SCREENING FOR MICROBIAL INHIBITORS AGAINST SIGNAL TRANSDUCTION IN EUKARYOTES AND *Mycobacterium*

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DECLARATION

I hereby declare that the materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

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ABSTRACT

This study involves the search for microbial inhibitors that disrupt Ras/Raf-1 interaction in the yeast two-hybrid system by the expression of *HIS3* and *lacZ* reporter genes. It is a continuation of the previous study where a *Streptomyces*, H7372 was identified as a possible inhibitor of Ras/Raf-1 protein-protein interaction. H7372 caused larger inhibition zone on histidine minus plates and reduce β-galactosidase activity. Radicicol was also positive in this screening. Radicicol and geldanamycin are two inhibitors of Ras/Raf protein-protein interaction by acting on HSP90 causing the degradation of Raf. H7372 also inhibited the growth of *Mycobacterium smegmatis*. Raf-1 is a serine-threonine kinase. *Mycobacterium* also possessed eukaryotic-like serine-threonine kinases. This creates the opportunity to investigate the possibility that the inhibitor of Raf-1 also inhibit the serine-threonine kinases of *Mycobacterium*. The first part of this study concerned isolating more actinomycetes for screening and the second part was to study H7372 in detail. Eighty actinomycete isolates were obtained by two selective isolation methods using soil samples from three forests in Sabah. No Ras/Raf-1 protein-protein interaction inhibitor was found. One false positive H11337 was identified. The extract was positive in the *HIS3* reporter gene but negative in the *lacZ* reporter gene. Crude freeze-dried extract of H7372 reduced p-ERK1/2 level in MCF-7 cells under insulin stimulation at 250μg/ml and 500μg/ml. A purified fraction, H7372PRE was isolated by cold precipitation method and produced one single peak at 26.03 min retention time by reversed-phase HPLC. H7372PRE inhibited both HIS3 and *lacZ* reporter expression. H7372PRE reduced the level of p-ERK1/2 at 75μg/ml and 100μg/ml and increased the level of p-MEK1/2 at 75μg/ml and 100μg/ml. HPLC fraction of H7372PRE reduced the level of p-ERK1/2 at 50μg/ml. Crude freeze-dried extract of H7372 decreased JNK/SAPK1c, MAPKAP1a and PKCa kinase activity to 54-63% of the original activity. The binding to HSP90 was not detected by surface plasmon resonance. The crude extract and fraction of H7372 consistently reduced p-ERK level, indicating its intervention to the MAPK pathway, possibly through disruption of Ras/Raf-1 interaction through HSP90. H7372PRE did not inhibit the growth of M. smegmatis. H9318 (*Penilicium sp.*) did not inhibit *M. smegmatis* but it's semi-purified fraction, H9318_S1 that inhibited PP2A and PP1 in vitro inhibited *M. smegmatis*, indicating that the inhibition was possibly through the eukaryotic-like phosphatase of *Mycobacterium*. This study demonstrated that the actinomycetes from forests in Sabah possess interesting compounds that affected the signal transduction pathways in eukaryotes and *Mycobacterium*.

