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Research article

Actinomycetes and fungi isolated from Maliau Basin, Sabah and the screening for novel secondary metabolites against eukaryotic signal transduction and *Mycobacterium*

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ABSTRACT: Maliau Basin is one of the world's hot spot of biodiversity. Maliau Basin Sabah has such forest diversity such as the heath forest and the submontane hill forest that contain the family dipterocarpaceae. The unique geological landscape which gives rise to the multitude of water falls. The Basin supports a myriad of flora, fauna and microbes. These microbes serve the main purpose of biodegrading organic materials and enriching the soil's nutrients. Actinomycetes and microfungi have been known to be prolific producers of bioactive secondary metabolites, which affect signal transduction and cell cycle. The search for novel pharmaceutical products requires a very large collection of new actinomycetes and fungi to be isolated from new micro niches. Certain microorganism may have a tendency to dominate or co-exist with other microorganisms in a certain niche based on the organic materials being degraded or its ability to produce certain compounds. Based on this assumption, we have sampled the soils just below the leaf litter under identified trees. We have successfully isolated 98 strains of actinomycetes and 254 strains of microfungi from the soil samples of Maliau Basin. Acetone extracts from these microbes have been tested against various yeast based screening systems. We screened for inhibitors against mitogen-activated protein kinase (MAPK) pathway by targeting the activity of MKK1 and MSG5 proteins, which are involved in the yeast cell wall integrity system. Overexpression, under induction by galactose utilizing the GAL1 promoter of a hyperactive mutant $MKK1^{P386}$ leads to cell lysis even in the presence of D-sorbitol (1M) as an osmoprotectant. No actinomycetes and filamentous fungi were found to inhibit against MAPK kinase (MKK1) in yeast and 3 actinomycetes strains H7897, H7944 and H7973 were shown to be toxic towards yeast in the $MKK1^{P386}$ screening system. The introduction of a dual specificity (tyrosine and serine/threonine) phosphatase MSG5 acting on Mpk1 (MAP kinase) into the $MKK1^{p386}$ system led to cell growth and inhibitor of this phosphatase will lead to growth inhibition (cell lysis). No actinomycetes and filamentous

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fungi were found to inhibit against protein phosphatase (MSG5) in yeast. Five actinomycetes strain H7897, H7944, H7970, H7973 and H8528 showed toxicity to yeast in MSG5 screening system. In the Ras/Raf protein interaction, the yeast two-hybrid system with cloned mammalian Ras and Raf proteins was used. No yeast growth will be observed on medium without histidine if the inhibitor blocks the Ras/Raf protein interaction as the histidine biosynthetic gene (*HIS3*) would not be transcribed. The screening done in the Ras/Raf protein interaction involves the inhibitory effect in which the protein-protein interaction was blocked. H7897, an actinomycete was found to be a potential inhibitor against Ras/Raf screening system and 4 actinomycetes strains (H7915, H7940, H7944 and H7976) were toxic against yeast in Ras/Raf screening system. Another strain, H7944, an actinomycete (a non-*Streptomyces*) from Maliau Basin soil was screened as toxic to yeast and found to have decrease phosphorylation of mammalian MEK1/2 and ERK1/2 kinases in the Western blotting analysis. Ras/Raf protein interaction may be affected as evidenced by lowered β-galactosidase synthesis. For the serine/threonine protein phosphatase type 1 (PP1) inhibitor screening, the yeast that possesses one catalytic subunit (Glc7p) was used as the target for the screening. The screening system uses two alleles of the Glc7p which are *GLC7*, the wild type allele (PAY704-1) and *glc7-10*, the temperature sensitive mutant (PAY700-4). The mutant allele causes cell wall integrity defects and cell cycle arrest at 37°C but rescued by high osmolarity (1M D-sorbitol). An inhibitor acting on Glc7p in the wild type would behaved same as the mutant phenotype as that caused by the mutant allele, with growth inhibition at 37°C but rescued by D-sorbitol (1M). Two *Penicillium* strains (H9307 and H9318) and 4 unknown fungi (H9114, H9213, H9316 and H9317) isolated from Maliau Basin soil showed up as positive in the screen. In *in vitro* system, three strains (H9307, H9317 and H9318) also decreased dephosphorylation by Glc7p of yeast as well as the mammalian PP1 gamma.

Mycobacterium

In the screening for inhibitors against isocitrate lyase (ICL) in *Mycobacterium*, there are 3 extracts (H7893, H7910 and H7944) shown toxicity against both the wild type *M. smegmatis* (H8000) and transformed *M. smegmatis* (H8012). These extracts inhibited the growth of *Mycobacterium* on both glucose and acetate plates.

INTRODUCTION

This paper concerns the search for soil actinomycetes and filamentous fungi from the humus layer and leaf litter from identified trees and other plants in different primary vegetation types in Maliau Basin, Sabah (Marsh, 1989). Maliau Basin is located in the centre of the southern part of Sabah in Borneo. Maliau Basin is approximately the shape of a saucer, perched on highlands. The rim of the elevated basin ranges from 1500m above sea level to 1900m. The almost circular Basin was delimited by cliffs on almost all sides. This peculiar shape had been moulded by faults and earth movements resulting in the Tanjung Formation during the early to middle Miocene age. A set of the Sungai Maliau tributaries fans out into the area. Sungai Maliau itself gorged out of the south-eastern comer of the basin into Sungai Kuamut which in turn feeds the longest and largest river in Sabah, the Sungai Kinabatangan (Maryati, 1998; Phillipps, 1997; Wong, 2001).

