Are there
“Magic Bullets”
for CancerTherapy?

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11 FEBRUARY 2011

Dewan Kuliah Utama
Fakulti Perubatan dan Sains Kesihatan
Universiti Putra Malaysia

Universiti Putra Malaysia Press
Serdang • 2011
http://www.penerbit.upm.edu.my

First Print 2011

UPM Press is a member of the Malaysian Book Publishers Association (MABOPA)
Membership No.: 9802

Perpustakaan Negara Malaysia Cataloguing-in-Publication Data

Seow, Heng Fong
Are there "Magic Bullets" for cancer theraphy / Seow Heng Fong
ISBN 978-967-344-203-4
1. Cancer--Treatment. 2. Signal Transduction. 3. Cancer--Hormone theraphy.
I. Title.
612.405

Reka letak teks : Ku Ida Mastura Ku Baharom
Reka bentuk kulit : Md Fairus Ahmad

Design, layout and printed by
Penerbit Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Tel: 03-8946 8855 / 8854
Fax: 03-8941 6172
http://www.penerbit.upm.edu.my

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FOREWORD

My interest in the field of signal transduction began while I was an undergraduate student at the University of London in 1979. I studied the impact of Philip Cohen's discovery in the early 1970s, of phosphorylation and dephosphorylation of proteins involved in glucose metabolism. Subsequently I pursued my PhD in 1982 focussing on the role of proteinases in the epidermal growth factor. At that time the signal transduction pathway was a simple linear pathway, though now it has exploded into a massive network of pathways with numerous proteins involved in the cascade. The 1980s, on the other hand, were filled with the excitement of the discoveries of tyrosine kinase receptors, tyrosine phosphorylation by tumour viruses and viral oncogenes which had counterparts in the human body.

Subsequently I left research in the area of signal transduction for the following 17 years. Initially, after my PhD, I took up a postdoctoral position at the Department of Cell Biology, Stanford School of Medicine, California, in Dr Peter Parham's laboratory. There I acquired skills in molecular biology and conducted many experiments in gene cloning as well as DNA sequencing. My colleagues and the research environment at Stanford University were a source of great inspiration for me and this enriching experience continues to inspire me. From USA, I moved on to work at the Institute of Molecular Biology in Singapore, CSIRO Melbourne, and the Burnet Institute (formerly the Macfarlane Burnet Institute), where my research involved me in the world of cytokines in infectious diseases and vaccines as well as the development of diagnostic tests and vaccines for Hepatitis E. In 1997, I returned to Malaysia and was employed at the Faculty of Medicine and Health

Sciences, Universiti Putra Malaysia, initially to teach the medical students Biochemistry and subsequently Virology and Immunology.

Over the last ten years, my research areas at UPM have covered the following: (1) elucidation of the aberrations in signal transduction pathway in colorectal, breast and nasopharyngeal carcinoma; (2) determination of the cytotoxic effects of purified compounds from Malaysian-derived soil bacteria; (3) generation of activated cytotoxic T lymphocytes towards adoptive immunotherapy for cancer therapy; (4) elucidating the immunomodulatory effects of mesenchymal stem cells towards cell-based therapy for cancer treatment; and (5) development of diagnostic tests and therapeutics in candidiasis and aspergillosis. All this was accomplished with contributions from my graduate students.

I will begin with my story on signal transduction pathways. This will be followed by the discovery of natural compounds from a soil-derived bacteria, Streptomyces, and then the field of adoptive immunotherapy using cytotoxic T lymphocytes. The ultimate goal of this multi-pronged approach is to obtain the 'magic bullets' to kill cancer cells. This will involve blocking the appropriate signal transduction pathways and enhancing sensitivity to chemotherapy with natural compounds. The potential of infusing activated cytotoxic T lymphocytes or mesenchymal stem cells needs to be further exploited. Since cancer cells are dynamic, heterogenous and undergo mutations in order to keep surviving until they kill the host, it is envisaged that this multi-pronged approach holds great potential in the fight against this devastating disease.

ABSTRACT

Despite continuing intensive research, cancer is still a devastating disease with high mortality rates. However, progress made in understanding the behavior of tumour cells has led to advances in the creation of 'magic bullets' for treating cancer as exemplified by imatinib, an inhibitor that targets an oncogenic kinase, namely, bcr-abl. This article will focus on the seminal discoveries made in oncogenes, growth factor receptors and tyrosine phosphorylation and contributions from my research on signal transduction, adoptive immunotherapy and drug discovery.

Signal transduction is a process whereby a cascade of proteins is activated inside the cell causing the cancer cell to grow uncontrollably and spread to other parts of the body. By blocking the signals with molecular-targeted drugs, the cancer cell growth can be controlled. The success of molecularly-targeted drugs depends on the target and knowledge of the signal transduction network. Due to the limited information on tumours available in Malaysia, we set out to determine the incidence of expression of proteins such as the epidermal growth factor receptor (EGFR), phosphatidyl-inositol-3-kinase (PI3K), pTEN, Akt and downstream targets such as β-catenin, pBAD, pGSK, pmTOR and pFHKR, by immunohistochemistry. This can provide insights into the possible mechanism of survival of colorectal, breast and nasopharyngeal carcinoma cells. The contributions of activating mutations such as PI3K in colorectal, breast and nasopharyngeal carcinoma were also compared. This approach revealed the potential therapeutic targets relevant to our population.

In the second phase of the research program, we attempted to isolate inhibitors of signal transduction from soil-derived *Actinomycetes*. The ability of natural compounds purified from extracts of soil-derived *Actinomycetes* (genus *Streptomycetes*)

to induce apoptosis, cytotoxic and cell cycle effects were also measured. This led to the discovery of the ability to arrest cells in the G1 phase in a breast cancer cell line, MCF-7. Further, the synergistic effect with a PI3K inhibitor was also revealed.

Another approach to cancer treatment is adoptive immunotherapy. Conditions for the generation of cytotoxic CD8+ T lymphocytes with recognition for HER2-peptide specific and Epstein Barr viral protein and latent membrane protein-2 (LMP-2) were established to obtain cells for treating breast and nasopharyngeal carcinoma patients. The existence of cells with T regulatory cell phenotype was detected in breast and nasopharyngeal carcinoma. An alternative state of the art approach using mesenchymal stem cells for cancer therapy was also attempted. Subsequently, the immunomodulatory effects of umbilical mesenchymal stem cells was demonstrated with K562, an undifferentiated erythromyeloblastoid leukemia cell line.

Lastly, the development of molecular diagnostic tests for ten species of Candida species was achieved. Monoclonal antibodies against *Candida glabrata* and *Aspergillus fumigatus* were also produced.

In summary, we have successfully demonstrated the aberrations of the signal transduction pathway in primary breast, colorectal and nasopharyngeal carcinoma. This study has given us an insight into the relevant biomolecules in the cascade. Results from the nasopharyngeal carcinoma tissues revealed that the PI3K/Akt/mTOR pathway is not a linear pathway. The potency of the natural compound for cancer treatment will need to be tested on animal models. The second approach of harnessing the immune system showed promising results. The effectiveness of the cytotoxic T cells and mesenchymal stem cells generated will need to be tested on animal models. Thus, further work is warranted to improve treatment outcomes for cancer patients.

BASICS IN MOLECULARLY-TARGETED THERAPIES AND CANCER

Introduction

> "Discovery consists of seeing what everybody has seen and thinking what nobody has thought"
>
> -Albert Szent-Gyorgyi, Hungarian American biochemist-

According to the American Cancer Society, cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled it can result in death. Cell growth and division in normal cells are tightly controlled. Cancer cells, on the other hand, have lost their ability to regulate their own growth or to respond to normal growth regulatory cues. In a paper by Hanahan and Weinberg (2000), which has been cited at least 6000 times todate, cancer cells were described as those that have acquired the capability to be self-sufficient in growth signals, are insensitive to antigrowth signals, can evade apoptosis, have unlimited replicative potential, have sustained angiogenesis and have the ability to invade tissue and metastasize. Our understanding of the cellular changes that convert normal cells to cancer cells have been due to advances in the 1970s and 1980s in the area of tumour virology, cell culture, oncogenes, growth factor receptors and tyrosine kinases. During the same period, advances in gene cloning, DNA sequencing and recombinant DNA technology contributed significantly to the progress of oncogene and growth factor receptor discovery (Figure 2). Oncogenes are defined as genes that contribute to the conversion of normal cells into cancer cells. Proto-oncogenes are present in normal cells and when mutated, these genes are called oncogenes.

A tumour is defined as an abnormal growth of tissue resulting from uncontrolled, progressive multiplication of cells. Conventional treatment regimens to eliminate tumours include surgery, radiotherapy and/or chemotherapy with cytotoxic drugs such as gemcitabine, oxaliplatin, paclitaxel and irinotecan. The side effects of chemotherapeutic drugs include nausea, vomiting, hair loss and immunosuppression. Further, autologous bone marrow transplantation can be used for treatment of leukemias and lymphomas. In 1977, tamoxifen, an antagonist of the oestrogen receptor, was approved for treatment of breast cancer in USA. However, not all breast cancer patients responded to tamoxifen and risk of recurrence was not uncommon. The advances made in the 1980s, in molecular and cell biology, provided information on the mechanisms that regulate survival or proliferation of the tumour cell. These studies led to innovations in targeted therapies. In May 2001, imatinib was approved by the United States Food and Drug Administration, for treating chronic myeloid leukemias. The cover of the TIME magazine hailed it as the ‘magic bullet’ for curing cancer. Another approach in targeted therapies is the application of humanized monoclonal antibodies. One major example is trastuzumab which is used to target the human epidermal growth factor receptor (HER)-2 on breast cancer cells. However, Cardiac dysfunction was observed in some patients treated with trastuzumab. Thus, the search for the ‘magic bullets’ continues. This review describes the early discoveries of oncogenes, growth factor receptors and tyrosine kinases that are of central importance to the development of targeted therapies. The efforts made by my research team in dissecting the aberrations of signal transduction pathways and inhibitors from natural compounds will be described, as well as the current status of cell-based therapies.