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ABSTRAK

PENYARINGAN PERENCAT MIKROBIAL TERHADAP TRANSDUKSI ISYARAT DALAM EUKARIOT DAN Mycobacterium

Kajian ini melibatkan pencarian perencat mikrobial yang merencat interaksi protein-protein Ras/Raf-1 dalam sistem dua-hibrid yis melalui pengekpresan gen pelapor HIS3 dan lacZ. Ia merupakan lanjutan daripada kajian sebelumnya dimana Streptomyces, H7372 telah dikenalpasti berkemungkinan besar merencat interaksi protein-protein Ras/Raf-1. H7372 menyebabkan zon rencatan pertumbuhan yang lebih besar dalam piring tanpa histidine dan merendahkan aktiviti β-galaktosidase. Radicicol juga menunjukkan keputusan positif dalam penyaringan ini. Radicicol dan geldanamycin merupakan dua perencat interaksi protein-protein Ras/Raf melalui HSP90 dengan menyebabkan degradasi Raf. H7372 juga merencat pertumbuhan Mycobacterium smegmatis. Raf-1 merupakan kinase serine-threonine. Mycobacterium juga mempunyai kinase serine-threonine seperti eukariot. Ini menghasilkan peluang untuk menyiasat kemungkinan perencat Ras/Raf-1 juga merencat kinase serine-threonine dalam Mycobacterium. Bahagian pertama kajian ini adalah berkenaan mengasingkan aktinomiset untuk penyaringan dan bahagian kedua adalah untuk mengkaji H7372 dengan lebih lanjut. Lapan puluh aktinomiset telah diperoleh dengan kaedah pemencilan selektif meggunakan sampel tanah dari tiga hutan di Sabah. Tiada perencat interaksi-protein-protein Ras/Raf-1 ditemui. Satu positif palsu H11337 dikenalpasti, ia positif dalam gen pelapor HIS3 tetapi negatif dalam gen pelapor lacZ. Ekstrak sejuk kering H7372 merencat p-ERK1/2 dalam sel MCF-7 di bawah rangsangan insulin pada 250μg/ml dan 500μg/ml. Satu fraksi, H7372PRE yang diasingkan melalui kaedah mendakan, menunjukkan puncak tunggal pada 26.03 min dalam analysis HPLC. Ia merencatkan ekspresasi kedua-dua gen pelapor HIS3 dan lacZ. H732PRE merencat p-ERK1/2 pada 75μg/ml dan 100μg/ml dan meningkatkan p-MEK1/2 pada 75μg/ml and 100μg/ml. Fraksi HPLC H7372PRE merencatkan p-ERK1/2 pada 50μg/ml. Ekstrak dan fraksi HPLC H7372 merencat p-ERK, ini menandakan ia mengakibatkan perencatan pada laluan MAPK, kemungkinan besar melalui perencatan interaksi Ras/Raf-1 melalui HSP90. Extract sejuk kering H7372 merendahkan aktiviti kinase JNK/SAPK1c, MAPKAP1a and PKCa kepada 54-63% daripada aktiviti asal. Penggabungan kepada HSP90 tidak dikesan melalui ujian 'surface plasmon resonance'. Ekstrak dan fraksi H7372 merencatkan p-ERK secara konsisten. Ini menunjukkan ia mengakibatkan perencatan pada laluan MAPK, kemungkinan merencat interaksi protein-protein Ras/Raf-1 melalui HSP90. H7372PRE tidak merencat pertumbuhan M. smegmatis. H9318 (Penilicium sp.) tidak merencat pertumbuhan M. smegmatis tetapi fraksi separa tulennya, H9318_S1 yang merencat PP2A and PP1 secara in vitro merencat M. smegmatis mc^2155, ini menunjukkan kemungkinan besar perencatan adalah melalui fosfatase yang menyerupai eukariot dalam Mycobacterium. Kajian ini telah menunjukkan bahawa aktinomiset dari hutan di Sabah mempunyai kompoun yang menarik yang memberi kesan kepada sistem transduksi isyarat dalam eukariot dan Mycobacterium.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
CK2	Casein kinase 2
CR1	Conserved domain 1
CR2	Conserved domain 2
CR3	Conserved domain 3
CRD	Cysteine rich domain
DAP	Diaminopimelic acid
DNA-BD	DNA-binding domain
DOTS	Direct Observed Treatment Short-course
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FT	Farnesyl transferase
G+C	Guanine plus cytosine
GA	Geldanamycin
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
H7372 FD	Crude freeze dried extract of H7372
H7372 PRE	H7372 extract purified by cold precipitation
HIS3	Histidine biosynthesis gene
HPLC	High performance liquid chromatography
HSP90	Heat shock protein 90
HV	Humic acid B-vitamins
ICL	Isocitrate lyase
lacZ	B-galactosidase gene
MBP	Myelin basic protein
MEK	Mitogen-activated protein kinase
MS	Malate synthase
OA	Oatmeal agar
ONPG	*O*-nitrophenyl-β-D-galactosidase
PAGE	Polyacrylamide gel electrophoresis
PAK	P21-activated kinase
PDGF	Platelet-derived growth factor
PMSF	Phenylmethylsulfonyl flouride
PP2A	Protein phosphatase 2A
RA	Radicicol
RBD	Ras binding domain
rRNA	ribosomal Ribonucleic acid
RTK	Receptor tyrosine kinase
SCN	Starch casein nitrate
SDS	Sodium dodecyl sulfate
SOS	Son of sevenless
SPR	Surface plasmon resonance