There were four major forest types in the Maliau Basin (Hazebroek, Adlin & Sinun, 2004). Firstly the lower montane heath (kerangas) forests was found fringing the north-western and western portion of the rim

and the south rim near where the base camp (Camel Trophy). It was characterised by the thick layer of moss on the forest floor, encircling most of the stunted trees. *Casuarina* and conifers dominated the heath forest with other unique plants such as pitcher plant (*Nepenthes sp.*). Other than the heath forest, especially in the inner portion of the Basin was the submontane hill forest. This was characterised by commercial trees such as *Agathis* and some species of the dipterocarp family (Maryati, 1998).

In an undisturbed situation, the erosion rate of the soil was very low due to the good porosity of soil. The porosity of the soil was high and therefore water was readily absorbed, giving very little run-offs. As such, there was little or no loss of top soil, which is vital as the nutrient content of the soil in this area was relatively poor. The poor condition of the soil was the basis of the heath forest, and affects plant behaviour. Nevertheless, there were landslides in some parts of the Basin and these were accounted for by the steepness of the slope as well as some run-offs. In these parts the rivers were fairly sedimented (Maryati, 1998).

Soil samples collections were carried out for the period of 18-24 May 2001 to the Maliau Basin in collaboration with the DANCED Maliau Basin project by Sabah Foundation. Maliau Basin was chosen for its fairly extreme poor nutrient, highly acidic and wet soils. The soil samples under identified plants (*Agathis sp.* and *Shorea sp.*) were collected by aseptic methods. Actinomycetes that can grow at pH3.5 or pH7.2 were especially isolated on humic acid (HV) – vitamin B plus cycloheximide. The fungi were isolated at pH4.5 on selective potato dextrose agar (PDA) and at the pH of 5.6 on DRBC medium. The pure isolates were grown aerobically and acetone extracts were produced.

Screening metabolites from soil microbes of the *Actinomycetales*, has led to the identification and isolation of a large number of antibiotics for example the anti-filarial avermectin from *Streptomyces avermitilis*. Anti-tumor antibiotics are amongst the most important of the cancer chemotherapeutic agents, which include daunomycin-related agents, daunomycin itself, adriamycin, the semi-synthetic derivatives idarubicin and epirubicin (Newman, Cragg & Snader, 2000). Except for the semi-synthetic compounds, many were isolated from various *Streptomyces* species. Members of the order *Actinomycetales* produce many commercially important metabolites, notably antibiotics and non-antibiotics. Some actinomycetes are also a source of inhibitors of eukaryotic cell cycle, for example, trichostatin A from *Streptomyces hygroscopicus* causes arrest of the cell cycle in synchronous cultures of normal rat fibroblasts in both the G1 and G2 phases by acting as a potent inhibitor of histone deacetylase (Yoshida, Horinouchi & Beppu, 1995). Staurosporine, an antifungal agent, is produced by *Saccharothrix aerocolonigenes* and was found to inhibit PKC. Its synthetic analogue, UCN01 (7-hydroxystaurosporine) also blocks cell-cycle-checkpoint-control kinase CHK1 (Cohen, 2002). A major advantage of the natural products approach to drug discovery is that it is capable of providing complex molecules that would not be accessible by other routes.

One of the crucial conditions in natural products approach to drug discovery is the target-directed screening system to identify specific inhibitor in the crude extracts. This screening system has been applied in the search for novel therapeutic agents (Kuo & Garrity, 2002). One such convenient screening system is to use the molecular targeted system in yeast (Grabley & Thiericke, 1999; Parsons *et al.*, 2003). These include the MAPK Kinase

(MKK1), MAP Kinase phosphatase (MSG5), Ras/Raf proteins interaction and serine/ threonine phosphatases (PP1) in yeast systems.

Screening for secondary metabolites that affect the eukaryotic signal transduction was carried out on all the isolated microbes (actinomycetes and filamentous fungi). These secondary metabolites are of interest as biochemical reagents in molecular cell biology and as potential drugs for cancer (Ho, 2003).

Mycobacterium

The abilities of *Mycobacterium tuberculosis* to infect macrophages, establishing a long term, persistent infection in the host have made it one of the most wide spread disease in the world. During latent infection, fatty acids are used as the main carbon source and in response; the glyoxylate shunt is up-regulated. In mice, the disruption of *icl* gene, which codes for the glyoxylate enzyme isocitrate lyase (ICL), attenuates *M. tuberculosis* persistent infection (McKinney *et al.*, 2000). The strategy for survival during the chronic stages of infection entails a metabolic shift in the bacilli utilization of carbon source to C_2 substrates, particularly in the form of acetyl-CoA generated by β-oxidation of fatty acids. Under these conditions, glyoxylate shunt allows anaplerotic maintenance of the tricarboxylic acid cycle and assimilation of carbon via gluconeogenesis.