Signal Transduction

Signal transduction is a process by which a cell responds to a stimulus outside the cell resulting in a cellular response and alterations in gene expression. Protein phosphorylation and dephosphorylation play key roles in signal transduction. A kinase is an enzyme that catalyzes the addition of a phosphoryl group to a protein, an event called phosphorylation. Tyrosine kinase adds a phosphoryl group to the tyrosine residue of a protein.

The external stimulus that triggers signal transduction is referred to as the ligand if it binds to a protein such as a cell surface receptor. Examples of external stimuli (ligand) include growth factors such as the epidermal growth factor (EGF), amino acid derivatives such as histamine or adrenaline, steroids, damaged DNA or gases such as nitric oxide. When the ligand binds to the receptor, signal transduction is initiated and the cell responds accordingly. Thus, the receptor and cytoplasmic tyrosine kinases act as the connecting bridge between extracellular signals in the form of ligands and intracellular signaling pathways.

The process involves modification of proteins by tyrosine phosphorylation through the protein kinase resulting in changes in activity location and association of proteins which are components of the signalling process. A cascade of proteins is phosphorylated in the signal transduction pathway.

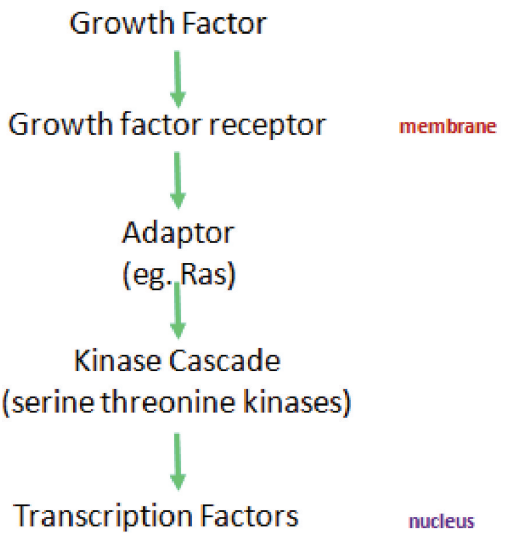


Figure 1 The figure above represents a generic signal transduction process whereby the steps are: (1) binding of the external stimulus (ligand) to the cell surface receptor; (2) activated receptor binds to the adaptor molecules such as Ras in the cytoplasm; (3) a series of proteins are phosphorylated resulting in cellular responses such as apoptosis or promote cell survival; and (4) translocation of some proteins to the nucleus or activation of transcription factor binding to specific DNA sequences resulting in alterations in gene expression such as those that increase proliferation, differentiation or cell migration.

In reality, a complex biochemically-related network working in a concerted fashion is used by regulatory biomolecules to mediate signals from the plasma membrane to the nucleus to cause cellular responses such as cell proliferation, apoptosis, differentiation, migration and angiogenesis.

Milestones in Oncogene and Growth Factor Receptor Tyrosine Kinases

The original discovery of the tumour virus that contributes to cancer was made by Peyton Rous in 1910 (Rous 1910). He successfully transplanted tumours from a Plymouth Rock hen to other chickens. Based on the work by Ellerman and Bang (1908), he showed that cell-free extracts from the tumour were able to induce solid tumours in other chickens (Rous 1911). His discovery was met with suspicion because at that time it was believed that cancer was of endogenous rather than of infectious origin. By the 1950s, various viruses, as listed in Table 1, were shown to cause tumours. In 1969, Hueber and Todaro showed that oncogenes from RNA tumour viruses in animals could cause cancer when the latent transforming genes were activated. Subsequently, the cellular origin of viral *src* was reported by Southern Blot hybridisation of normal avian DNA with a DNA probe specific for viral *src* (Stehelin *et al*., 1976). The src protein was later identified in 1977 (Beemon *et al*., 1977) and shown to be a protein kinase (Collett and Erikson, 1978). In recognition of his work, Rous was awarded the Nobel Prize in 1966, 56 years after the discovery of the Rous sarcoma virus.

In the 1950s, Levi-Montalcini and Stanley Cohen purified the nerve growth factor from large quantities of snake venom and mouse salivary gland extracts. In 1978, Cohen identified the epidermal growth factor receptor (EGFR) and associated tyrosine kinase activity. He also proposed that the phosphorylation of membrane components and associated proteins might be crucial in the generation of intracellular signals that regulate proliferation (Ushiro and Cohen 1980). In 1980, Hunter and Sefton discovered that the transforming protein of the Rous sarcoma tumour virus, v-src, had tyrosine phosphorylation activity. This milestone discovery linked deregulated protein tyrosine phosphorylation to tumorigenesis. In

1980, Michael Bishop and Harold Varmus raised the question on whether the oncogenes that initiate signal transduction pathways through tyrosine phosphorylation could also induce cancer. In recognition of their work, Levi-Montalcini and Stanley Cohen shared the 1986 Nobel Prize for Medicine and Physiology, while Michael Bishop and Harold Varmus were awarded the Nobel Prize in 1989.

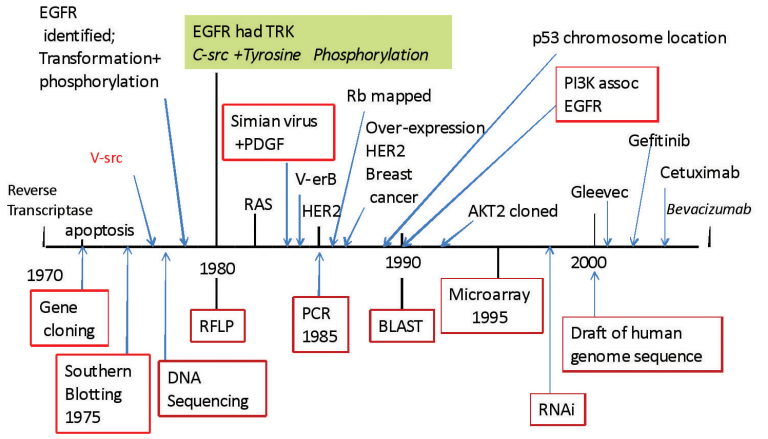


Figure 2 Key milestones in the discovery of oncogenes, tumour suppressors, tyrosine phosphorylation, molecular targeted drugs and recombinant DNA technology

The discovery of the growth factor receptor tyrosine kinases (RTKs), tyrosine phosphorylation by tumour viruses together with the finding that animal retroviral oncogenes are of cellular origin, led to the concept that oncogenes initiating signaling pathways through tyrosine phosphorylation induced human cancer. A review of the timelines of these discoveries has been published (Gschwind et al.,

2004). Table 1 shows the animal viruses with ability to transform cells and associated oncogenes.

Table 1 Animal viruses with oncogenic activity

Oncogene	Retrovirus	Origin	Oncogene product
abl	Abelson murine leukemia virus	mouse	Protein tyrosine kinase
Erb-B	Avian erythroblastosis virus	chicken	Truncated EGF receptor
src	Rous sarcoma virus	chicken	Protein tyrosine kinase
fos	Murine sarcoma virus	mouse	Transcription factor AP-1 complexes with fos
jun	Avian sarcoma virus	chicken	Transcription factor AP-1 complexes with jun
sis	Simian sarcoma virus	monkey	Truncated PDGF (b chain)
myc	Myelocytoma virus 29	chicken	DNA binding protein
fes	Feline sarcoma virus	cat	Protein tyrosine kinase

As the cDNA cloning technologies improved in the early 1980s, the EGF receptor and oncogenes such as *Ras* (Shih and Weinberg, 1982), *sis* (Doolittle *et al*., 1983; Waterfield et al., 1983), *v-erb*B (Debuire *et al*., 1984) and *Akt* (Staal, 1987) were cloned. Ras was shown to mediate transformation by Src signaling (Smith *et al.,* 1986) and Ras/MAPK and PI3K were parallel signaling pathways

initiated for the transformation process (Pineul and Martin, 1999). Homologues of cellular proto-oncogenes and growth factor receptors were also discovered. Meanwhile, the Epidermal growth factor receptor was found to have sequence homology to a known avian erythroblastosis virus onocgene, v-*erbB***.** Other growth factor receptors with homology to oncogenes include the human epidermal growth factor receptor-2 (*c-erb2*), steel factor receptor (*c-kit*), insulin growth factor-1 (*c-ros*), fibroblast growth factor receptor (*fig*), colony stimulating factor-1 receptor (*c-fms*) and the neurotrophic growth factor receptor (*trk).*

The seminal discovery by Hunter and Sefton (1980) supported the idea that small changes in the regulatory pathway had a great impact in conversion of a normal cell into a cancer cell, triggered by a burst of activity in signal transduction. A number of papers in the area of signal transduction started to appear in the late 1980s. The figures show that there was a 5-fold increase from 1990-1995 (approximately 500 in 1990 as compared to 2500 in 1995). From 1995, the number of papers published continued to rise steadily with 3000 and 3500 publications in the year 2000 and 2005, respectively. A total of 48,377 papers were published from 1977 to 2007, of which 11,211 were reviews. This indicates that signal transduction is an area of intensive research. Interestingly, the outcome of this research has been translated into clinical applications. As of 2009, there are now 16 drugs targeting protein kinases in signal transduction pathways and 153 protein kinase inhibitors undergoing clinical trials, with 23 in phase III clinical trials. Examples of these molecularly-targeted drugs are trastuzumab, Gleevec, gefitinib, cetuximab and bevacizumab. The timeline for their approval and applications are as shown in Figure 2 and Table 3.