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Ste11	Yeast homologue of Raf
TAD	Transcription activation domain
TBS	Tris-buffered saline
TEMED	N,N,N,N-tetramethylethylenediamine
VEGF	Vascular endothelia growth factor
WHO	World health organization
WSSP	Whole cell sugar pattern
PP1	Protein phosphatase 1
β	Beta

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CHAPTER 1

INTRODUCTION

1.1 Research background

Bioactive compounds are on a new level of pursuit. Unlike the conventional ways, screenings are now targeted not only at growth inhibition of pathogenic microorganisms but also against signal transduction pathways believed to be key players regulating vital cellular functions. Actinomycetes, high G+C, Gram positive bacteria play the central role in drug discovery. The *Streptomyces* and *Micromonospora* are abundant actinomycetes in soil. *Streptomyces* are best known as producers of bioactive compounds like streptomycin and doxorubicin and are currently use as anti-infective and anticancer drugs. Gentamicin is known to be produced by *Micromonospora*. The major differences between the two is the absent of aerial mycelia of *Micromonospora*, extremely slow growth of the bacteria and the differences in cell wall constituents (Kudo, 1997; Yokota, 1997).

Chemical novelty associated with natural product is high and metabolites produced by bacteria provide great structural diversity. More than half of known antibiotics are of microbial origin like kanamycin, streptomycin and erythromycin, whereas daunorubicin, doxorubicin and mitomycin are important antitumor agents (Demain, 1999). Regulatory factors like A-factor, implicated in both morphological differentiation and secondary metabolism, is thought to be responding to environmental and physical stresses (Bibb, 2005). Kinase

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inhibitors produced by actinomycetes are like staurosporine, a broad range kinase inhibitor and K252a, protein kinase C inhibitor. There are also protein phosphatase inhibitors like tautomycin, fostriecin and FK506 of *Streptomyces sp.*

Borneo is the second largest tropical island in the world harbouring nature's precious biodiversity resources where its potential has yet to be fully explored. The actinomycetes diversity and abundance is high in the tropical forest region (Wang *et al.*, 1999). The search for biologically active compounds has led us to exploit the forest area in the hope for finding higher microbial diversity that provides better chance at obtaining a hit. Geldanamycin, leptomycin, mitomycin, manumycin, luminacins are all produced by *Streptomyces sp.* and have anti tumour activities by interfering with cell signalling.

In this study, actinomycetes were isolated from soil samples collected from just below the leaf litter as the degradation of the leaf litters and other dead organic material were aided by microbes in which many of them are fungi and actinomycetes. These microbes were selectively isolated and the acetone extracts produced by shake flask fermentation of these isolates were screened against Ras/Raf-1 protein-protein interaction screening using the yeast two-hybrid screening, which was designed to screen for inhibitors affecting mammalian Ras/Raf-1 interaction. Potential inhibitor of the screening affects the interaction directly or indirectly, as demonstrated by radicicol (Ki *et al.*, 1998).

The Ras/Raf/MEK/ERK pathway is important in governing cell proliferation, differentiation, transformation and apoptosis. Growth factors bind to their associated receptors (which are receptor tyrosine kinase) on the cell membrane to activate cascades of signals, relaying cellular information from the cell membrane to the nucleus (Kolch, 2000). The activation steps of Ras protein includes localization of Ras protein to the membrane, ras dimerization and

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involving guanine nucleotide exchange factor (GEF), which serve to accelerate the exchange of RAS-GDP to RAS-GTP. RAS-GTP. The active Ras attained a conformation enabling it to bind to Raf-1. In order to return to it's resting state RAS-GAP interacts with GTPase activating protein (GAP) (Inouye *et al.*, 2000). Activated Ras will then activate Raf, a process involving many other proteins and critically phosphorylation at S338 (Edin & Juliano, 2005) and dephosphorylation at S259 by PP2A which leads to membrane localization, for subsequent activation step (Kubicek *et al.*, 2002), and S471 phosphorylation for MEK binding (Zhu *et al.*, 2005). Raf activates MEK (Lee & McCubrey, 2002) and subsequently ERK (Zheng & Guan, 1993). The RAS-MAPK pathway is a key signalling mechanism that regulates many cellular functions such as cell growth, transformation and apoptosis. Regulation of both Ras and Raf is crucial in the proper maintenance of cell growth as oncogenic mutations in these genes lead to high transforming activity.