A rapid drug screening strategy adapted from Sharma *et al.* (2000) is being conducted in our laboratory. This screening strategy essentially searches for potential inhibitors of ICL. The system is principally based on the growth inhibition of an *icl*-transformant grown on acetate carbon source. The *icl*-transformant employed is a *Δicl M. smegmatis* mutant complemented with the *M. tuberculosis* ICL normal gene.

MATERIALS AND METHODS

a) Soil sampling and isolation of microbes (actinomycetes and fungi)

A total of 146 soil samples were collected under the leaf litter of identified trees from heath forest Maliau Basin (Table1). The maps in Figure 1 and Figure 2 showed the approximate sampling areas in Maliau Basin Conservation Area, Sabah. Figure 3 showed the view of Giluk Falls with some soil sampling near the Giluk Falls, Maliau Basin located at north-western of the Camel Trophy camp. Figure 4 showed the Maliau Basin heath vegetation dominated by small and medium size of trees and plants especially *Tristoniopsis grandifolia* (Palawan) with peeling bark. A total of 10 soil samples were collected from inside and outside the Maliau Basin rim for the determination of soil pH of Maliau Basin (Table 3). Soils were especially collected under resins (dammar) producing dipterocarps, *Shorea* spp. (Figure 5) and *Agathis* spp. (Figure 6) with profuse secretion of white and black resins.

Actinomycetes was isolated by suspending 0.1g of soil into 10mL of sterile distilled water and later diluted up to 10^{-3}. 100µL of the suspension is spreaded on humic-acid (HV) – vitamin B plus cycloheximide (Nonomura & Hayakawa, 1988) with pH 3.5 and pH 7.2 respectively. Isolated strains were transferred from HV medium on to similar pH Oatmeal Agar (OA) plates and incubated at 28 °C for 14 days. Colour determination should be made under normal daylight for the mass colour of mature culture (sporulating aerial surface growth on the agar), the colour of the substrate mycelium (as viewed from reverse side of the plate) and diffusible soluble pigments. The ability of sporulation was also observed and recorded. Fungal strains were isolated from soil source using the direct inoculation of soil onto the plated followed by spreading. The amount of soil to be plated out on the isolating plate was

limited to one pinch of the forceps. Soil samples that were collected were processed for fungi isolation within 2 weeks. Two types of media were used for isolation of microfungi. The initial medium was PDA with chloramphenicol (50µg/ml-final concentration) and high concentration of NaCl (7.5 %) with the pH at 4.5, followed by DRBC medium with NaCl (2.5%) with the pH at 5.6. A pinch of soil was spread evenly on the isolating agar plate using a sterile glass rod spreader. The isolating plate was then left for incubation from 3 to 7 days at 28°C. Colonies that appear on the isolating plates were transferred to new purification plates of normal PDA using a sterile forceps. The transfer would be a pinch of a small portion of the edge of the colony. The transfer is ideally done without isolating media agar attached. This is to avoid contamination of bacteria or fungi that did not germinate in the harsh condition of the isolating media. Colonies of fungi at the edge of the isolating plates were avoided as there could be a possibility of contaminated fungi from the air growing on the isolating plates. The removal of multiple fungal strains from the isolation plates was based on the visible characteristics of the colonies. The characteristics were shape of the colony, colour of the top and bottom of the colony and the extra cellular pigmentation produced by the colonies. Colonies that differ in one or more of these characteristics were identified and transferred on to new purification PDA plates. The colours of the aerial mycelium and the substrate mycelium of the purified fungi colonies were observed and recorded. Any extra cellular pigmentation produced was also recorded. These observations were done 7 to 8 days after incubation. The growth rate of the microfungi was also noted. Some of the microfungi strains which have given positive results of screening tests were further observed under optical microscopy using lactophenol stain.

b) Production of microbial extracts

Actinomycetes were aerobically grown in 10ml of mannitol-peptone liquid medium (D-mannitol 2g, peptone 2g, D(+)glucose 1g, distilled water 100 ml) in 125ml Erlenmeyer flask at pH 7.2 and incubated shaking at 220rpm for 5 days at 28°C. An equal volume of acetone was added to the incubated culture to produce microbial extract and stored at 4°C.

Fungi were aerobically grown in 15ml of yeast extract-peptone-sucrose liquid medium (yeast extract 10g, peptone 10g, sucrose 10g, KH_2PO_4 1g, $MgSO_4.7H_2O$ 0.3g, distilled water to 1 litre) pH 5.6, contained in 125ml Erlenmeyer flasks and incubated shaking at 220rpm for 7 days at 28°C. An equal volume of acetone was added to the incubated culture to produce microbial extract and stored at 4°C.

c) Screening for specific inhibition of molecular targets in cell based system

1. MAPK kinase (MKK1) and MAP kinase phosphatase (MSG5) in cell wall integrity system in yeast (Gustin *et al.*, 1998; Watanabe, Irie & Matsumoto, 1995; Yashar *et al.*, 1995)

The screening for inhibitor against MKK1 and MSG5 followed the method as described in Ho *et al.* (2001). An inhibitor of Mkk1 will result in a halo of yeast growth around the paper disk containing the extract in the galactose medium while growth will cover the whole plate in the glucose medium. This behaviour is caused by the inhibitor cancelling the lethal (lysis) effect of the overexpressed mutant $MKK1^{P386}$ kinase under galactose induction through the Gal-1 promoter in the cell wall integrity system.

