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Discovery and Characterisation of Novel Anticancer Compounds Acting on Sphingosine Kinase

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: Date:

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ABBREVIATION

°C	Degrees centigrade
ABA	Abscisic acid
ABC	ATP binding cassette
Abs	Absorbance
ADP	Adenosine 5'-diphosphate
AKAP	Protein kinase A anchoring protein
APCI	Atmospheric pressure chemical ionization
ATP	Adenosine 5'-triphosphate
BACE	β-site APP cleaving enzyme
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CCT	Chaperonin containing t-complex polypeptide
Cdc42	Cell division control protein 42
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CIB1	Calcium- and integrin-binding protein
DAG	Diacylglycerol
DAPI	4',6- diamidoino-2-phenylindole
DHS	D,L-*threo*-dihydrosphingosine
DMEM	Dulbecco's Modified Eagle Medium
DMS	N,N-dimethylsphingosine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid

EFCS	European foetal calf serum
EGF	Epidermal growth factor
ELK1	E twenty-six-like transcription factor 1
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
ESI	Electrospray ionisation
FC	Flash chromatography
Fig	Figure
FKBP-12	FK-binding protein 12
FRIM	Forest Institute of Malaysia
g	Gram
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HER2	Human epidermal growth factor receptor 2
hr(s)	Hour(s)
HRP	Horseradish peroxidise
hSK	Human sphingosine kinase
HTS	High throughput screening
Hz	Hertz
IFN-γ	Interferon-γ
IL	Interleukin
IP	Immunoprecipitation
IP_3	Inositol triphosphate

JNK	c-Jun N-terminal kinase
kDa	Kilodalton
K_i	Inhibition constant
K_{ic}	Competitive inhibition constant
K_{iu}	Uncompetitive inhibition constant
K_m	Michaelis constant
M	Molar
MAPK	Mitogen activated protein kinase
mg	Milligrams
min(s)	Minute(s)
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
mSK	Murine sphingosine kinase
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NES	Nuclear export signal
NF-κB	Nuclear factor-κB
ng	Nanograms
NLS	Nuclear localization signal
nm	Nanometre
NP-40	Nonidet P-40
PA	Phosphatidic acid
PAK1	p21-activated kinase 1
PARP	Poly(ADP-ribose)polymerase

PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule 1
PHB	Prohibitin
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
pmol	Picomole
PMSF	Phenylmethanesulfonyl fluoride
PVP	Polyvinylpyrrolidone
rDNA	Ribosomal deoxyribonucleic acid
RIP	Receptor interacting protein
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
S1P	Sphingosine-1-phosphate
$S1P_{(1-5)}$	Sphingosine-1-phosphate receptor (1-5)
SAR	Structure-activity relationship
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGE	Skin polyphenolic extracts
SIDR	Strathclyde Innovations in Drug Research
SIR	Sirtuin
siRNA	Small interfering RNA
SK	Sphingosine kinase

SKIP	Sphingosine kinase interacting protein
SOC	Super optimal broth with catabolite repression
TBST	Tris buffered saline with Tween20
TCP1	T-complex protein 1
TGF-β	Transforming growth factor beta
TLC	Thin layer chromatography
TMS	N,N,N-trimethylsphingosine
TNF-α	Tumour necrosis factor-α
TRAF2	TNF receptor-associated factor 2
TRiC	TCP1 ring complex
UV	Ultraviolet
V	Volts
VEGF	Vascular endothelial growth factor
V_{max}	Maximum rate of reaction
WB	Western blotting
µg	Micrograms
µl	Microlitre
µM	Micromolar

Publications

Lim, K. G., Tonelli, F., Li, Z., Lu, X., Bittman, R., Pyne, S., & Pyne, N. J. (2011). FTY720 analogues as sphingosine kinase 1 inhibitors: enzyme inhibition kinetics, allosterism, proteasomal degradation and actin rearrangement in MCF-7 breast cancer cells. *J Biol Chem*, *286*(21), 18633-18640.

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Pyne, N. J., Tonelli, F., **Lim, K. G.,** Long, J., Edwards, J., & Pyne, S. (2011) Sphingosine-1-phosphate signalling in cancer. ***Biochemical Society Transactions***. Submitted.