Hsp90 is present in the cytoplasm of eubacteria, yeast and multicellular organisms, it plays an important role in the conformational regulation of key signalling molecules including various steroid hormon receptors, kinases including Raf-1 and Ste11 and other proteins (Buchner, 1999). The disruption of Hsp90 by an ansamycin, geldanamycin leads to destabilization of Raf and thereof prevented the association of Ras with Raf (Schulte *et al.*, 1995). Radicicol, a macrolide antibiotic was also affecting Raf-1 through Hsp90 (Schulte *et al.*, 1999). Novobiocin, unlike geldanamycin and radicicol, act on the carboxyl terminal ATP binding site, also disrupting Hsp90 function (Marcu *et al.*, 2000).

Tuberculosis is reemerging world wide at alarming rate. Apart from the multi drug resistance tuberculosis (MDR-TB), latent persistent infection is posing greater fear as the infected individual will not show any symptom until his

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immune system becomes compromised. The bacteria reside in the human lung for decades unnoticed. Several evading mechanism of this bacteria is being noticed, the (isocitrate lyase) ICL and malate synthase (MS) of the glyoxylate cycle are two potential targets.

There are 11 eukaryotic-type serine/threonine kinases (PknA, B, D, E, F, G, H, I, J, K and L) and 4 phosphoprotein phosphatases in *Mycobacterium tuberculosis* genome (Cole *et al.*, 1998). Their presumptive roles includes cell division and differentiation regulation, receptor for environmental sensing and most importantly PknG was found to be vital in mediating intracellular survival of mycobacteria (Walburger *et al.*, 2004). Inhibition of these eukaryotic like kinases or phosphatases is therefore an attractive target. H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine), a protein kinase C inhibitor was found inhibit *M. smegmatis* and *M. bovis* BCG growth (Drews *et al.*, 2001).

Mycobacterial serine/threonine phosphatase (mstp) role is implicated in cell division regulation. Mstp is a transmembrane protein that dephosphorylates phosphorylated PknA and PknB on serine/threonine residues. The presence of mstp in slow growing mycobacterial species, its transmembrane localization, and ability to dephosphorylate phosphorylated PknA and PknB implicates that Mstp may play a role in regulating cell division in *M. tuberculosis* (Chopra *et al.*, 2003).

H7372 is a *Streptomyces* isolated from soil from root of *Bruguiera sp.*, a plant found in the mangrove swamp ecosystem. It was found to inhibit Ras/Raf-1 protein-protein interaction, H7372, using yeast two-hybrid screening. This isolate was aerobically cultured to produce bioactive compounds. The crude freeze-dried extract was found to reduce p-ERK and p-MEK level in NIH3T3 cells. H7372 extract was also found to inhibit ICL of the *Mycobacterium* (Cheah, 2003). The compound of interest is therefore pursued.

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H9318 was a *Penicilium sp.* Isolated from soil. The extract was picked up from yeast based screening against PP1 (protein phosphatase 1). The crude extract was found to positively inhibit PP1 and PP2A (protein phosphatase 2A) by the *in vitro* test (Voo, 2004). Two fractions, H9318_S1 and H9318_S2 were isolated by HPLC of ethyl acetate extract of H9318. H9318_S1 and H9318_S2 were later identified to have similar bioactive properties by the yeast based screening and the *in vitro* dephosphorylation assay (Ong, 2005).

1.2 Objectives of study

a. To isolate diverse new actinomycete from forest soils of Sabah using selective isolation methods for actinomycetes particularly *Streptomyces* and *Micromonospora*.

b. To screen for inhibitors of mammalian Ras/Raf-1 protein-protein interaction by the yeast two-hybrid system from the actinomycetes isolates and compare them with geldanamycin or radicicol. Geldanamycin from *Streptomyces hygroscopiscus* (actinomycete) and radicicol from *Monosporium bonorden* (fungus) are two inhibitors that affect Raf-1 through HSP90.

c. H7372 was identified as Ras/Raf-1 protein-protein interaction inhibitor by yeast two-hybrid system in previous study. H7372 protein kinases inhibition and Hsp90 binding was assessed by *in vitro* kinase assay and surface plasmon resonance (SPR) assay in comparison to geldanamycin or radicicol.

d. To purify bioactive fraction of H7372 actinomycetes extract that effected the mammalian Ras/Raf-1 interaction in the yeast two-hybrid system in both the *HIS3* and *LacZ* reporter.