An inhibitor of MSG5 will show a growth inhibition halo around the paper disk containing the extract in galactose medium as the protein phosphatase of MSG5 will nullify

the lethal effect of overexpression of mutant MKK1^{P386} kinase.

2. Ras/Raf protein-protein interaction (Ki *et al.*, 1998; Vojtek, Hollenberg & Cooper, 1993)

The screening utilized the yeast two-hybrid (cloned mammalian Ras and Raf) system. The LZ (H10014) is a transformant of L40 (H10006) *MATá trp1 leu2 his3 LYS::lexA-HIS 3 URA3::lexA-lacZ* with pLexA-RAS^{V12}(H10,011) and pVD16-RAF (H10,012). This transformant is able to grow on medium lacking histidine and produces β-galactosidase since the transcription of these two reporter genes are stimulated by the formation of the complex pLex-RAS^{V12} and pVP16-RAF (Ki *et al.*, 1998).

If this protein-protein interaction is interrupted by the inhibitor, the transformant can only grow in the presence of histidine but not in the absence of histidine. Expression of the *lacZ* reporter gene is also not induced.

β-Galactosidase assay (quantitative test) (Ki *et al.*, 1998; Miller, 1972)

For quantitative β-galactisidase assay, LZ (H10014) was grown in the SD liquid medium until it's concentration reached OD_{600} ~ 1. Then, 0.2ml of the cells were added to 1.4 ml of Z buffer (Miller, 1972) containing 0.75mg/ml 5 bromo-4-chloro-3-indolyl-β-galactopyranoside and 0.27% β-mercaptoethanol. The cells were permealized by mixing (vortex mixer) with 0.2ml 0.05% SDS in 50% of $CHCl_3$ and then added with 0.32ml of an ONPG (*O*-nitrophenyl β D-galactopyranoside) solution. The mixture was agitated (vortex mixer) for 10s and incubated for 1 hour at 30°C. The reaction was terminated by adding 0.8ml of 1M Na_2CO_3 . The mixture (sample) was centrifuged to pellet the cell debris. Then, a portion of supernatant fraction was removed for OD measurement at 420nm. Miller units were calculated with formula below:

$$\text{Miller units: } \frac{OD_{420} \times 1000}{OD_{600} \times \text{reaction time (min)} \times \text{volume of LZ cells (ml)}}$$

There were two controls used in this test. One control was blank with no ONPG to correct for colour due to the sample (LZ cells). Another control was no sample in condition to correct for spontaneous hydrolysis of ONPG.

3. GLC7, the catalytic subunit of serine/ threonine protein phosphatase type I (PP1) in yeast (Sassoon *et al.*, 1999; Shenolikar, 1994; Stark, 1996)

The screening system (Ong *et.al.*, 2006) used the temperature sensitive mutant GLC7-10 (PAY700-4) with cell cycle arrest at 37°C but the lethal effect was suppressed by high osmolarity (1M D-sorbitol) (Andrews and Stark, 2000). The inhibitor that acts on GLC7 protein in the wild type (PAY704-1) should have the same characteristic as that caused by the temperature sensitive mutation (*glc7-10*), namely with growth inhibition at 37°C but with growth with the addition of 1M D-sorbitol.

d) Western blotting

Western blotting analysis on phosphorylation of mammalian MEK1/2 and ERK1/2

Antibody and cell culture. An anti-phospho-p44/42 ERK 1/2 (Thr202/Tyr204) antibody and an anti-phospho-MEK1/2 (Ser217/221) antibody were purchased from Cell Signaling Technology Inc. An anti- MEK1 antibody was purchased from BD Transduction Laboratories. An anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey) were purchased from Amersham Pharmacia Biotech Ltd. NIH3T3 cells were maintained in DMEM (Nissui Pharmaceutical Co) containing 10% heat-inactivated fetal bovine serum (FBS) (Cell Culture Laboratories).

Western blot analysis. NIH3T3 cells were grown to 60% confluence in 10 cm tissue culture dish (Falcon Labware) in complete medium. Cells were serum-starved (0.5% fetal bovine serum) overnight prior treated with each compounds at 5% final concentration for 30 min and then to incubated with EGF 1 ng/ml for MAPK family induction for 30 min. The cells were washed 2 times with ice-cold PBS and lyzed with ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% (v/v) glycerol, 0.1 mM PMSF, 10 mM 13-glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate, and 10 mM leupeptin. The lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and protein concentrations of the supernatants were determined by Bradford method (Bio-Rad Laboratories). Equal amount of protein was added with 5 X Laemmli sample loading buffer followed by boiling for 5 min. Proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Milipore) by electroblotting. The membranes were blocked with 3% skim milk in 10 mM Tris-HCL (pH 7.5), 150 mM NaCl and 0.1% Tween 20 for 30 min at RT, and incubated with primary antibody (1:1000) with same buffer except for 5% BSA gentle agitation overnight at 4°C. For detection, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature. The antigen-antibody complexes were visualized with an ECL Western blotting detection kit (Amersham Pharmacia Biotech) according to manufacture's instruction. MEK1 of the blots were subjected to immunoblotting for corresponding nonphospho antibody. The same membrane was reported by stripping buffer. (Restore TM Western Blot Stripping buffer, PIERCE)

Western blotting analysis on phosphorylation of mammalian p38 MAP kinase and SAPK/JNK

Antibody and cell culture. An anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody and An anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody were purchased from Cell Signaling Technology Inc. An anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey) were purchased from Amersham Pharmacia Biotech Ltd. NIH3T3 cells were maintained in DMEM (Nissui Pharmaceutical Co) containing 10% heat-inactivated fetal bovine serum (FBS) (Cell Culture Laboratories).