Lim, K. G., Gray, A. I., Pyne, S., Pyne, N. J. (2011) Resveratrol dimers are novel sphingosine kinase 1 inhibitors and affect sphingosine kinase 1 expression and cancer cell growth and survival. ***Carcinogenesis***. Submitted

Abstract

There is a critical need to develop novel anticancer therapeutics. Accumulating evidence has demonstrated that sphingosine kinase (SK) is a promising target for the treatment of cancer. There are two SK isoforms, termed SK1 and SK2. SK phosphorylates sphingosine to form sphingosine-1-phosphate which drives cancer cell proliferation and migration. Therefore, the aims of this project were to discover hit compounds that can be developed into effective chemical tools and/or anticancer drug leads targeting SK. Compounds from both synthetic and natural origins were screened using SK enzymatic assays. Natural compounds were isolated using bioassay-guided fractionation and structural elucidation was achieved using nuclear magnetic resonance and mass spectrometry. Cell-based assays were employed to assess cellular activity of these inhibitors. Cell growth was determined using [^{3}H]-thymidine incorporation assays. Immunoprecipitation was used to study SK1 oligomerisation whereas fluorescence microscopy was used to examine the effects of SK inhibitors on actin distribution. Three key findings emerged from these studies. First, SK1 inhibitor kinetic characterisation with sphingosine revealed that (*S*)-FTY720 vinylphosphonate is a novel uncompetitive inhibitor whereas FTY720 and SKi (2-(*p*-Hydroxyanilino)-4-(*p*-chlorophenyl)thiazole) are competitive and mixed inhibitors respectively. These inhibitors also inhibited proliferation, induced proteasomal degradation of SK1 and apoptosis of cancer cells. Over-expression of an inactive SK1 mutant inhibited the catalytic activity of wild type SK1. Together with the discovery of two activators for SK1, a model was proposed in which SK1 contains allosteric site(s) that auto-inhibits catalytic activity. Second, (*R*)-FTY720-OMe was discovered as a selective SK2 inhibitor, which also inhibited MCF-7 cell growth and S1P-induced actin rearrangement, demonstrating SK2 as a pro-survival and migratory protein. Third, novel SK inhibiting scaffolds including resveratrol were identified from a plant hit. Balanocarpol (a resveratrol dimer) might act like a chelator to inhibit multiple SK1 molecules, giving new insights into diversity-oriented biosynthesis of resveratrol oligomers. Together, these findings provide strong impetus for the development of inhibitors targeting SK1 and/or SK2 as effective anticancer agents.

Chapter 1 General Introduction

Cancer is a leading cause of deaths worldwide. Despite advances in diagnosis and treatment, recurrence and resistance of the disease are still common. Therefore, research into new therapeutic targets and development of novel compounds with improved selectivity are essential. A key discovery is that sphingosine kinase (SK) and its enzymatic product sphingosine-1-phosphate (S1P) contribute to cancer growth and progression. SK inhibitors have been shown to be effective in retarding tumour progression thus offering hope for novel treatments in cancer. Yet, selective SK1 or SK2 inhibitors are lacking in the field. The mechanisms of action of these inhibitors in retarding cancer cell growth are also not completely understood. Besides, natural SK inhibitors have been isolated from bacteria and fungi. However, SK inhibitors from plants have not been discovered.

Natural products are one of the best sources for drug discovery because of their vast diversity. Many contemporary medicines have been developed through research on metabolites produced by plants, animals and microorganisms. For thousands of years, natural products have been widely used in traditional medicines to treat various ailments. Today, natural products still play a major role in drug discovery especially in anticancer drug development. Particularly, research in medicinal plants has yielded several important anticancer compounds including taxanes and vinca alkaloids which are widely used clinically. Though highly efficacious, most anticancer drugs invariably cause serious side effects which limit their use. Therefore, research into cancer cell biology is equally important to offer a better understanding of the disease and to reveal novel targets for drug treatment.

This introduction will give some background information on cancer followed by discussions on SK/S1P signalling pathway and its role in cancer. An overview of S1P receptors ligands and SK inhibitors will also be presented. Finally, the introduction will include a review of plants in drug discovery and the aims of this project.