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e. To examine the effects of H7372 crude extracts and purified fraction towards phosphorylation level of MAPK pathway key proteins in MCF-7 human fibroblast cell line through immunoblotting approach.

f. To test the inhibitory effects of H7372 purified fraction against *Mycobacterium smegmatis* mc^2155 through paper disc susceptibility test. H7372 was found to be inhibitor of ICL in *M. smegmatis* mc^2155 based assay by previous work.

g. To test the inhibitory effects of H9318_S1 and H9318_S2 [HPLC fractions of *Penicilium sp.* extract, identified through yeast based screening and *in vitro* dephosphorylation assay as inhibitors of mammalian protein phosphatases (PP1 and PP2A)] against *Mycobacterium smegmatis* mc^2155 through paper disc susceptibility test.

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CHAPTER 2

LITERATURE REVIEW

2.1 Actinomycetes

Actinomycetes are Gram positive, high G+C content bacteria. They are morphologically characterized by the type and stability of the mycelium, the number, type and disposition of the spores and among others formation of sporangia and flagellate elements. The chemical characterizations include the cell wall and whole cells sugars compositions alongside with type of lipids found. Cell wall type I has LL-DAP (LL-2,6-diaminopimelic acid) and glycine with 11 members of different genera like *Streptomyces, Streptoveticillium, Nocardioides, Microellobosporia* and *Arachnia* they are further differentiated based on morphology and physiological differences. There are a few genera having type II cell wall, all five of them are *Micromonospora, Actinoplanes, Amorphosporagium, Ampullariella* and *Dactylosporangium*. The cell wall contains *meso*-DAP and glycine. Xylose and arabinose are the diagnostic sugars of the whole cell sugar pattern (WSSP). Type III and IV cell wall also contain *meso*-DAP. Actinomadura and Frankia are members of the Type III group along with 13 others with WSSP with or without madurose and with diverse physical morphology. As for Type IV, the cell wall and WSSP contains arabinose and galactose with *Mycobacterium* and *Nocardia* as the more well known members. There are up to 9 cell wall type with different cell wall compositions and other members of the actinomycetes (Lechevalier & Lechevalier, 1981).

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Actinomycetes on a solid medium forms the substrate mycelium or the primary mycelium, which penetrate into the agar. The aerial mycelium, which is permanently in contact with air is formed later (Vobis, 1997). The colonies consistencies varies: raised, flat, hard, soft, leathery, smooth, wrinkled, granular etc. There are a wide spectrum of colours for the colonies including white, yellow, orange, red, purple, blue, green, brown, grey and black. Growth of filamentous actinomycetes is completely different, in the sense both of biomass increase and cell division, from the patterns of cell enlargement and binary fission that occur in unicellular bacteria. Vegetative hyphae grow into long, often multiply branched masses of tangled threads without undergoing cell division. The result is large, essentially multinucleate cells. Separation of cytoplasm and nuclei by septa occurs sporadically and without apparent pattern. Reproduction occurs by a variety of mechanisms of fragmentation of hyphae to form haploid spherical cells in the nonsporeforming genera such as *Nocardia* or by differentation of the fragments to spores in other genera (Ensign, 1999).

Actinomycetes can be found easily in humus rich soil, the ecological niche of most actinomycetes is probably the aerobic zone of soil whereby they are actively involved in the degradation of leaf litters and other organic substrates. It was estimated that there are more than one million of them per gram of soil (Nonomura & Hayakawa, 1988). Actinomycetes can also be isolated from river mud, lake bottom and also swampy area. Some corynebacteria, mycobacteria and species of *Actinomyces* are normal microbial flora of the human skin and also found in gingival tissue of the mouth.

Secondary metabolism is brought on by exhausion of a nutrient, biosynthesis or addition of an inducer, and/or by a growth rate decrease. The deep-seated reason for the production of these secondary metabolites that are

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