Western blot analysis. NIH3T3 cells were grown to 60% confluence in 10 cm tissue culture dish (Falcon Labware) in complete medium. Cells were treated with each compound at 5% final concentration for 30 min and anisomycin 100 ng/ml as a positive control. Cells were washed 2 times with ice-cold PBS and lyzed with ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% (v/v) glycerol, 0.1 mM PMSF, 10 mM 13-glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate, and 10 mM leupeptin. The lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and protein concentrations of the supernatants were determined by Bradford method (Bio-Rad Laboratories). Equal amount of protein was added with 5 X Laemmli sample loading buffer followed by boiling for 5 min. Proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Milipore) by electroblotting. The membranes were blocked with 3% skim milk in 10 mM Tris-HCL (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 for 30 min at RT, and incubated with primary antibody (1:1000) with same buffer except for 5% BSA

gentle agitation overnight at 4°C. For detection, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at RT. The antigen-antibody complexes were visualized with an ECL Western blotting detection kit (Amersham Pharmacia Biotech) according to manufacturer's instruction.

Western blotting analysis on phosphorylation of mammalian MKK3/6

Antibody and cell culture. An anti-phospho-MKK3/6 kinase (Ser189/207) antibody was purchased from Cell Signaling Technology Inc. An anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey) were purchased from Amersham Pharmacia Biotech Ltd. NIH3T3 cells were maintained in DMEM (Nissui Pharmaceutical Co) containing 10% heat-inactivated fetal bovine serum (FBS) (Cell Culture Laboratories).

Western blot analysis. NIH3T3 cells were grown to 60% confluence in 10 cm tissue culture dish (Falcon Labware) in complete medium. Cells were treated with each compound at 5% final concentration for 30 min and anisomycin 100 ng/ml as a positive control. Cells were washed 2 times with ice-cold PBS and lyzed with ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% (v/v) glycerol, 0.1 mM PMSF, 10 mM 13-glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate, and10 mM leupeptin. The lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and protein concentrations of the supernatants were determined by Bradford method (Bio-Rad Laboratories). Equal amount of protein was added with 5 X Laemmli sample loading buffer followed by boiling for 5 min. Proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Milipore) by electroblotting. The membranes were blocked with 3% skim milk in 10 mM Tris-HCL (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 for 30 min at RT, and incubated with primary antibody (1:1000) with same buffer except for 5% BSA gentle agitation overnight at 4°C. For detection, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at RT. The antigen-antibody complexes were visualized with an ECL Western blotting detection kit (Amersham Pharmacia Biotech) according to manufacturer's instruction.

Mycobacterium

e) *ICL screening system*

Screening for ICL inhibitors using *M. smegmatis* H8000 (wild type) and H8012 (transformant) was done by paper disk susceptibility test method. H8012 is a *Δicl M.smegmatis* complemented with *M. tuberculosis* normal ICL gene. Both H8000 and H8012 will be plated on minimal medium containing either glucose or actetate as the limiting carbon source. An ICL inhibitor is expected to cause growth inhibition of *M. smegmatis* on acetate plate but not on the glucose plate (Sharma *et al.*, 2000).

RESULTS AND DISCCUSION

The soils and plants of Maliau Basin

146 soil samples (Table 1) were collected from 144 sites (Figure 1 and 2) inside and outside the Maliau Basin rim which could be divided into 6 sampling areas. There were two repeats in the same sites, which were MB46 and MB147. Out of this number, 4 of the soil samples collected were not directly associated with any particular plant. Therefore 142 soils samples were collected from under 140 separate plants (Table 2). The bulk of the plant

identification was carried out through the leaves and bark characteristics at the Sabah herbarium, Forest Research Center, Sandakan while the rest were identified in the field. Out of the 141 plants, 132 were identified and further subdivided to 24 plant families. Nine plants were unknown although there may be shrubs and mosses growing along with the tree where the soil was collected, only the tree was identified. Tree was considered as the main factor in the modification of the soil which leads to the selection and diversity of the soil microbes. There was also one sample (MB101) collected from water with foam from the Giluk Falls.

As shown in Table 3 the soils of Maliau Basin was found to be highly acidic. The soil collection could be divided into two categories, the soil collected outside the Basin and the soil collected within the basin which includes the areas enclosed by the Basin rim. Overall the soil in the Basin has been shown to be slightly more acidic than the soil surrounding the Basin. Two out of 4 soil samples collected outside the Basin has been found to be above pH 4, while all the soil samples collected in the Maliau Basin has shown pH below 4. Beside this distinction, there is also a difference in the altitude of the sampling areas which dictates the forest types of the area. The forest surrounding the basin is dominated by dipterocarp trees while within the Basin with higher elevation, the vegetation consist of heath forest and submontane forest.