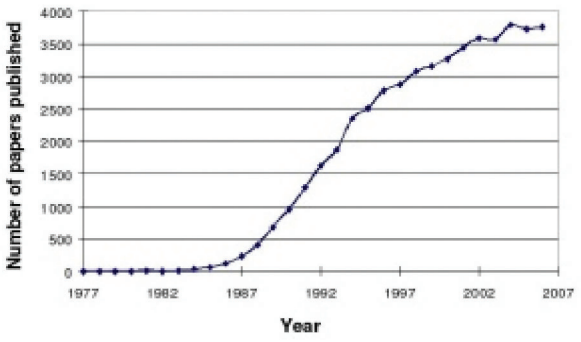


Figure 3 The figures above shows the number of papers containing the specific term "signal transduction" published in the MEDLINE database from 1977 until 2007 (http://www.wikimediafoundation.org/wiki)

Why Do Cancers Arise?

Over the past 50 years, the paradigm in cancer research is that cancer begins with a single mutation in a somatic cell followed by successive mutations. Activating mutations of oncogenes or deactivation of tumour suppressor genes occur resulting in loss of control of the cell cycle and escape from the normal biochemical systems that regulate the balance between apoptosis and survival. Cancer cells achieve independence from growth signals by constitutively overexpressing growth factor receptors, activating downstream signal molecules or possessing activating mutations. In addition, gene amplifications (eg.cyclin D amplification) or chromosomal translocations (eg. *Abl-bcr* in acute lymphoblastoid leukemia) can cause deregulation of the signal transduction pathway resulting in induction of cell proliferation independent of growth receptor activation.

Under physiological conditions, cell proliferation is induced by growth factors such as the epidermal growth factor (EGF). In

the 1980s, overexpression of EGFR and deregulated signalling were described in many cancers (Gullick 1991). Downstream of the growth factor receptor, the common transduction proteins are RAS and phosphatidylinositol-3-kinase (PI3K). RAS was first identified as the cellular homologue of the viral oncogene of the transforming retroviruses (Shih and Weinberg, 1982). One of the best described signaling pathway downstream of the receptor tyrosine kinase is the RAS/RAF/MEK/ERK pathway (Chang *et al*. 2003). The signaling cascade downstream from growth factor receptors (RAS, RAF, MEK, ERK) can directly or indirectly (by the induction of survival signals) inhibit cell death.

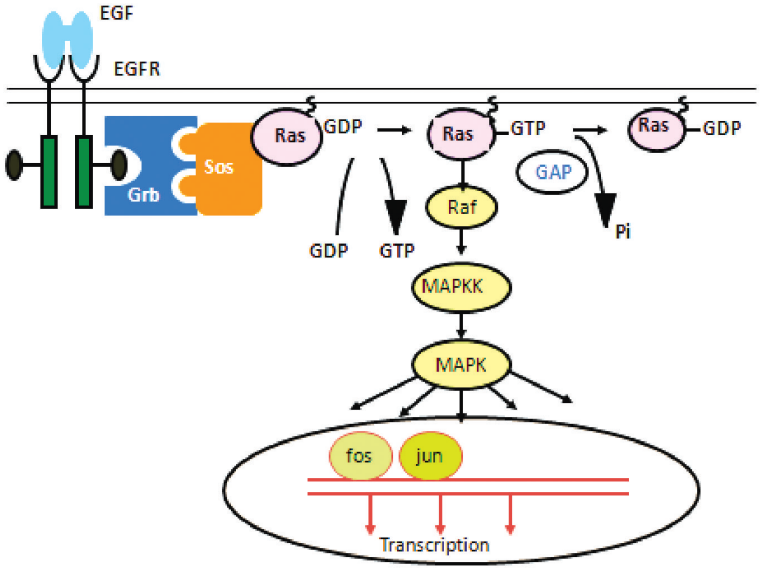


Figure 4 A schematic representation of the RAS/RAF/MEK/ERK pathway activated by the epidermal growth factor (EGF)

The human epidermal growth factor receptor 2 (HER2), also known as c-erbB2 or neu, is a tyrosine kinase receptor. The *HER2* gene was reported to be overamplified in 20-30% of breast cancers and is correlated with relapse and poor survival (Slamon *et al*., 1987). HER2 overexpression has also been reported in colon, prostate, ovarian and pancreatic cancers. Akt can be activated by HER2 overexpression in transformed cells (Yu *et al*., 1998). Akt1, a serine/threonine protein kinase was first discovered as the human homologue of a viral oncogene from thymomas in AKR mice (Staal 1987). Akt1 was shown to be amplified in gastric adenocarcinoma (Staal 1987). In humans, Akt2 gene amplification has been reported in ovarian, cervical, pancreatic and non-Hodgkin's lymphoma (1992). Figure 5 below is a schematic representation of the HER2 signalling pathway involving RAS/RAF/MEK/ERK and PI3K/Akt pathways.

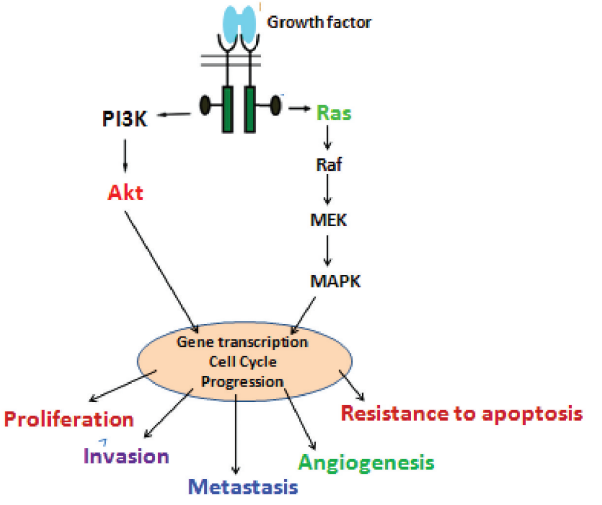
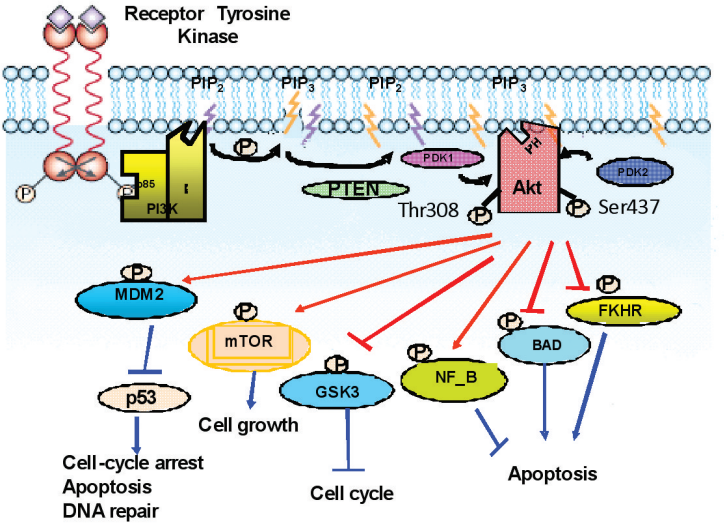


Figure 5 A simple representation of the signal transduction pathway activated by HER2 and the downstream effects.

Another major pathway turned on during neoplasia is the phosphoinositide 3-kinase (PI3K) pathway. PI3K is a lipid kinase that regulates a host of cellular functions, including proliferation, survival, shape and mobility, which play a critical role in facilitating the transformation of a cell into a malignant cell. PI3K was found to be associated with EGFR signaling (Bjorge et al., 1990).



Activation of the receptor tyrosine kinase, eg. EGFR, by ligands such as EGF results in the autophosphorylation of the receptor which is recognized by phosphoinositide-3-kinase, abbreviated as PI3K. Figure 6 shows activated PI3K catalyses of the turnover of the phospholipids resulting in the production of the PIP3, which is essential for the recruitment of AKT to the plasma membrane. Akt is then phosphorylated at two sites. Phosphorylation of these two sites can be detected by antibodies specific to the two residues, i.e. Thr 308 and Ser437. Activated Akt phosphorylates a number

of proteins, such as BAD, resulting in inhibition of apoptosis and stimulation of cell proliferation and growth. pTEN is a phosphatase that shuts off PI3K/Akt signaling.

Figure 6 is a schematic representation of the PI3K/Akt signaling pathway. Akt is a serine threonine protein kinase. Activated Akt phosphorylates many cellular proteins, such as BAD, FHKR and mTOR, resulting in resistance to apoptosis, stimulation of cell growth and dysregulation of the cell cycle. Activation of Akt can be prevented by a phosphatase, namely, pTEN.

