dibutil phthalate (IC_{50} =3.1 μM) menujukkan rencatan aktif terhadap GSK-3β, manakala cylco-leu-pro mempunyai rencatan sederhana (IC_{50} =12.94 μM). Kesimpulannya, perencat GSK-3β telah berjaya dikenal pasti daripada strain aktinomiset H11809, dan berpotensi untuk menjadi calon ubatan yang baik untuk dikembangkan kerana perencat yang tidak bersaing dengan ATP adalah sasaran utama dalam penemuan ubat-ubatan.

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LIST OF ABBREVIATION

1,3PG	1,3-Bisphosphoglycerate
2PG	2-Phosphoglycerate
3PG	3-Phosphoglycerate
ADP	Adenosine diphosphate
AIA	Actinomycetes isolation agar
AKT	Protein kinase B
Ala	Alanine
ANNOVA	Analysis of variance
APCI	Atomic pressure chemical ionization
Arg	Arganine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
C18 column	Carbon 18 column
CALK	Aurora-like kinase
CBP	CREB-binding protein
CC	Column chromatography
CDKs	Cyclin-dependent kinase protein
ChCl3	Chloroform
CREB	cAMP response element-binding protein
DAP	L-diaminopimelic acid
DBH	Debromohymenialdisine
DBP	Dibutyl phthalates
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EI	Electron ionization
ESI	Electrospray ionization
F6P	Fructose-6-phosphate
FRAT1/2	requently rearranged in advanced T-cell lymphomas ½
FRZ	Frizzled
FT-IR	Fourier transform infrared spectroscopy
G6P	Glucose 6-phosphate
GAP	Glyceraldehyde 3-phosphate
GC-MS	Gas chromatography–mass spectrometry
Gln	Glutamine
Glu	Glutamic acid
GLUT4	Glucose transporters 4
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase-3

HD	Hymendialdisine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hex	Hexane
HIV	Human immunodeficiency virus
HMKs	Halomenthylarylketones
HPLC	High-performance liquid chromatography
HVA	Humic acid agar + Vitamin B
ICL	Isocitrate lyase
Ile	Isoleucine
ISP3/4	International Streptomyces Project 3/4 agar
LC-MS	Liquid chromatography–mass spectrometry
Leu	Leucine
LLE	Liquid-liquid extraction
Lys	Lysine
MAPK	Mitogen-activated protein kinases
MAPs	microtubule-associated proteins
Met	Methionine
MKK1	Mitogen-activated protein kinase kinase 1
MSG5	Tyrosine protein phosphate 5
OA	Oatmeal agar
PBS	Phosphate buffer solutions
Phe	Phenylalanine
PKA/B/C	Protein kinase A/B/C
PP1	Protein phosphatase 1
PPT	Protein precipitation
Pro	Proline
Ser	Serine
SPE	Solid phase extraction
TDZDs	Thiadiazolidinones
Thr	Threonine
TLC	Thin layer chromatography
Tyr	Tyrosine
Val	Valine
VRE	Vancomycin resistant

LIST OF SYMBOLS

%	Percentage
[I]	Inhibitor concentration
[S]	Substrate concentration
µg	Microgram
µL	Microliter
µM	Micromolar
A	Absorbance
g	Gram
H_o	Null hypothesis
H_1	Alternative hypothesis
IC_{50}	Half-maximal inhibitory reaction
K_i	Inhibitory constant
K_m	Michaelis-Menten equation
M^+	Molecular ion
m/z	Mass per charge
mg	Milligram
mg/mL	Milligram per milliliter
mL	Milliliter
mm	Millimeter
ng	Nanogram
OD	Optical density
^oC	Degree Celsius
R_f	Retention value
RLU	Relative light unit
rpm	Revolutions per minute
v/v	Volume per volume
V_{max}	Maximum velocity
v_o	Velocity
α	Alpha
β	Beta

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Actinomycetes are gram positive bacteria commonly found in soil and sediment. They are regarded as the most promising source for new drugs due to their ability to produce active compounds with unique structural features and various biological activities (Takahashi and Omura, 2003; Toume *et al.,* 2014). For example, antimicrobial agents (streptomycin and erythromycin) (Schatz *et al.,* 2005; Shangavi *et al.,* 2014), anticancer agents (nonactin, tetracenomycin D, resistomycin and 1-hydroxy-1-norresistomycin) (Kock *et al.,* 2005; Jeong *et al.,* 2006) and antiparasitic agents (phenzamine 12 and Trioxacarcins) (Maskey *et al.,* 2004; Dashti *et al.,* 2014). Actinomycetes also gained interests to search for small molecule inhibitor targeting kinase proteins; especially glycogen synthase kinase-3 (GSK-3) (Ojo *et al.,* 2011).

GSK-3 is a serine/threonine kinase discovered in the 80's as a key regulator of glucose metabolism. It is expressed as two isoforms in human; GSK-3α and GSK-3β (97 % of similarities) which only differ on the length of their N-terminal (Woodgett, 1990). Extensive studies revealed that, GSK-3 involved in numerous signaling pathways and processes in human such as Wnt and Akt signalling, cell differentiation and cell survival (Jope and Johnson, 2004; Klann *et al.,* 2004; Grimes and Jope, 2011). Due to its diverse role in cells, its dysregulation may cause diseases such as cancer, neurodegenerative disease and diabetes; often due to overexpression of GSK-3β (Elder-Finkelman *et al.,* 1999; Cohen and Goedert, 2004; Baylin and Ohm, 2006). Thus, molecule that can inhibit the activity of this enzyme is regarded as a potential curative way (Elder-Finkelman and Martinez, 2011). The example of GSK-3β inhibitor which is clinically in used is lithium to treat neurodegenerative disease (Sun *et al.,* 2002). However, lithium is lack in specificity which raised concern that it might lead to other diseases such as cancer since it will