Isolation of microbes (actinomycetes and fungi) from Maliau Basin

A total of 98 isolates of actinomycetes (Table 4 and 5) and 254 isolates of fungi (Table 6) were isolated from soil samples from different sites in the forest of Maliau Basin, Sabah.

Microfungi isolation. A total of 254 microfungi strains were isolated from Maliau Basin. Two isolation media were used for the isolation of fungal strains from Maliau Basin. The first isolation medium used was PDA with 7.5% NaCl and 50µg/ml chloramphenicol with the pH of 4.5. Due to Maliau Basin's soil low pH, the isolation medium with a pH of 4.5 was used as opposed to the usual 5.6. By using a lower pH an increase of agar concentration was needed for the isolation plates to solidify. The agar concentration was increased from 1.8% to 2.5%. As this isolating medium may impose high selective condition and may limit the variety of fungi that could be isolated a second isolating medium was used. This isolating new medium was the DRBC medium with a pH of 5.6 and 2.5% of NaCl. Fungi from soil MB 29 to MB 50 and MB 101 to MB 150 were isolated using the first isolation medium. Out of the 102 soil samples processed using the first isolation medium, 69 soil samples have produced fungal strains. The soil samples MB fungi from 1 to MB 28 were isolated using the second isolation medium which was the DRBC agar with 2.5% NaCl. 10 out of the 28 soil samples produced the required distinguishable fungal strains. A further 7 soil samples that was previously plated on PDA with successful isolation (except MB 36), produced distinguishable fungal strains using the DRBC medium.

Screening for specific inhibition of molecular targets in cell based system

Screening for inhibitors against the eukaryotic signal transduction was carried out on selected microbial extracts.

a) MAPK kinase (MKK1) screening system and screening results

Watanabe, Irie & Matsumoto (1995) constituted a plasmid pNV7-MKK1 containing GAL1p-MKK1^{P386}, which was introduced to

wild type yeast strain at the trp1 locus. $MKK1^{P386}$ is a hyperactive mutation of MKK1, resulting from the change of serine to proline at the position 386. This mutation site lies between protein kinase sub-domains VII and VIII close to the putative phosphate acceptor residue, which is thought to be phosphorylated by Bck1. The $MKK1^{P386}$ mutation is able to suppress both the Pkc1 and Bck1 deletion, indicating that the mutationally activated MKK1 kinase transmit signal even in the absence of Pkc1 and Bck1 upstream kinases. On the other hand, the $MKK1^{P386}$ mutation fails to suppress defects associated with Mpk1 null mutations, consistent with the model in which MKK1 acts upstream of Mpk1. $MKK1^{P386}$ inhibited growth when overexpressed by fusion to the strong GAL1 promoter. Thus, overexpression of this gene can be achieved by inducing it with the addition of galactose in the media. This growth inhibitory effect was suppressed by the Mpk1 deletion, indicating that hyperactivation of the Mpk1 pathway is toxic to the yeast cells. The growth inhibitory effect was more severe when cells were grown in the presence of D-sorbitol (Watanabe, Irie & Matsumoto, 1995). In the MAPK kinase screening system, yeast utilizes the glucose and grows normally on glucose plates. On the galactose plates, GAL1 promoter is induced strongly resulting in overexpression of $MKK1^{P386}$, thus causing growth inhibitory effect on the yeast. So, if there is an enzyme inhibitor in the paper disc, there would be yeast growth around the disc on the galactose plate.

No MKK1 inhibitor was found. There were 3 extracts (H7897, H7944 and H7973) showing toxicity against $MKK1^{P386}$ (H10068) yeast on glucose plate (Table 8).

b) MAP kinase phosphatase (MSG5) screening system and screening results

MAPK phosphatase have been identified in species ranging from yeast (Doi *et al.*, 1994) to human suggest that the mechanism for inactivation of MAPKs are highly conserved, as are the mechanisms for their activation. Watanabe, Irie & Matsumoto (1995) also found the MSG5 gene that encodes a protein phosphatases which negatively regulates the Mpk1 pathway. The MSG5 gene was selected by using yeast genomic library cloned in multiple copy vector, Yep13 (containing *S. cerevisiae* LEU2). The library was transferred into the wild type strain which carries a GAL1-$MKK1^{P386}$ construct. The transformants were screened for ability to grow in the presence of D-sorbitol and in the absence of leucine. Hence, the screening medium is without leucine. The MSG5 gene was identified as a multicopy suppressor of growth arrest caused by a *gpa*1 mutation. MSG5 encodes a dual-specificity protein phosphatase that dephospholylates and inactivates Fus3 MAPK acting in the mating pheromone signal transduction Mpk1 (Doi *et al.*, 1994). Overexpression of MSG5 suppresses the $MKK1^{P386}$ overexpression toxicity by dephosphorylating and inactivating Mpk1. Thus, MSG phosphatase appears to act on Mpk1 under overexpressed conditions and maintain the cell wall integrity of yeasts. So, on the galactose plate, there is growth of yeast. If there is an inhibitory zone around the disc containing the extract on the galactose plate but none on the glucose plate, it could well be the action of an inhibitor is at the MS65 (Watanabe, Irie & Matsumoto, 1995).