Table 2 Functions of APC, Akt and downstream targets

Biomolecules	Functions
APC	Tumor suppressor gene; regulates the level of β-catenin; mutations in mutation cluster region (nt. 3000-4800) represents ~ 60% of somatic mutations in CRC (Miyoshi et al., 1992)
β-catenin	Binds with LEF/TCF transcriptional factor and transactivates downstream target genes; mutations in exon 3 have been detected in colorectal cancers
p-Akt (Thr308)	Phosphorylation of Akt at Thr308 in the activation loop, activates PI3K/Akt signaling pathway. Expression of p-Akt (Thr308) in colorectal cancer was unknown when we commenced our research project
p-Akt (Ser473)	Phosphorylation of Akt at Ser473 within its C-terminus activates PI3K/Akt signaling pathway. Expression of p-Akt (Ser473) in colorectal cancer has been previously reported (Roy et al., 2002)
p-BAD (Ser136)	pro-apoptotic member of Bcl-2 family; downstream target of PI3K/Akt pathway; phosphorylation of BAD at Ser136 by Akt inhibits BAD mediated apoptosis (Datta et al., 1997)

cont. Table 2

p-GSK3β	Downstream target of PI3K/Akt pathway; important in the regulation of β-catenin stability
FHKR	FHKR belongs to the FOXO Forkhead transcription factor family. It promotes transcription of pro-apoptotic genes such as FasL and Bim. Increased Akt activity leads to export of the forkhead transcription factor, FHKR from the nucleus to the cytoplasm, thus, preventing FHKR from promoting expression of the pro-apoptotic genes.
mTOR	A serine/threonine kinase that serves as a molecular sensor that regulates protein synthesis based on the availabillty of nutrients
P70S6K	Regulator of protein translation; phosphorylates ribosomal protein S6 to increase translation of messenger RNAs; downstream target of mTOR

Why Study Signal Transduction?

Deregulated signal transduction leads to aberrant cellular behavior and, as a consequence, can induce or contribute to various disorders such as cancer. Examples of aberrant cellular behaviour include increased proliferative potential, sustained angiogenesis and inhibition of apoptosis. Hence, understanding the signalling cascades has contributed to the numerous molecular targets for cancer treatment. After nearly 30 years of intensive research, one of the most exciting developments in cancer research has been the clinical validation of molecularly targeted drugs that inhibit the action of tyrosine kinases. These molecularly targeted drugs, which include trastuzumab (an antibody against human epidermal growth receptor-2), bevacizumab (an antibody against the vascular endothelial growth factor), cetuximab and panitumumab,

(antibodies against the epidermal growth factor receptor) and imatinib (a small molecule targeting the abl-bcr kinase), which are designed to target signaling pathways hold promise in treating human diseases. Their targets are usually proteins that play a role in malignant transformation or normal receptors or signalling proteins that regulate apoptosis or cell cycle. Blocking the action of these proteins arrests uncontrolled growth and ultimately leads to the death of the tumour cell. VEGF is a critical growth factor for angiogenesis which is a process that allows the tumour to grow and recruit blood vessels in order to spread to other parts of the body. Thus, blocking VEGFR activity can inhibit angiogenesis.

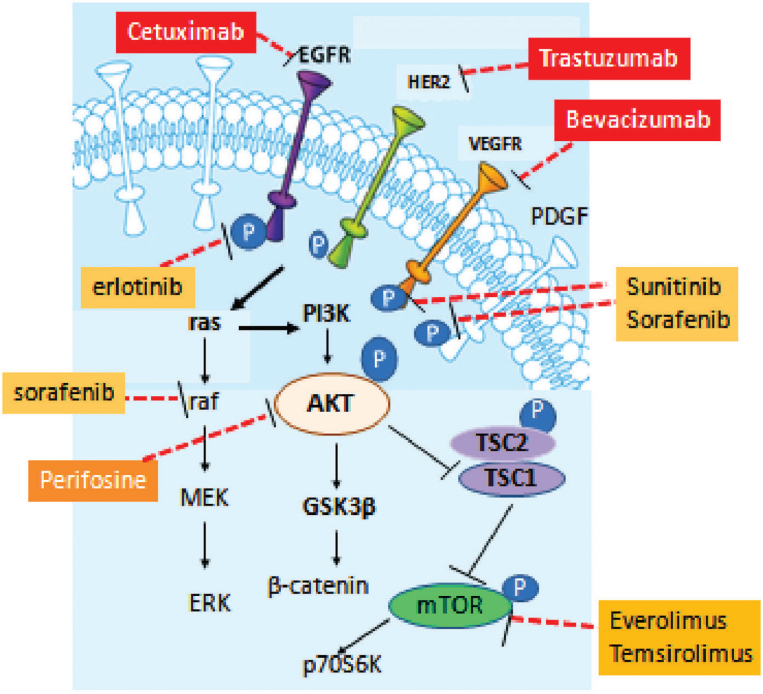


Figure 7 illustrates the sites targeted by molecularly-targeted drugs. Monoclonal antibodies that are antagonists of EGFR, HER2 and VEGFR include cetuximab, trastuzumab and bevacizumab, respectively. Erlotinib and sunitinib are small molecule tyrosine kinase inhibitors of EGFR and VEGF. These small molecules block the ATP pocket of the receptor, thereby inhibiting phosphorylation and downstream signal transduction. Sorafenib inhibits the tyrosine kinase activity of PDGF and raf has been used in treatment of advanced renal cancer. Further, Oral mTOR rapamycin analogues, everolimus and temsirolimus have displayed encouraging antitumour activity.

Figure 7 shows examples of chemicals or drugs known to act on specific proteins of the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signal transduction pathways. Akt is an indirect regulator of mTOR through phosphorylation and inactivation of tuberin (TSC2) complex

Table 3 Examples of molecularly-targeted drugs and their applications

Cancer	Drug	Targeted molecule
Breast	Trastuzumab	HER-2
B cell lymphoma	Retuximab	CD20
Colon, lung	Cetuximab, gefitinib (TK inhibitor)	EFGR
Colorectal	Bevacizumab	VEGF
CML, GIST, DFSP	Imatinib (Glivec)	BCR-ABL, c-kit, PDGFRB

Imatinib deserves a special mention as it was considered the 'magic bullet' for the treatment of chronic myeloid leukemia with *bcr-abl* fusion protein (Druker *et al.,* 2001). The *bcr/abl* fusion gene is formed when the *c-abl* tyrosine kinase gene on chromosome 9 is translocated to chromosome 22 and fused with part of the *bcr* gene on that chromosome. The resulting hybrid chromosome, the Philadelphia chromosome, encodes a new protein called Bcr/Abl. This new protein has increased kinase activity and drives proliferation causing transformation. In general, these specific inhibitors were expected to have less side effects than conventional chemotherapeutic drugs and have improved efficacy and selectivity of cancer treatment. However, they were found to be more complex and some extent of Side effects were also observed. Efficacy required appropriate selection of patients based on the presence of targeted molecules and problems of intrinsic drug resistance was common. Thus, understanding the interactions of the signaling molecules is of central importance in order to translate the use of these specific inhibitors to clinical applications. However, the ability of the cancer cell to develop drug resistance and to constantly undergo mutations or use alternative signaling pathways should be noted. Thus it will continue to be a challenge for clinical management. Consequently, it necessary to identify the appropriate biomarkers that can be used to accurately assess and individualise therapy.

Cancer Incidence in Malaysia

According to the Second Report of the National Cancer Registry 2003 (Lim and Halimah, 2004) the ten leading types of cancer amongst males in Peninsular Malaysia were lung, nasopharynx, colon, leukemia, rectum, prostate, stomach, lymphoma, skin and liver cancers. Amongst females, the ten leading cancers were breast,

cervical, colon, rectum, ovary, leukemia, lung, stomach and other skin cancers.

In Malaysia, the incidence of the different types of cancer differs between races. In Chinese men, nasopharyngeal cancer is the second common cancer, while in Malay men, it only ranks sixth. The top three most common cancers among Malay men are lung, leukaemia and rectal cancer. For Malay women, cancer of the breast, cervix and leukaemia are the most common.

The top three most common cancers in Chinese men are lung, nasopharynx and colon cancer. In Chinese women, breast, cervical and colon cancer are the most common. For Indian men, cancer of the stomach, prostate and leukaemia are the most common, in contrast to breast, cervical and oral cancers among Indian women.

In Malaysia, colorectal cancer is the third most common type of cancer. The age-standardized incidence rates for colon cancer were 13.9 and 11.2 per 100,000 persons, for men and women respectively; The age-standardized incidence rates for rectum cancer were 11.7 and 6.7 per 100,000 persons for men and women respectively.

In United States, colorectal cancer is ranked fourth in overall frequency and second in terms of cancer mortality. The age-standardized incidence rates were 30.68 and 40.56 per 100,000 persons for women and men respectively in year 2000. In the European Union, the age-standardized incidence rates was 29.36 per 100,000 persons for both sexes (Parkin *et al*., 2001). Thus, higher incidence rates of colorectal cancers were recorded for United States and European Union.