1.1 Cancer

Worldwide, around one in eight deaths is due to cancer. Cancer caused more than 7.6 million deaths in 2007 alone (Garcia *et al*., 2007). In the developed countries, prostate and breast cancers were the most prevalent cancers for males and females, respectively. The leading cause of cancer deaths, however, was lung cancers for men and breast cancers for women. Interestingly, it has been estimated that about half of all new cancer cases and deaths can be prevented (Garcia *et al*., 2007). Early diagnosis and life-style measures (e.g. healthy eating and avoiding tobacco use) play important roles in the prevention and treatment of cancers. It is hoped that with the advancement in cancer diagnosis/treatments, the outlook for patients will be improved. However, no cures have been found and cancer remains one of the most challenging diseases.

A multitude of factors contribute to cancer initiation and progression. Cancer is caused by uncontrolled cell proliferation and spread of diseased cells, which take several steps to develop. Both external (e.g. radiation, chemicals and microorganisms) and internal factors (e.g. genetic predisposition, hormonal and metabolic disorders) contribute to cancer development (Garcia *et al*., 2007). The abilities acquired by cancer cells in the process of tumourigenesis have been extensively reviewed by Hanahan and Weinberg (2000). Accordingly, emerging cancer cells must acquire certain functional capabilities (denoted as hallmarks) to breach the boundaries that restrict normal cells from overgrowing. Such capabilities include self-sustained proliferation, insensitivity to growth suppressors, resistance to apoptosis, an immortalised phenotype, self-inducing angiogenesis and metastasis. After a decade, the authors have revisited the hallmarks and proposed two additional capabilities of cancers; these are, altered energy metabolism and evasion of immune destruction (Hanahan and Weinberg, 2011). Underlying these hallmarks are two important enabling properties: genomic instability and tumour-promoting inflammation which propagate or expedite the transformation of normal cells into cancers.

Involvement of oncogenes, tumour suppressor genes and other genetic factors suggest that cancer is a genetic disease; the cause of which can always be traced to one or more aberrant genes in the genome (Albertson and Pinkel, 2003). Cancer can be considered as a proteomic disease because the phenotype of different cancers can be similar even though the underlying genetic aberrations are different. Since the disease manifests itself at the protein level where molecular signalling occurs, it is possible to target a particular protein pathway in the treatment of many cancers (Jones, 2008). Drugs that target signalling pathways rather than genetic aberration alone can also provide economic benefits in drug discovery because they may act on the same target for different cancers, thereby expanding their use (Jones, 2008). However, certain cancers do have a strong correlation between single genetic mutation and an aberrant phenotype. Personalized therapy or specific treatment regimens targeted at a particular genetic mutation still play a major role in reducing toxicity and improving efficacy. Emphasis is now placed on employing several drugs that target multiple signalling pathways in order to prevent relapse and adaptive resistance in cancers (Hanahan and Weinberg, 2011).

There has been a huge amount of effort to improve the understanding of the pathology and treatment of cancers. Better insights into cancer cell biology enable more drug targets to be identified and a large number of therapeutic compounds have been developed. However, not all identified targets are suitable for therapeutic targeting. A detailed understanding of the targets in relation to cancer development is essential. The study of these putative targets has led to the use of combination cancer chemotherapy which has largely transformed the outlook for cancer patients. Conventional anticancer drugs such as antimetabolites, DNA alkylators, microtubules inhibitors, DNA topoisomerase inhibitors and hormone antagonists are used routinely for cancer treatment because they are more economic than newer agents including small molecule inhibitors and biologics (monoclonal antibodies). Acting through different mechanisms, anticancer drugs can be classified as cell cycle specific or non cycle specific. Generally, cell cycle specific drugs either inhibit DNA synthesis (S-phase specific) e.g. anti-metabolites or inhibit mitosis (M-phase specific) e.g. microtubule inhibitors. Some cell

cycle specific drugs such as anthracycline antibiotics can act on multiple phases of the cell cycle. Non cell-cycle specific drugs include DNA alkylators and DNA topoisomerase inhibitors. Such classification is useful for the design of combined drug regimen where the drug is administered when the cells are most sensitive to it (Shapiro and Harper, 1999). Two prominent proteins implicated in deregulated cell cycle control are telomerase and p53 which regulate cell senescence and cellular arrests/apoptosis, respectively. Novel therapeutic strategies may employ molecules that inhibit telomerase (hence reducing replicative potential) or MDM2, an intracellular protein which induces proteasomal degradation of the tumour suppressor protein, p53 (Hartwell and Kastan, 1994; Kubbutat *et al*., 1997).