In the screening for MAPK phosphatase MSG5 inhibitor in yeast, there were 5 extracts (H7897, H7944, H7970, H7973 and H8525) showing toxicity against MSG-5 in yeast (H10069) (Table 9). No inhibitors among the extracts were found against MSG5.

c) Ras/Raf yeast two hybrid screening system and screening results

Protein-protein interaction between two proteins have generally been studied using

biochemical techniques such as cross-linking, co-immunoprecipitation and co-fractionation by chromatography. Fields and Song (1989) have generated a novel genetic system of the yeast *Saccharomyces cerevisiae* to identify protein-protein interaction *in vivo* through reconstitution of the activation of a transcription activator. The method is based on the properties of the yeast GAL4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation. Plasmids encoding two hybrid proteins, one consisting of the GAL4 DNA-binding domain fused to protein X and the other consisting of the GAL4 activation domain fused to protein Y, are constructed and introduced into yeast. Interaction between proteins X and Y leads to the transcriptional activation of a reporter gene containing a binding site for GAL4 (Chien *et al.*, 1991).

Construction of two hybrid system for Ras/ Raf Interaction

Regarding to the two hybrid system used in this screening, one hybrid is a fusion between the LexA DNA –binding domain (amino acids 1 to 211) and a protein of interest (oncogenic-Ras^{V12})

The second hybrid is a fusion between a nuclear localized VP16 acidic activation domain and a second protein of interest (Raf-1). Individually, these hybrids are unable to activate transcription: one does not contain a DNA-binding domain, and the other does not contain an activation domain.

These two hybrids are co-expressed in an *Saccharomyces cerevisiae* strain that contains two integrated reporter constructs, the yeast *HIS3* gene and the bacterial *lacZ* gene, which contain binding sites for the Lex A protein. Transactivation of the reporter constructs is dependent on the formation of a complex between the proteins fused to LexA and VP16. If the two hybrids are able to interact, then transactivation of the reporter constructs occurs; the yeast strain expressing both two hybrid proteins which is prototrophic for histidine and contains detectable β-galactosidase activity (Vojtek, Hollenberg & Cooper, 1993). If the Ras/Raf interaction is blocked by an inhibitor, the yeast strain (LZ) which transformed with pLexA-RAS^{V12} and pVP16-RAF are unable to activate the *HIS3* and *lacZ* reporter genes. So far, radicicol is reported as an inhibitor of an *in vivo* Ras-Raf interaction with the yeast two hybrid screening system (Ki *et al.*, 1998). This system will be developed to screen for Ras/Raf Interaction inhibitors.

In the screening for inhibitors against Ras-Raf protein interaction with the yeast two-hybrid system, there was 1 extract (H7897) showing 4mm larger in zone of inhibition on plate without the addition of histidine to that with histidine. Four actinomycetes extract (H7915, H7940, H7944 and H7976) showed toxicity against LZ yeast (H10014) (Table 10).

d) Result of β-galactosidase assay (quantitative test): Ras-Raf interaction

Acetone crude extract (20µl) from H7944 showed no inhibitory activity on the β-galactosidase synthesis (Table 11). In an independent experiment, H7944 crude freeze-dried extract at final concentration of 1mg/ml reduced β-galactosidase activity of LZ cells to 73.73% compared to the original activity (control). These results indicated that H7944 exert inhibitory activity at higher concentration.

e) Serine/threonine protein phosphatase type I (PP1) screening system and screening results

The gene *GLC7* encodes the catalytic subunit isoform of PP1. Its catalytic domain is more

than 80% similar to mammalian PP1. *Saccharomyces cerevisiae* has only one gene that is the *GLC7*. A variety of studies using *glc7* mutants have revealed that the yeast PP1 is required for many key cellular functions including glucose repression (Tu & Carlson, 1994; 1995), kinetochore function (Bloecher & Tatchell, 1999; Sassoon *et al.*, 1999) vesicle fusion during protein secretion (Peters *et al.*, 1999) glycogen synthesis (Feng *et al.*, 1991), meiosis (Bailis & Roeder, 2000; Tu, Song & Carlson, 1996) and cell wall integrity (Andrews & Stark, 2000). It is the function of Glc7p on cell wall integrity that this research utilized to find novel compounds that affect the PP1 serine/theronine phosphatsase.

Two *Penicillium* strains (H9307 and H9318) and 4 unknown fungus (H9114, H9213, H9316 and H9317) isolated from Maliau Basin soil, have been found to inhibit the growth of the wild type yeasts at 37°C and the presence of D-sorbitol (1M) relieved this inhibition. In *in vitro* system, three strains (H9307, H9317 and H9318) also decreased dephosphorylation by Glc7p of yeast as well as the mammalian PP1 gamma, as determined by Prof. Michael Stark at Dundee University, Scotland. While 5 other actinomycetes strains (H7897, H7940, H7944, H7970 and H7973) showed toxicity against PP1 screening system (Table 12).

Western blotting analysis

Western blotting analysis on phosphorylation of mammalian MEK1/2 and ERK1/2

In this test, the effect of extract from H7944 (MBA94-2) on the phosphorylation of MEK/ERK signalling pathway was examined. Epidermal growth factor (EGF) was used as an inducer of MEK/ERK pathway. Untreated control NIH3T3 cells were constitutively expressed high levels of phosphoryalted ERK1/2 (Figure 7). Result from antibodies identifying phosphorylated MEK1/2 and ERK1/2 confirm that extract from H7944 showed inhibitory effect in the ERK pathway Extract H7944 also inhibited the phosphorylation of MEK1/2 beside ERK1/2. This means that H7944 was able to inhibit phosphorylation of MEK1/2.