The annual incidence of new cases of cancer in Malaysia has been estimated to be 30,000. In 2002, 4337 cases of breast cancer were reported to the National Cancer Registry with an incidence rate of 52.8 per 100,000 and accounting for 30.4% of all diagnosed malignancies among Malaysian women. It has been estimated that one in 9 Malaysian women has a chance of developing breast

cancer. Breast cancer incidence in Malaysia is intermediate between the rates of industrialized countries like the US, 91/100,000 and developing countries like India, 19/100,000 (**Omar and Yip, 2005).**

ELUCIDATION OF WNT AND PI3K/AKT PATHWAYS IN COLORECTAL, BREAST AND NASOPHARYNGEAL CARCINOMA

When we commenced on this research in 2002, limited studies had previously been conducted to determine the aberrations of signal transduction in tumours among patients in Malaysia. We set out to determine the relevance of Wnt signalling in hepatocellular carcinoma and colorectal cancer and then PI3K/Akt pathways in breast, colorectal and nasopharyngeal carcinoma. The relationships between the biomolecules in these pathways were determined where some of the questions addressed were:

i) Is there a relationship between Wnt and the Akt pathway in colorectal cancer?

ii) Is Akt activated in breast cancer and is there a relationship between the oestrogen receptor and HER2 positivity?

iii) Is PI3K/Akt activated in nasopharyngeal carcinoma?

In addition, mutations in PI3K and pTEN genes were also determined to elucidate the mechanism of oncogenicity in each type of cancer.

APC, Beta-Catenin, pAkt and COX-2 Expression in Colorectal Cancer

APC (adenomatous polyposis coli) is a well-known tumour suppressor (Kinzler and Vogelstein, 1996) found to be mutated in

80% of sporadic colorectal cancers. Immunohistochemical staining with an antibody to the C-terminus of APC revealed that only 24 /47 (51.1%) of colorectal carcinoma tissue and 40/40 adjacent normal tissue were immunoreactive to this antibody [**p< 0.001 (**Mann-Whitney's test)]. The lack of immunoreactivity could be explained by the mutations of APC that resulted in a premature stop codon. A sampling of 11 tumours showed that 5/11 (50%) of the tumours harboured the mutations that resulted in the premature stop codon.

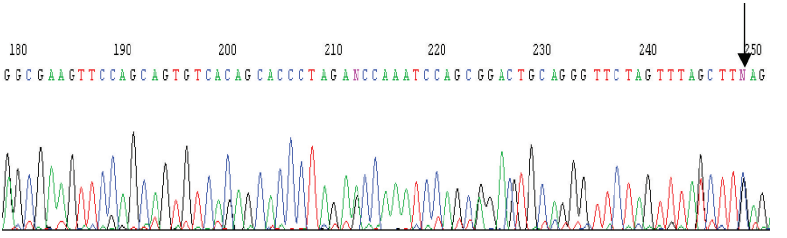


Figure 8 An electrophoregram showing the sequence of the partial *APC* gene. A heterozygous genotype of *APC* where TCA= serine or TGA=stop codon (N= G or C) is as indicated by the arrow shown.

The accumulation of β-catenin is an indicator of the activation of Wnt signalling. Majority of tumour cases (97%) express nuclear β-catenin, suggesting that strategies to block nuclear β-catenin accumulation is likely to be a useful therapeutic strategy. Our published data also showed that phosphorylation of Akt was detected in 63% of tumours (Ser 473=27% , Thr 308=16% , both =20%) (Khor *et al*., 2004). Accumulation of cytoplasmic β-catenin was also detected in hepatocellular carcinoma (Ban *et al*., 2003; Ban *et al.,* 2004). Further, COX-2 was overexpressed in 80% of CRC cases (Hong et al., 2004). COX-2 inhibitors have been shown to reduce mortality by 40-50% and reduce the number of intestinal polyps(Taketo, 1998). In conclusion, our study revealed that

strategies to block accumulation of β-catenin, phosphorylation of Akt and expression of COX-2 are likely to be useful in colorectal cancer treatment.

Akt Activation in Breast Carcinoma

Surgery, radiotherapy and chemotherapy remain the treatments of choice for breast cancer patients. An overview of the history of chemotherapy (Chabner and Roberts, 2005) and interesting developments in breast cancer treatment is summarized in the table below (Table 4).

Table 4 History of chemotherapy

Year	Drugs
1946	Mustine was used for non-Hodgkin's lymphoma
1958	Methothrexate for choriocarcinoma; vinca alkaloids and antioestrogenic action of MER-25 was discovered
Late 1970s	Successful development of tamoxifen Several clinical trials with combination chemotherapy [CMF (cyclophosphamide, methotrexate, fluorouracil), FAC (fluorouracil, adriamycin, cyclophosphamide), AC (cyclophosphamide, doxorubicin)] were conducted
1970s-1990s	Discovery of anthracylines (topoisomerase II inhibitor), camptohecins, fluorouracil, cisplatin
1987	Paclitaxel (Taxol) found to be effective for ovarian cancer
1990s	Clinical trials with aromatase inhibitors were conducted
1997	Trastuzumab approved by US FDA
1999	Docetaxel was the reference agent for treating metastatic breast cancer

For decades, tamoxifen was the most important hormonal agent for treatment of breast cancer. It significantly reduces the risk of recurrence and death. Further, Aromatase inhibitors were found to be useful for postmenopausal women. In 1997, trastuzumab, a monoclonal antibody to the human epidermal growth factor receptor 2 present on the plasma membrane of cells, was approved by the US FDA. Tamoxifen and trastuzumab caused a lot of excitement as they were thought to be the 'magic bullets' for breast cancer treatment. It has since been established that the existence of the oestrogen receptor and HER2 serve as basic markers for predicting drug response. However, drug resistance remains a problem and recurrence of the disease can still occur. One of the proposed mechanisms of drug resistance is the activation of Akt (Clark *et al*., 2002).

Akt is activated by many receptor-stimulated pathways involved in breast cancer, including ERa, IGF-IR, epidermal growth factor receptor and erbB2 (Clark, *et al.*, 2002). *In vitro* studies show that activated PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) which is essential for the phosphorylation of Akt at Ser473 and Thr308. Activated Akt then phosphorylates many cellular proteins resulting in diverse cellular processes which are critical for tumorigenesis such as cell proliferation and survival. p-Akt is a powerful promoter of cell survival as it antagonises the components of the apoptotic cascade such as the Bcl-2 associated death domain (BAD) protein. Akt is also implicated in angiogenesis and metastasis which are the two important processes in cancer development. Thus, it is not surprising that frequent activation of Akt occurs in a wide spectrum of cancers such as prostate, breast and ovarian carcinomas. *In vitro* studies suggest that the PI3K/Akt pathway is transduced when the ERa and HER2 are activated (Clark *et al*., 2002).

We addressed the question of whether Akt is activated in our breast cancer samples and whether Akt activation correlates with ER positivity and c-erbB2 expression. Further, we determined the incidence of Akt phosphorylation in breast tumours and the relationship between the expression of ER-α, ER-β, HER2, Ki-67 and phosphorylated Bcl-2 associated death domain (p-BAD). Immunohistochemical staining was performed to detect these molecules on 43 paraffin-embedded breast tumour tissues with commercially available antibodies. Eighteen (41.9%), 3(7.0%), 23 (53.5%), 35 (81.4%), 21 (48.8%), 29 (67.4%), and 34 (81.0%) of the breast tumours were positive for nuclear ER-α, nuclear ER-β, membranous HER2, p-Akt (Thr308), p-Akt (Ser473), p-BAD and Ki-67, respectively. The p-Akt (Ser473) correlated with increased levels of p-BAD (Ser136) ($p=0.012$). Cytoplasmic localization p-Akt ^{T308} staining was found to be significant in tumour tissues 35/43 (81.4%) positive ($p<0.001$). BAD is phosphorylated and inactivated by p-Akt resulting in inhibition of apoptosis tumor cells proliferating inappropriately without compensatory apoptosis ($p = 0.006$, $r = 0.366$). Correlation analysis revealed that c-erbB2 signalling could lead to activation of Akt via phosphorylation of Akt T308 ($p = 0.014$, $r = 0.0366$). To summarise, Akt activation is independent of oestrogen receptor status and overexpression of p-AktT308 correlated with c-erbB2.

The significance of our study is in that it showed that Akt is likely to be a relevant target for breast cancer drug discoveries (Seow *et al*., 2009). Our study does not support the simple model of linear ERa/PI3K/Akt pathway in breast cancer. This data differs from that observed with breast cancer cell lines (Clark *et al*., 2002). Further studies will be required to determine the alternative signaling pathway utilized by ERa positive breast tumours.

Aberrant PI3K/Akt of Signal Transduction Pathway in Nasopharyngeal Carcinoma

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor. Upon binding of EGF, EGFR initiates signal transduction cascades that promote cell division, migration and angiogenesis, and inhibits apoptosis. EGFR is abnormally expressed and activated in cancer cells in many tumor types such as breast and colon cancers. However, it was unknown whether EGFR is abnormally expressed in nasopharyngeal carcinoma.

