SCREENING FOR INHIBITORS FROM MICROBIAL SECONDARY METABOLITES FOR PHOTODYNAMIC THERAPY AND GLYCOGEN SYNTHASE KINASE-3β (GSK-3β)

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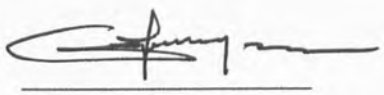
(Dr. Ivy Wong Nyet Kui) 

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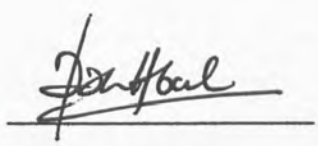
(Dr. Roziah Kambol) 

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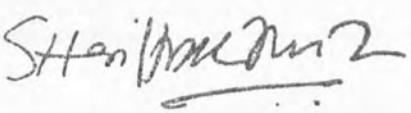
(Supt(K) Assoc. Prof. Dr. Shariff A. Kadir S. Omang) 

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ABSTRACT

Photodynamic Therapy, a promising novel treatment for cancer, is a technique based on the preferential accumulation of photosensitizers in tumour cells. Following irradiation with appropriate light wavelength, reactive oxygen species ($^1O_2$) is generated which leads to apoptosis of the tumour cells. Presently an ideal photosensitizer with regards to chemical purity, tumour selectivity, chemical and physical stability as well as rapid body clearance has not been found. GSK-3β, a serine/threonine protein kinase has been found highly active in some tumours, namely colorectal cancer. GSK-3β has been associated with tumour cell survival and proliferation, where inhibition causes apoptosis of the tumour cell. This research focused on the possibility of fungal secondary metabolites having potential photosensitizers and GSK-3β inhibitors. 13 pure cultures of fungi were selected from stock cultures of fungi originating from Sabah's tropical rain forests. These fungi strains produced pigmented secondary metabolites and were non-toxic to *Saccharomyces cereviseae*. These pure strains were grown aerobically to produce secondary metabolites. The acetone extract of these microbes were screened for photocytotoxic activity against a human leukaemic cell line HL60 using an MTT-based cell viability assay and also for GSK-3β inhibitors using a mutant yeast strain H10075. All extracts exhibited marginal photocytotoxicity at 20 μg/ml with no cytotoxicity when cells were kept in the dark, while for GSK-3β screening, only one strain H9042 showed marginal inhibition of yeast H10075 when it was incubated at 37°C without inhibiting at 25°C. One strain, H9969 W was chosen for investigation as PDT photosensitizers and partially purified fractions tested showed slight increment in activity by 2.6%. Further investigation on the potential of H9969 W as a photosensitizer is worth pursuing using pure compounds of H9969 W.

ABSTRAK

Terapi Fotodinamik adalah penemuan terbaru untuk rawatan kanser. Teknik ini berasaskan pengumpulan fotosensitizer terutamanya dalam sel-sel tumor. Sel ini diradiasikan dengan gelombang cahaya yang berpatutan untuk menghasilkan spesies oksigen reaktif ($^1O_2$) yang menyebabkan sel tumor mengalami apoptosis. Pada masa kini, fotosensitizer yang memuaskan dalam aspek kimia, pemilih sel tumor, kestabilan secara kimia dan fizikal serta kebolehannya penyingkiran yang pantas belum lagi ditemui. GSK-3β sesuatu kinase protein serine/threonine didapati aktif dalam sel tumor, terutamanya, kanser usus besar. GSK-3β terlibat dalam kewujudan dan pembiakan sel tumor dan pencegahan GSK-3β ini menyebabkan sel tumor mengalami apoptosis. Kajian ini bertumpu pada penyelidikan bagi kebolehan metabolit sekunder daripada fungi untuk digunakan sebagai fotosensitizer dan pencegah GSK-3β. 13 kultur tulen fungi telah dipilih daripada kultur stok fungi berasal daripada hutan rimba Sabah. Strain- strain fungi ini menghasilkan metabolit sekunder yang berwarna dan tidak toksik terhadap *Saccharomyces cereviseae*. Strain tulen ini telah dikultur secara aerobic untuk menghasilkan metabolit sekunder. Ekstrak aceton mikrob-mikrob ini seterusnya disaring untuk activiti fotositotoksik terhadap sel leukemia manusia HL60 dengan menggunakan ujian berasaskan MTT untuk menguji daya hidup sel, dan untuk penyaringan pencegah GSK-3β, strain yis mutan H10075 digunakan. Semua ekstrak menunjukkan kefotositotoksikan yang sedikit sahaja pada kepekatan 20 μg/mL dimana sel yang dieram dalam gelap telah menunjukkan kefotositotoksikan. Untuk penyaringan GSK-3β pula, hanya satu strain, H9042 yang menunjukkan kebolehan untuk mencegah GSK-3β dengan yis yang dieram dalam suhu 37°C tanpa mencegah pada suhu 25°C. Satu strain fungi, H9969 W telah dipilih untuk tinjauan seterusnya untuk PDT. Sebatian hampir tulen ekstrak ini telah diuji sekali lagi dan keputusannya ialah peningkatan dalam aktiviti fotositotoksik sebanyak 2.56%. Tinjauan seterusnya untuk menemui sebatian aktif dalam H9969 W amat disyorkan sebagai kajian masa hadapan.