Ras/Raf protein interaction is at the upstream of MEK1/2 and ERK1/2. The possibility that extract from H7944 shows inhibition to mammalian Ras/Raf protein interaction was first excluded by negative results obtained from Ras/Raf interaction for histidine biosynthesis in yeast two-hybrid system (Table 10) and β-galactosidase synthesis (Table 11). However, later experiments using high concentration of crude freeze-dried extract did lower the level of β-galactosidase (Table 11).

Western blotting analysis on phosphorylation of mammalian p38 MAP kinase and SAPK/JNK

In this test, the effect of extract H7944 (MBA94-2) was examined on the phosphorylation of mammalian p38 MAP kinase and SAPK/JNK pathway. Anisomycin 100 ng/ml was used as a positive control. Experimented data revealed that extract of H7944 has no effect on the phosphorylation of mammalian JNK. Extract H7944 also has no effect on the phosphorylation of mammalian p38 (Figure 8). JNK is as SAPK (stress activated protein kinase), which is activated to various cellular stress substances.

The protein synthesis inhibitor, anisomycin can act as potent signalling agonist even at subinhibitory concentrations. Anisomycin can strongly and selectively activate intracellular kinase such as the JNK/SAPK. Anisomycin is an extremely potent and selective activator of JNK/SAPKs, eliciting no detectable activation of the ERKs, and may prove extremely useful in unraveling the

complexity of circuitry upstream of the JNK/SAPKs (Cano & Mahadevan, 1995).

Western blotting analysis on phosphorylation of mammalian MKK3/6

In this test, the effect of extract H7944 (MBA94-2) was examined on the phosphorylation of mammalian MKK3/6. Anisomycin had 3 bands because MKK3/6's apparent molecular weights were 40 and 41 kDa but the main one was 40 kDa's. The other band (39 kDa's) was the non-specific band. All extracts including H7944 did not stimulate phosphorylation of mammalian MKK3/6 (Figure 9). Since MKK3/6 function upstream of the p38 pathway, all extracts did not show any effect on p38 pathway.

Isocitrate lyase of glyoxylate pathway in Mycobacterium (ICL) screening and screening results

In the screening for inhibitors against isocitrate lyase (ICL) in *Mycobacterium*, there are 3 extracts (H7893, H7910 and H7944) shown toxicity against both the wild type *M. smegmatis* (H8000) and transformed *M. smegmatis* (H8012) (Table 13). These extracts inhibited the growth of *Mycobacterium* on both glucose and acetate plates on repeated tests.

Important findings from actinomycetes isolated from Maliau Basin.

Extract from strain H7944 showed toxicity against MAPK kinase, MAP kinase phosphatase (MSG5) and Type 1 protein serine/threonine phosphatase (GLC7) screening systems. In the Ras/Raf interaction with the yeast two-hybrid system, H7944 lowered β-galactosidase activity without affecting the *HIS3* reporter. The most interesting point is that the extract from H7944 showed inhibition of phosphorylation of mammalian ERK1/2 and MEK 1/2. Extract from H7944 has no activation effect on the phosphorylation of mammalian JNK and also no effect on the phosphorylation of mammalian MKK3/6. Ras-Raf protein interaction is upstream of MEK1/2 and ERK1/2, so it is possible that compound from extract H7944 inhibited mammalian Ras-Raf protein interaction. Strain H7944 belong to non-*Streptomyces* based on the presence of *meso*-DAP in it's peptidoglycan cell wall. Table 14 showed the summary of the screening results

CONCLUSION

In this study, a total of 98 actinomycetes and 254 fungi strains were isolated from Maliau Basin. The interesting strains are the following: H7897 showed potential inhibitor against Ras/Raf protein interaction with the yeast two-hybrid system but need to be confirmed by inhibition of β-galactosidase synthesis. Extract from H7944 was found to decrease phosphorylation of mammalian MEK1/2 and ERK1/2 kinases in the Western blotting analysis and inhibited Ras/Raf protein interaction as evidenced by lowered β-galactosidase synthesis. Therefore, H7944 may be inhibiting the interaction. Two *Penicillium* strains (H9307 and H9318) and 4 unknown fungi (H9114, H9213, H9316 and H9317) isolated from Maliau Basin soil showed inhibition towards the ser/thr protein phosphatase type 1 of *Saccharomyces cerevisiae*. In *in vitro* system, three strains (H9307, H9317 and H9318) also decreased dephosphorylation by Glc7p of yeast as well as the mammalian PP1 gamma (Ong *et al.*, 2006).

Mycobacterium

In the screening for inhibitors against isocitrate lyase (ICL) in *Mycobacterium*, there are 3 extracts (H7893, H7910 and H7944) shown toxicity against both the wild type *M. smegmatis* (H8000) and transformed *M. smegmatis* (H8012). These extracts inhibited the growth of Mycobacterium on both glucose and acetate plates.

Maliau Basin certainly contain microbes of pharmaceutical interest. It needs total conservation, in view of threats from future coal mining (The DPA group International Inc, 1992).

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