The objectives of our study were: (i) to elucidate the expression rate of pEGFR and the downstream targets, namely, pAkt, pBAD, pFKHR, pGSK-3β, p-mTOR in NPC and non-malignant nasopharyngeal tissue specimens; (ii) to determine the relationship between these biomolecules; and (iii) to determine whether *PI3KCA* mutations contribute to aberrations of the PI3K/Akt pathway in NPC

Immunohistochemical staining results suggest that the activation of the PI3K/Akt pathway is mainly contributed by activation of epidermal growth factor receptor (EGFR) rather than c-erbB2(HER-2). Forty-one per cent (26/64) of NPC expressed the phosphorylated form of EGFR. EGFR phosphorylation correlated with phosphorylation of Akt at Thr308 but not Ser 473. Phosphorylation of Akt was positively correlated with phospo-BAD ($p=0.004$), p-FKHR ($p=0.001$) and Ki-67($p=0.004$). No correlation was found between phosphorylation of Akt and phosphorylation of mTOR and GSK-3β (Yip *et al*., 2008). Our results suggest that the pathogenesis of NPC is more complicated than the linear model of the PI3K/Akt/mTOR pathway and that phosphorylation of mTOR and GSK-3β occurs via other pathways. Over the last few years there have been advances in the use of monoclonal antibody to EGFR such as cetuximab for treating non-small lung

and colorectal cancers. Immunohistochemical staining to evaluate EGFR protein levels is a convenient method for analyzing clinical samples. However, studies show that there is a lack of correlation between EGFR overexpression and response to cetuximab (Chung *et al.* 2005; Scartozzi *et al.* 2004). This could be explained by the possiblilty of there being downstream signals which are activated by other pathways or oncogene mutations such as K-ras mutations (ref). Thus, the clinical benefit of drugs that target EGFR for nasopharyngeal carcinoma patients is unclear at this stage. Further investigation of the other possible EGFR downstream signals needs to be identified in nasopharyngeal carcinoma.

To further understand the mechanisms in aberrations of the PI3K signal transduction pathway, the somatic mutations of the *PIK3CA p110*α gene were examined. *PIK3CA* mutations have been frequently found in various human cancers. Most of these mutations are clustered in exon 9 (helical domain) and exon 20 (kinase domain). By using PCR with DNA extracted from tumour tissues followed by DNA sequencing, no "hotspot" mutations of *PIK3CA* were detected in 25 of our nasopharyngeal carcinoma tissues. In parallel, mutations of breast carcinoma tissues were also studied. Six out of 20 (30%) of our breast carcinoma tissues were found to harbour *PI3KCA* mutations (Leong *et al.,* 2009). Further studies on elucidation of the *PIK3CA* gene copy number and the role of mTOR in NPC is currently being investigated. These studies have important implications in the selection of currently available targeted drugs and future development of molecularly-targeted therapies.

ANTICANCER EFFECTS OF PURIFIED COMPOUNDS FROM SOIL-DERIVED ACTINOMYCETES (GENUS STREPTOMYCES)

Introduction

The discovery of new drugs targeted at specific molecules of the signal transduction pathway is a 'hot' field of research. These molecularly targeted drugs may have fewer side effects and thus, may be useful alternatives. Secondary metabolites, especially from actinomycetes such as *Streptomyces,* may be among the most important sources for novel anti-cancer agents. Microbes have a good history of being the source of useful drugs, as exemplified by antibiotics such as penicillin or kanamycin and the immunosuppressive drug, tacrolimus. Among others, an antifungal agent, Staurosporine, produced by the actinomycete *Saccharothrix aerocolonigenes,* was found to be effective in inhibiting PKC (protein kinase C); and the synthetic analogue, UCN-01 (7-hydroxystaurosporine) was found to block the cell cycle (Cohen, 2002). The use of microbes as source of drugs is more advantageous than the use of plants as harvesting of plants destroys the environment and at least another 10 years will be required to use synthetic biology to produce large amounts of material for clinical trials.

Elucidation of the Mode of Action of Compounds from the H7372 Strain

We have been successful in identifying a strain of *Actinomycetes* (genus *Streptomyces)* that can kill cancer cell lines *in vitro*. These extracts have been screened using the yeast hybrid system and were found to interfere with Ras-Raf interaction. The results have been presented (Lai *et al.*, 2009).

(a) (b)

Figure 9 (a) Growth of strain H7372 on a broth culture plate (b) A representative picture showing the mangrove swamp where the actinomycetes H7372 was collected.

We were able to detect the presence of inhibitors from actinomycetes, strain H7372, in nasopharyngeal, colon and breast cancer cell lines. Preliminary results indicated that the crude extracts could induce apoptosis of a breast cancer cell line and reduce the activation of the Akt pathway. We hypothesize that inhibition of the Ras pathway by H7372 will also inhibit other signal transduction pathways such as the phosphoinositide-3-kinase (PI3K)/Akt pathway, resulting in anti-proliferation and apoptosis (ie. death of the tumour cell). Since there is cross-talk between the signal transduction pathways and their network, the compound could alter multiple oncogenic pathways. This is advantageous provided they do not perturb normal cells. Drug companies are making active efforts to discover drugs of this nature. Information on the precise targets of the drug is important to ensure that no serious side-effects are exerted by these new drugs. Thus, the discovery of new compounds is essential for the development of new drugs or biologicals.

The research approach uses cancer cell lines to test their responses to the fractionated extracts containing the antitumour active compound. The viability of the cell lines in response to the fractions are tested using the MTT assay. Todate, we have identified a compound from a soil-derived bacteria, namely, *Streptomyces,* which has the ability to reduce the viability of a number of cancer cell lines such as the MCF-7(breast), TWO-6 (nasopharyngeal), HT-29 (colon), liver (HepG2), prostate (PC3), and cervical(HeLa) cancer cell lines (Yip *et al*., 2010). Effects on the cell cycle and protein expression were determined by flow cytometry and Western Blotting, respectively (Yip *et a*l., 2010). We will also determine whether the active fraction can exert synergistic effects with other molecularly-targeted drugs such as the antioestrogen drugs, tamoxifen, the c-erbB2 inhibitor (trastuzumab) and the epidermal growth factor (cetuximab). Resistance to commercially available drugs is a problem. Thus, the mode of action of the combination of our microbial-derived fraction and the commercial drugs will be determined and this could give better insight into the mechanisms of drug antagonism and how to overcome it. To summarise, we have conducted a fundamental study on the action of the active fraction with and without a combination of commercially available drugs. A glycogen-synthase-3-kinase inhibitor was also discovered from another strain of *Streptomyces* (Cheenpracha *et al*., 2009).

Conventional chemotherapy has long been thought to be cytotoxic to both tumour and normal cells. However, recent evidence shows that these cytotoxic drugs can defeat immunosuppression and increase the efficacy of active immunotherapy. A number of studies performed more than 20 years ago showed that cyclophophosphamide, a cytotoxic drug, when given before a cancer vaccine, allowed more effective activation of antitumour immunity. It was thus hypothesized that this could be due to the

inhibition of immunosuppressor cells. However, it was not until the late 1990s that the existence of these suppressor cells came to light when the identification and isolation of T regulatory cells was reported (Sakaguchi *et al*., 1995). These T regulatory cells have been found to be sensitive to cyclophosphamide (Ghiringhelli *et al*., 2004). We are currently studying whether the compounds isolated from *Streptomyces* (genera *Actinomycetes*) have an effect on immunosuppression.

GENERATION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY IN BREAST AND NASOPHARYNGEAL CARCINOMA

Introduction

Cytotoxic T lymphocytes (CTLs) are lymphocytes that kill other cells, virally infected cells, allografts or tumour cells. Each CTL has a unique T cell receptor (TCR) that is specific to a particular target antigenic peptide bound to a Major Histocompatibility Complex (MHC) Class I protein on the surface of a tumour cell, as depicted in Figure 10. The engagement of the TCR by a MHC/peptide complex on the target cell triggers the CTL effector functions and can result in destruction of the target cell. CTLs have cytoplasmic granules that contain the proteins perforin and granzymes. When the CTL binds to its target, the contents of the granules are discharged by exocytosis and kill the target cell. Thus, killing tumour cells by using the immune system, particularly with CTLs, is a rational approach.

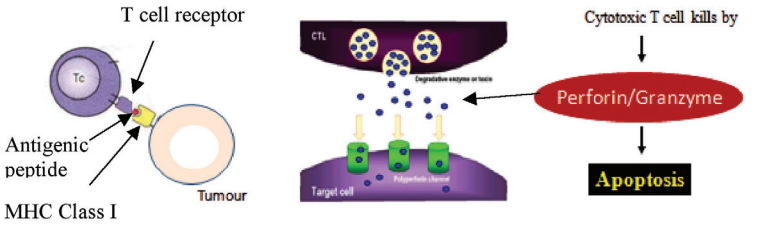


Figure 10 A simplistic diagram to show the key players in the killing of tumour cells by activated cytotoxic T lymphocytes. (a) The antigenic peptide (as indicated in red) is loaded onto the MHC Class I molecule (as indicated in yellow) of the tumour cell which is presented to the T cell receptor. (b) Activated cytotoxic T lymphocytes release perforins and granzymes(as indicated by blue dots) resulting in the death of the tumour cells by apoptosis.

The infusion of CTLs into cancer patients is known as adoptive cellular therapy. Adoptive cellular therapy is also sometimes referred to as adoptive immunotherapy. It involves taking cells from the patient, either from the blood or tumour, and manipulating these cells in the laboratory. After *in vitro* manipulation, eg. stimulating them with cytokines or antigen, the cells are expanded to sufficient numbers and then infused back into the same patient.