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LIST OF SYMBOLS, UNITS AND ABBREVIATION

ADP	Adenosine diphosphate
APC	Adenomatous polyposis coli protein
ATP	Adenosine triphosphate
Carif	Cancer Research Initiatives Foundation
DNA	Deoxyribonucleotide
Dvl	Dishevelled protein 1
Fz1	Frizzled protein 1
GSK-3β	Glycogen Synthase Kinase- 3β
GSK-3α	Glycogen Synthase Kinase- 3α
HPD	Hematoporphyrin derivative
HPLC	High Performance Liquid Chromatography
IR	Infra-red
MHC	Multi-histocompatibility complex
MS-2	Mouse Sarcoma 2
MTT	3-4,5-dimethylthiazol-2,5-diphenyl-2*H*-tetrazolium hydrobromide
OM	Organic medium
PBS	Phosphate-buffered saline
PDA	Potato dextrose agar
PDT	Photodynamic Therapy
PS	Pthalocyanine disulphonate
ROS	Reactive Oxygen Species
SC-Ura	Synthetic Complete minus Uracil
SiO_2	Silicon Dioxide
SST	School of Science and Technology
TLC	Thin Layer Chromatography
UDPG	Uridine diphosphoglucose
UMS	Universiti Malaysia Sabah
°C	Degrees Celcius
w/v	Weight over volume

CHAPTER 1

INTRODUCTION

Cancer has been the focus of scientific research for the past century due to its lethality and indestructible persistence when it infects. Cancer is a disease that affects the world regardless of race, cast and creed; it is the leading cause of death in developed countries. Studies have been done on this disease to understand the mechanism of its trigger and tumour morphology. Scientific research done on all diseases is to ultimately discover a cure or at the very least develop a satisfactory treatment while search for the cure continues. Similarly, in the case of cancer, the quest has been going on for centuries for the ultimate triumph over cancer.

It is now understood that DNA mutation resulting in defective genetic expression of proteins involved in the cell cycle is the cause of uncontrollable tumour proliferation. The molecular comprehension of the cause of cancer has indicated that many of the cancer genes involve proteins kinases and phosphatases. These proteins are key components of signal transduction pathways in the cell cycle. When DNA mutations cause these signals to become defective, signal transduction pathways become disorderly; leading to cancer. Inhibition of these proteins has been observed to cause immediate

cessation of cellular proliferation as it triggers apoptosis. Therefore, these protein kinases and phosphatases have become the latest therapeutic target for cancer treatment as they can be used to stop tumour proliferation (Ho, 2003).

Conventional cancer treatments are not satisfactory in eradicating and controlling cancer. The side effects suffered by most make the treatments something to be feared of. But the most important observation is that those treatments are not able to stop recurrence of cancer in successfully treated patients. The ideal cancer therapy would not only destroy the primary tumour, but at the same time trigger the immune system to track down and destroy any remaining tumour cells, be they at or near the site of the primary tumour or distant metastases with minimal or no side effects (Castano, 2006). Conferring of the immunity against the tumour would also help fight tumour rechallenge in the patient.