Anticancer drugs act on different cellular targets (Fig. 1.1) to inhibit cell growth or to induce apoptosis. Antimetabolites such as arabinosides and 5-flurouracil are purine and pyrimidine analogues respectively whereas folate antagonist inhibits dihydrofolate reductase. Together, these drugs inhibit DNA synthesis. Camptothecins (irinotecan, topotecan) and etoposide inhibit DNA topoisomerase I and II respectively. Inhibition of DNA topoisomerase prevents DNA religation and leads to trapping of DNA cleavage complexes which can induce apoptosis (Burden and Osheroff, 1998; Pommier *et al*., 1998). Platinum compounds, such as cisplatin, alkylate DNA leading to activation of p53 and induction of apoptosis (Siddik, 2003). Taxanes and vinca alkaloids promote and inhibit tubulin polymerisation respectively resulting in cell cycle arrest at M phase (Rowinsky *et al*., 1990). It is worth noting that newer agents may be more selective as they have been rationally designed to target specific receptor or kinase in cancer cell. For example, gefitinib binds to the adenosine 5'-triphosphate (ATP)-binding site on epidermal growth factor receptor, blocking ligand-induced activation of tyrosine kinase activity (Ciardiello *et al*., 2000). Imatinib inhibits the kinase activity of BCR-ABL, an intracellular kinase which is implicated in pathogenesis of chronic myelogenous leukaemia (Ren, 2005). Alternatively, drugs can act directly on receptors to block the access of endogenous ligands. For example, tamoxifen is an oestrogen receptor antagonist used in breast cancer. Trastuzumab inhibits the ectodomain of human

epidermal growth factor receptor 2 (HER2) and induces immune cell-mediated cytotoxicity, which results in blockade of growth factor receptor signal transduction (Molina *et al*., 2000). Together, by targeting multiple targets essential for cell growth and survival, anticancer drugs retard tumour progression.

Even though cancer chemotherapy is effective, serious side effects are inevitable because most of the drugs act indiscriminately against all cells in the body. The selectivity only resides in targeting cells that grow fast. Therefore, cancer cells can be targeted but this also exposes normal cells to cytotoxicity, especially those with a high growth rate. Therapeutic strategies that selectively target an aberrant gene or its product implicated in cancers are therefore valuable. It is now known that small molecule inhibitors can offer better selectivity as more intracellular oncogenic targets are identified. Targeted therapeutics that focus on developing small molecule inhibitors also have higher success rates in cancer drug development (Walker and Newell, 2008). Interestingly, this study also shows that drugs are less likely to succeed into phase III from phase II clinical trials, highlighting the importance for novel drugs to act on validated targets involved in established oncogenic pathway. Of note, chemotherapy is only part of the treatment for cancer because surgery, radiotherapy and complementary treatments are equally important in the treatment of cancer depending on the types and grades of the tumours. With the advent of new developments in cancer therapy, it is hoped that cancer patients will receive better treatment and enjoy a higher quality of life.