Since the last decade, the strategy of adoptive transfer of *ex vivo* generated tumour specific $CD8^+$ effector T cells has been developed and utilized in either alternative therapeutic intervention (for pre-existing tumour eradication) or preventive vaccination, to create immunological protection against subsequent tumour challenge (Rosenberg SA, 2004). Progress was made with the infusion of autologous lymphokine-activated killer cells (Rosenberg *et al*., 1985) and CD8+ T cell clones (Yee *et al*., 2002) as well as in improving methods for generating *ex-vivo* cytotoxic T cells with

artificial antigen presenting cells (Maus *et al*., 2002) and genetic engineered T lymphocytes (Sadelain *et al*., 2003). These attempts were made to improve the quantity, persistence and efficacy of adoptively transferred cytotoxic T lymphocytes. The preventive vaccinations, which involve the use of inactivated tumour cell injection and gene therapy with tumour-specific antigens, are effective in eliminating the inoculated tumour cells with the pre-existing anti-tumour immunity (Chan *et al*., 2002; Chan, 2010). Todate, the results show that these therapeutic approaches are relatively effective in most haemopoietic-derived tumours such as lymphoma (Rooney *et al*., 1998) but are less effective in established solid tumours such as nasopharyngeal carcinoma, which may largely be due to the existence of immune invasion mechanisms in the tumour microenvironment and the use of effector T cells that have heterogeneous properties.

For nasopharyngeal carcinoma (NPC), immunotherapy may serve as either an alternative or an additional treatment to improve the clinical outcomes of the NPC patients as compared to conventional therapeutic approaches, namely radiotherapy and chemotherapy, which usually result in severe long lasting side effects. Various forms of immunotherapy strategies, both active and passive, are being developed and modified to treat NPC. The application of adoptive transfer of autologous cytotoxic T lymphocytes that recognize Epstein-Barr viral (EBV) proteins, namely, LMP-1, LMP-2 or EBNA-1, has been tested but with limited success (Lee *et al.,* 1997). Another approach using dendritic cells pulsed with peptides derived from LMP-2 (Chua et al., 2001) was used but again with limited success. Several clinical trials have also been conducted (Lin *et al*., 2002; Comoli *et al*., 2004; Comoli *et al*., 2005; Ma & Chan, 2008; Merlo *et al*., 2008). The outcomes of these clinical trials are relatively inconsistent and unsatisfactory.

However, some promising outcomes have been shown in adoptive transfer of effector T cells that are specific for EBV antigens, including nuclear antigens (EBNA-1, -3A, -3B and -3C) and latent membrane proteins (LMP1 and LMP2), for the treatment of EBV associated lymphoproliferative diseases in transplant patients. Overall, some progress has been made in adoptive T cell therapy in NPC and it has been shown to be relatively safe and capable of eliciting specific immune response to control the disease in advanced NPC (Comoli *et al*., 2005; Straathof *et al*., 2005). The milestones in cancer immunotherapy and current trends indicating the usefulness of combining chemotherapy with adoptive cellular therapy have been clearly stated in a review (Leong and Seow, 2006).

It is still unclear whether the immune system can be harnessed for the purpose of immunotherapy. The types of immune cells and phenotype optimal for killing tumour cells *in vivo* have not been definitely identified. It is also unclear how tumours can tolerise the immune system and reduce antitumour immunity. In the last decade, there has been a resurgence of studies on immunosuppression mediated by T regulatory cells. The effectiveness of adoptive cell transfer to treat cancer patients requires a clear understanding of the functions of the cells in the tumour microenvironment. We have detected the presence of cytokines (Chia *et al*., 2002) and infiltrating T regulatory cells in breast and nasopharyngeal tumours (Leong *et al*., 2006; Yip *et al*., 2009). The influence of cytokines and T regulatory cells on the success of adoptive cellular transfer of *ex-vivo* activated CTLs is still unknown.

Generation of Cytotoxic CD8+ Lymphocytes Stimulated with LMP-2 Specific Peptides

When we commenced on this research, the conditions for generation of cells and phenotype *of in vitro* generated cells for optimal

successful adoptive immunotherapy was not well characterized. Our research addressed the issue of the appropriate conditions to support growth and stimulate the generation of less differentiated effector cells with the ability to kill target tumour cells. Latent membrane protein (LMP)-2 specific peptides were designed using the PEPVAC software. However, the human leukocyte antigen (HLA) type of the lymphocytes present in peripheral blood of patients need to be determined first and HLA typing methods had to be established beforehand (Leong et al., 2010). Finally the phenotype and cytotoxic capability of the cultured cells were successfully determined (manuscript in preparation).

This area of work posed a great challenge as generation of sufficient numbers of effector T cells was difficult. Other challenges faced include the ability to sustain the persistence of these cells and to direct them to the tumour site. These questions will need to be further addressed in future experiments to ensure the efficacy of the *ex vivo* activated T cells in killing tumour cells.

IMMUNOMODULATORY EFFECTS OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSC) are multipotent non-hematopoietic stem cells, which reside in the bone marrow and other tissues, together with the hematopoietic stem cells (HSC) (Bobis *et al*., 2006). The multipotency of MSCs was first identified in 1976 (Friedenstein *et al*., 1976) whereby, MSC in culture were demonstrated to have the ability to differentiate other cells such as osteocytes, chondrocytes and adipocytes. Their potential application in regenerative medicine, prevention of graft versus host reaction in transplants and anti-cancer therapy has recently generated a great deal of interest .

Our study showed that MSCs derived from bone marrow, but not umbilical cord blood, inhibited T cell proliferation (Ramasamy *et al*., 2008; Tong et al., 2008). We also found that MSCs from human umbilical cord blood were capable of inhibiting the growth of K562, an undifferentiated erytholeukemia cell line derived from a chronic myeloid leukemia patient (manuscript in preparation). The procedure for generation of MSCs from human umbilical cord tissue was established in our laboratory (Tong *et al*., 2010). Further studies are currently underway to identify the factors responsible for tumour inhibitory activity.

DEVELOPMENT OF DIAGNOSTIC TESTS FOR DETECTION OF CANDIDA AND ASPERGILLUS FUMIGATUS

Introduction

In the last decade, the incidence of opportunistic fungal infections has increased dramatically. An invasive, disseminated, life-threatening form of candidiasis is seen in patients in intensive care as well as in post-surgical and neutropenic patients. The case fatality rate of systemic fungal infections, of 70% - 90%, is nearly three times higher than that for bacterial infections. The Candida species of clinical importance reported at the University Hospital, University of Malaya, Kuala Lumpur (Ng et al., 2000) differs from Western data whereby *C.albicans* represents 40 % of systemic candidiasis. Thus, the patterns of Candida prevalence in other hospitals in Malaysia is worthwhile investigating. However, this requires accurate, rapid, sensitive and cost-effective diagnostic tests with the ability to detect many medically important Candida species. Current commercial diagnostic tests have a number of limitations including the inability to detect more than five Candida species,

being time consuming and in some cases, the tests are technically demanding.

Early detection of systemic infection has a great impact on the clinical outcome of candidiasis. The Candida species needs to be identified as some of the Candida species are inherently resistant to standard antifungal drugs. For example, *C. krusei* is resistant to fluconazole and *C. glabrata* is resistant to fluconazole and itraconazole. The wide spectrum antifungal drug, amphotericin has side effects. Thus, it is important to identify the Candida species in order to prescribe the most suitable antifungal drug for the management of the patient. Current laboratory diagnosis relies on blood culture, biochemical tests and morphological identification. These procedures take at least 3-7 days as the Candida is slow to grow and the tests are time-consuming and laborious. Consequently, when these results are obtained, it is too late to save the life of the patient. Thus, there is a dire need for development of improved rapid diagnostic tests.

Summary of Molecular Approaches and Monoclonal Antibody Production

Successful detection was achieved by the use of seminested PCR utilizing ribosomal RNA (rRNA) region, successfully detecting and identifying sixteen fungi species; ten *Candida* species (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. rugosa* and *C. tropicalis*), five *Aspergillus* species (*A. flavus*, *A. fumigatus*, *A, nidulans*, *A. niger* and *A. terreus*) and *Cryptococcus neoformans* (patent submitted). Besides rRNA, primers designed within the isocitrate lyase gene was designed for semi-nested and realtime PCR to detect the ten *Candida* species. In addition, monoclonal antibodies produced using *in vivo* mice method obtained from hybridoma clones,

designated as C6(3), F8(3) and F4(2) of antibody isotype IgG class and IgG2b subclass, were generated. They showed specificity towards *Candida glabrata*. Five monoclonal antibodies were also generated for detection of *Aspergillus fumigatus*.

In conclusion, molecular diagnostic tests for ten species of Candida were successfully developed and monoclonal antibodies to *Candida glabrata* as well as *Aspergillus fumigatus* were also produced. Further validation of these tests with clinical specimens will need to be performed.

PATENTS

Title:	**Rapid semi-nested PCR method for detection of ten medically important Candida species.**
Inventors:	Than L, Chong PP and Seow HF.
Applicant:	Universiti Putra Malaysia
Application No.:	PI2008 3143, PCT/MY2009/000109
Abstract:	This invention relates to nucleic acid sequences used for detecting DNA from ten medically important Candida species by semi-nested PCR

Title:	**Nucleotide Probes for Detection and Species Differentiation of Candida Infections.**
Inventors:	Chong PP, Seow HF and Ng KP.
Applicant:	Universiti Putra Malaysia

Abstract: This invention relates to nucleic acid sequences that were used for development of an array-based assay to detect DNA from eight medically important Candida species.