In this project, the focus was on acquiring secondary metabolites from fungal source to inhibit the GSK-3β, a serine/threonine protein kinase and also compounds with pigmentation that could be a potential essential component of a novel model for cancer treatment, which was the Photodynamic Therapy (PDT). The expression of the human homologue of GSK-3β was done in yeast *Saccharomyces cerevisiae* because several of the key enzymes in signal transduction and cell cycle are homologous in yeast and human (Ho, 2003). For Photodynamic Therapy, the tests to screen for potential photosensitizers were done using cancer cell lines, HL-60, HSC-2 and HSC-3 in Cancer Research Initiatives Foundation (Carif), Subang Jaya Medical Centre in Selangor, Malaysia.

Fungal samples selected for this project were revived from the stock cultures made by previous students of University Malaysia Sabah. The fungal samples were isolated by them from various places in Sabah, such as Tabin, Maliau Basin, Imbak Valley, and Crocker Range Park (Lee 2003 and Voo 2004). The selection of fungi for this project was based on their observations of the fungal extracellular pigmentation in culture media to correspond with the objectives of this project.

The objectives of this study were as follows:

1. To extract fungal secondary metabolites with red or other pigments that could have Photodynamic Therapy (PDT) potential.
2. To extract fungal secondary metabolites with GSK-3β inhibitory potential
3. To screen of the fungal extracts for potential photosensitizer for Photodynamic Therapy (PDT) with cancer cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The world of microorganism brings its bane to humans and other organisms by creating diseases, but the bane to its very existence is also derived from itself. Novel compounds such as antibiotics discovered on microorganisms have altered the medical world in our times where discovering the remedy for diseases is a matter of time and the perfect compound. As time passes, more and more use for microbial secondary metabolites is being discovered that has made microorganism indispensable to us.

2.2 Cancer Origins

Cancer is a class of disease or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis (Sompayrac, 1999). Metastasis is defined as the stage at which cancer cells are

transported through the bloodstream or lymphatic system (Sompayrac, 1999). Cancer may affect people of all ages, but risk tends to increase with age, due to the fact that DNA damage becomes more apparent in aging DNA.

The unregulated growth that characterizes cancer is caused by damage to DNA, resulting in mutations to genes that encode for proteins controlling cell division. However, many mutation events may be required to transform a normal cell into a malignant cell. These mutations can be caused by chemicals or physical agents called carcinogens, by close exposure to radioactive materials, or by certain viruses that can insert their DNA into the human genome. Mutations occur spontaneously, or are passed down generations as a result of germ line mutations (Sompayrac, 1999).

Cell division or cell proliferation is a physiological process that occurs in almost all tissue and under many circumstances. Normally the balance between proliferation and programmed cell death is tightly regulated to ensure the integrity of organs and tissues. Mutations in DNA disrupt these orderly processes and this leads to cancer. The uncontrolled and often rapid proliferation of cells can lead to either a benign tumor or malignant tumor which is cancer. Benign tumors do not spread to other parts of the body or invade other tissues, and they rarely are a threat to life unless they extrinsically compress vital structures. Malignant tumors on the other hand are life threatening because of their ability to invade other organs and metastasize (Sompayrac, 1999).

Carcinogenesis, which means the initiation or generation of cancer, is the process of derangement of the rate of cell division due to damage to DNA. Ultimately, cancer is a disease of genes. In order for cells to start dividing uncontrollably, genes regulating cell growth must be damaged. Proto-oncogenes are genes which promote cell growth and mitosis, a process of cell division, and tumor suppressor genes discourage cell growth, or temporarily halt cell division in order to carry out DNA repair. Usually a series of several mutations to these genes are required before a normal cell transforms into a cancer cell. Proto-oncogenes promote cell growth in a variety of ways such as by producing hormones, being part of the signal transduction system or produce mitogens and being part of DNA transcription for protein synthesis. Hormones are chemical messengers between cells that encourage mitosis, the effect which depends on the signal transduction of the receiving tissue or cells. Signal transduction is the process where chemical signals are passed from the external source through a series of transducers to the DNA to induce mitosis or gene translation to produce proteins. Some of the proto-oncogenes are responsible for the signal transduction system and signal receptors in cells and tissues themselves, thus controlling the sensitivity to such hormones (Sompayrac, 1999).