Title: Detection of Epstein-Barr Virus in nasopharyngeal cancer

Inventors: Seow HF, Yap YY and See HS.

Applicant: Universiti Putra Malaysia

Application No.: PI20071547

Abstract: This invention relates to novel primers used to quantify latent membrane protein-1 DNA in peripheral blood of suspected nasopharyngeal carcinoma patients.

Title: Possible diagnostic markers as a guide to drug responsiveness in acute leukemia treatment

Inventors: Maha, Abdullah; Cheong, Soon Keng; Leong, Chui Fun, Seow, Heng Fong

Applicant: Universiti Putra Malaysia, filed Aug 2003.

Application No.:

Abstract: Drug resistance is a major obstacle to successful treatment of acute myeloid leukaemia. The precise molecular events involved in the development of drug resistance remain largely unknown. We found induction therapy induced phosphorylation of mediators of signaling pathways. Molecules

of the PI3 kinase and MAP kinase pathways were found to increase or decrease phosphorylation depending on whether the cells were responding or resistant to chemotherapy. PI3 kinase activates Akt which then phosphorylates FKHR and Bad. Sequestration of these latter two molecules leads to survival of the cell. This was observed in cells that were found to be non-responsive to chemotherapy. In patients who achieved complete remission, p38 and Jnk, members of the MAP kinase pathway, were found to be phosphorylated. Observation of these changes suggests that these proteins may provide more specific targets for human therapeutic and diagnostic purposes.

Title:	**Peptides that inhibit the association of the surface and core antigens of hepatitis B virus.**
Inventors:	Tan, W.S., Seow H.F., Yusoff, K. and Ho, K.L.
Applicant:	UPM. Filed on 28th September 2001.
Application Number:	PI 20014538.
Abstract:	This invention relates to recombinant phages that bind tightly to hepatitis B core particles and also peptides that inhibit the assembly of the hepatitis B virus.
Title:	**Immune response modulators and uses thereof**
Inventors:	Wood Paul and Seow Heng Fong
Applicant:	Commonwealth Scientific and Research Organisation

Application No.: WO1996AU00360 19960614

Abstract: The present invention relates to a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an ovine IL-5 and IL-12 cytokine molecule. The invention further provides recombinant isolated ovine IL-5 and IL-12 polypeptides which are useful as immune response modulators in livestock animals.

Title: Adjuvant

Inventors: Corner Leigh Austin, Rothel James Stuart, Wood Paul Richard, McWaters Peter and Seow Heng Fong

Applicant: Commonwealth Scientific and Research Organisation Australia

Application No.: US19970732398 19970211

Abstract: The present invention relates generally to adjuvants which comprise a combination of at least two cytokines or functional derivatives thereof. More particularly, the present invention is directed to an adjuvant such as a vaccine adjuvant comprising at least two cytokines or functional derivatives thereof wherein the cytokines selected from IL-1 beta and TNF alpha or IL-1 beta and GM-CSF. The present invention is farther directed to genetic adjuvants encoding at least two cytokines or derivatives thereof, either separately of fused together.

The present invention also contemplates a method for enhancing an immune response to an antigen comprising the administration of at least two cytokines which act in synergy to enhance an immune response to said antigen. The present invention is particularly useful in pharmaceutical vaccines and genetic vaccines in humans and livestock animals.

Title:	**Ovine Cytokine Genes**
Inventors:	Rothel James Stuart, Wood Paul Richard, Seow Heng Fong
Applicant:	Commonwealth Scientific and Research Organisation Australia
Application no.:	WO1991AU00419 19910912
Abstract:	The present invention relates to a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding of an ovine cytokine-like molecule. The preferred cytokine-like molecules include recombinant IFN-gamma, GM-CSF, IL=1, IL-2, IL-4, TNF alpha and TNF beta.

CONCLUSION

Our data on signal transduction revealed the heterogeneity of the individual specimens in terms of expression of the biomolecules. Nevertheless, correlation analysis confirmed the positive relationship between pAkt and downstream targets such as pBAD and pGSK in colorectal cancer. In nasopharyngeal carcinoma,

results suggest that the PI3K/Akt/mTOR pathway is not a simple linear pathway. Cytostatic and cell cycle arrest by a purified compound from a soil-derived *Actinomycetes* (genus *Streptomyces*) was demonstrated. The efficacy of the compound will need to be tested in animal models.

Similarly, the successful generation of cytotoxic $CD8^+$ T lymphocytes and mesenchymal stem cells for adoptive immunotherapy will require further experimentation in animal models.

In conclusion, the 'magic bullets' for cancer therapy that can be used to treat metastatic diseases and to overcome problems of drug resistance, toxicity and disease relapse are still at its infancy. Future studies on identification of feedback mechanisms and redundancies in the signalling network is essential for the formulation of multiple targeted drugs for cancer treatment to overcome the problem of drug resistance. This will require in depth understanding of how the complex signal transduction pathways and networks interact as well as the effects on the feedback mechanisms in the presence of one inhibitor or multiple inhibitors. Innovative strategies for effective design and delivery of cell-based therapies are highly desirable for treating cancer. It is envisaged that in the next decade further advances will be made in the field of siRNA and tumour initiating cells, sometimes referred to as 'cancer stem cells' research.

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BIOGRAPHY

Professor Dr. Seow Heng Fong was born in Melaka and was a student at the Convent of the Holy Infant Jesus (1960-1975) and Melaka High School (1976-1977). She graduated from the University of London, United Kingdom with a BSc (Hons) in Biomedical Sciences in 1982 and a PhD degree in Biochemistry from the University of Auckland, New Zealand in 1985. She was awarded a postdoctoral fellowship at the Stanford School of Medicine, Stanford University, California in 1986 where she acquired skills in molecular biology, focussed on gene cloning and DNA sequencing, and also received training in the area of immunology and the genetics of the human leucocyte antigen (HLA) genes.

She has working experience as senior research fellow at the Institute of Molecular and Cell Biology, National University of Singapore and senior scientist at CSIRO Australia and the Macfarlane Burnet Institute for Medical Research, Melbourne, Australia. At CSIRO, she was appointed to lead the Cytokines project. The project was aimed at formulating novel adjuvants for vaccine development and identification of cytokine profiles in infectious diseases. At the Macfarlane Burnet Institute, she was the Acting Head of the Hepatitis Unit and supervised projects on Hepatitis B therapeutics as well as Hepatitis E diagnostics and vaccine development.

She joined Universiti Putra Malaysia in May 1997 as Associate Professor at the Faculty of Medicine and Health Sciences. In 2003, she spent her sabbatical leave at the Gene and Stem Cell Therapy Lab, Centenary Institute, University of Sydney, Australia. She was promoted to Professor, Department of Pathology, Faculty of Medicine and Health Sciences, UPM in 2003.

She has been the project leader for 3 projects under the MOSTI Priority Research Scheme and program leader for a project under

the MOSTI National Biotechnology Directorate. In June 2010, she commenced duties as the Deputy Dean (Learning Support and Student Affairs) at the School of Graduate Studies, UPM.

Her field of specialization is in molecular biology and cancer. Her research interests include mechanisms of drug resistance in cancer, aberrations in signal transduction in cancer and cancer immunology. She has also led projects in the discovery of novel compounds with anti-cancer properties and the development of diagnostic tests for *Candida* and *Aspergillus.*

Dr. Seow's 220 publications include 108 full articles and 9 patents, including papers in high impact journals such as Nature and Nature Genetics. During her 11 years at UPM, she has supervised at least 50 postgraduate and undergraduate research students. Her graduates have been successfully employed at local companies and universities as well as overseas institutions such as the Mayo Clinic at Minnesota USA, MD Anderson Cancer Centre, University of Texas USA, Rutgers University, USA and the National Cancer Centre in Singapore.

Her society memberships include the American Association for Cancer Research and the Australasian Society for Immunology. She is also a member of the editorial board of the Malaysian Journal of Pathology and Journal of Tropical Conservation. She also serves as a reviewer for journals including the International Journal of Cancer, Journal of Translational Medicine, Stem Cells International and Journal of Immunological Methods.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to the following funding bodies for their support:

i) MINISTRY OF SCIENCE, TECHNOLOGY AND INNOVATION :

Priority Research, MOSTI

- Molecular studies on disease biomarkers and pathogenesis of cancer: colorectal carcinoma as a model (06-05-01-003BTK/ER018) - 2003-2005
- Identification of nasopharyngeal carcinoma specific Epstein-Barr virus antigens towards the development of cytotoxic T lymphocyte-based immunotherapy (06-02-01-0000-PR0054/06-04) - 2003-2008
- Identification of novel therapeutic targets for treatment of breast cancer (06-02-04-0373-PR002/07-07) - 2002-2004

National Biotechnology Directorate, MOSTI

- Molecular Approaches to the Diagnosis and Management of Systemic Fungal Infections (06-02-04-005 BTK/ER/023) - 2003-2008

eScience

- Discovering signal transduction inhibitors from Actinomycetes for potential anticancer drugs (02-01-04-SF0347)

ii) MINISTRY OF HIGHER EDUCATION: FRGS

Characterisation of the mode of action of signal transduction inhibitors from Actinomycetes extracts

iii) UPM: RESEARCH UNIVERSITY GRANT SCHEME (RUGS)

Immunomodulatory effects of mesenchymal stem cells: implications for cancer therapy

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