Mutation of a proto-oncogene creates an oncogene which have modified expression and function which increases the amount of activity of the product protein. Consequently, the cells have higher chance to divide excessively and uncontrollably due to the prolific production of growth proteins. Tumor suppressor genes that code for anti-proliferation signals and proteins suppress mitosis and cell growth. They are activated by cellular stress or DNA damage and upon activation by free-floating genetic material

resulting from DNA damage, cell cycle progression is arrested to allow DNA repair to prevent mutations from being passed on to daughter cells. Damage to tumor suppressor gene hinders DNA repair and this inevitably also leads to cancer.

2.2.1 Cellular Signaling In Cancer

The signaling system in multicellular organisms is complex and has to be coordinated between functioning cells which are distanced from each other. The components involved in cellular signaling comprise of various chemical signals that form a cascade of signaling to reach the target such as the DNA within the nucleus. The signaling cascade is called signal transduction (Ho, 2003).

The signal transduction pathways are numerous and have equally different ways to cause cell proliferation or mitosis, protein synthesis and so on that are involved in the cell cycle. As stated earlier, DNA mutation specifically in the genes that are involved in synthesizing proteins in the cell cycle resulting in the defectiveness of signaling pathways. This then contribute to the malignant transformation of cells (Ho, 2003).

2.3 Glycogen Synthase Kinase- 3β (GSK-3β)

Glycogen Synthase Kinase- 3β (GSK-3β) is a serine/ threonine protein kinase first discovered to be a mediator in the glycogen metabolism involving insulin. A protein

kinase such as the GSK-3β transfers the terminal phosphate group from ATP to a hydroxyl group in an amino acid residue of a protein as shown in Figure 2.1.

Figure 2.1 Protein Kinase mechanism (Joyce, 2006)

In this case, GSK-3β catalyzes the transfer of the terminal phosphate group from ATP to the hydroxyl group in a serine or threonine residue of another protein. There are two types of isoforms of GSK, which are the GSK-3β and GSK-3α. The only difference between the two isoforms is the presence of glycine rich N-terminus in the α- isoform. In this study, the β-isoform of GSK3 gains credence over the α-isoform because the original study conducted on GSK3 was obtained from *Drosophila* which expressed a single form of GSK3 very similar to GSK-3β. For these reasons, it has been widely assumed that the GSK-3β isoform is ubiquitous in nature compared to the α-isoform of GSK3 (Cohen and Frame, 2003).

2.3.1 Roles of GSK-3β

Initially GSK-3β was determined as the protein that phosphorylates and inhibits glycogen synthase, an enzyme that catalyzes the transfer of glucose from UDPG to glycogen (Cohen and Frame, 2003). Later on it was discovered to play key roles in other

physiological responses including protein synthesis, gene expression, sub-cellular localization of proteins and protein degradation in mammalian cells by phosphorylating many substrates, including neuronal cell adhesion of molecule, neurofilament, synapsin I, tau transcription factors, and in the Wnt pathway involved in embryonic development (Andoh *et al.*, 2003).

2.3.2 GSK-3β in Wnt Signaling Pathway

The proto-oncogenic effects of wnt were discovered years ago inciting an investigation into the role of wnt genes in human cancer (Nusse and Varmus, 1982). Subsequently a signaling pathway was assembled which was found to contain cancer causing genes that lead to over-expression and accumulation of β-catenin and promote neoplastic transformation of cells. (Shimizu *et al*, 1997)

Figure 2.2 shows GSK-3β being involved in Wnt signaling pathway as the inhibitor of β-catenin, a proto-oncoprotein in Wnt pathway. Signaling in this pathway is initiated by the secreted wnt proteins, which then binds to a G-protein, Fz1, at the membrane. G-proteins are a class of seven-pass transmembrane receptors encoded by the frizzled genes and the activation of the receptors leads to the phosphorylation of the disheveled protein, Dv1, which through its association with axin, keeps GSK-3β in its inactive form (Bhanot *et al.*, 1996). Thus GSK-3β is prevented from phosphorylating critical substrates such as β-catenin of this pathway (Yang-Snyder *et al.*, 1997).

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