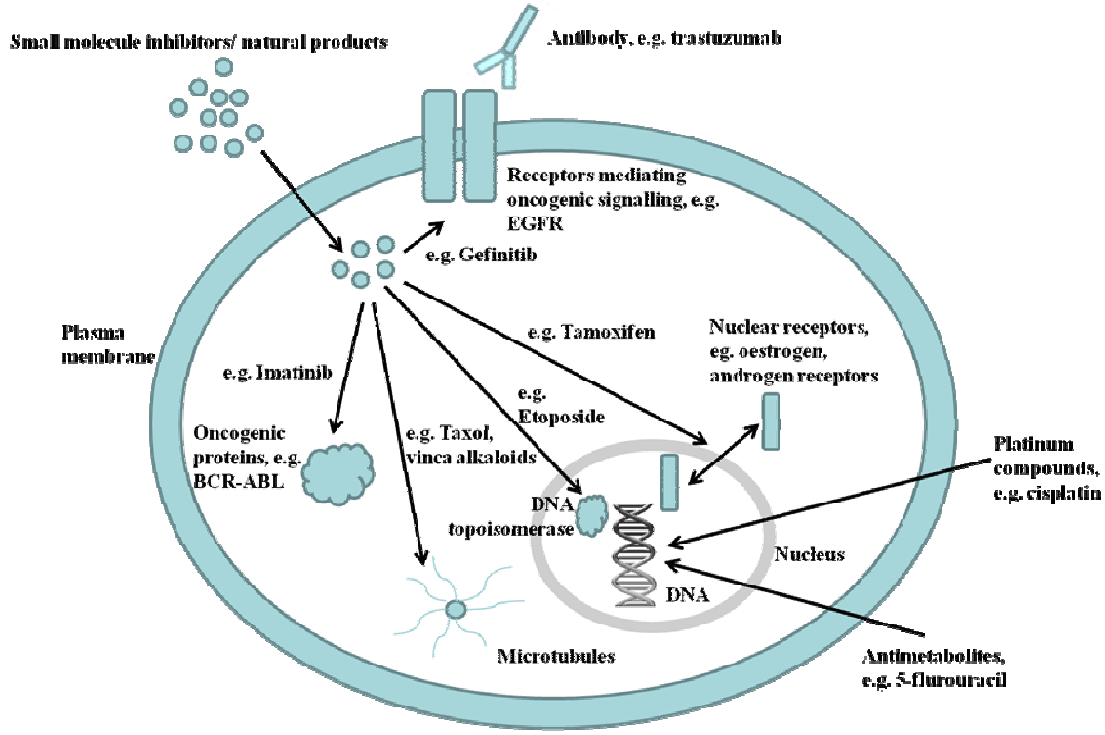


Figure 1.1 Cellular targets of current anticancer drugs. Most anticancer drugs act on intracellular targets and they are well represented by small molecule inhibitors (e.g. imatinib, gefitinib, 5-flurouracil and tamoxifen) or compounds derived from natural products (e.g. taxol, vinca alkaloids and etoposide). Newer agents such as biologics are antibodies that target extracellular receptors (e.g. trastuzumab). Representative drugs and targets are presented. See text for references.

1.2 Sphingosine kinase and sphingosine-1-phosphate receptors

Apart from maintaining cell membrane integrity, it has long been proposed that sphingolipids may play important roles in the regulation of many cellular processes including cell growth and differentiation (Hannun and Bell, 1989). Indeed, the production of a bioactive metabolite, S1P can be induced by growth factors such as platelet-derived growth factor (PDGF) to act as a second messenger that promotes cell proliferation (Olivera and Spiegel, 1993) and prevents apoptosis (Cuvillier *et al*., 1996). It is well established that S1P is an endogeneous ligand for five G protein-coupled receptors (GPCR) that mediate a variety of functions including cell motility (Okamoto *et al*., 2000). The mitogenic and anti-apoptotic effects of S1P are important in the context of cancer progression since the hallmarks of cancer include uncontrolled proliferation and evasion of apoptosis (Hanahan and Weinberg, 2011). Moreover, there is now substantial evidence that S1P is implicated in cancer progression (Pyne and Pyne, 2010). Therefore, the following section will provide an overview of the discovery and functional studies of SK and S1P receptors.

1.2.1 Sphingosine kinase

SK is a ubiquitous enzyme which is expressed in many tissues and cell types. Similar to other kinases, SK phosphorylates its substrate (sphingosine) to produce a bioactive signalling molecule, S1P in an ATP dependent manner. Apart from independently regulating cellular functions, sphingosine is produced by ceramidase from ceramide, a messenger molecule derived from sphingomyelin by the action of sphingomyelinase. *De novo* synthesis at the endoplasmic reticulum (ER) can also produce ceramide which leads to the formation of S1P (Fig. 1.2). The catabolism of S1P can be modulated by different enzymes such as S1P lyase, S1P phosphatases and the non-specific lipid phosphate phosphatases (Fig. 1.2). These enzymes have been shown to be important in the regulation of the S1P signalling pathway since the phosphatases are also involved in converting S1P back to its precursors which are also bioactive.

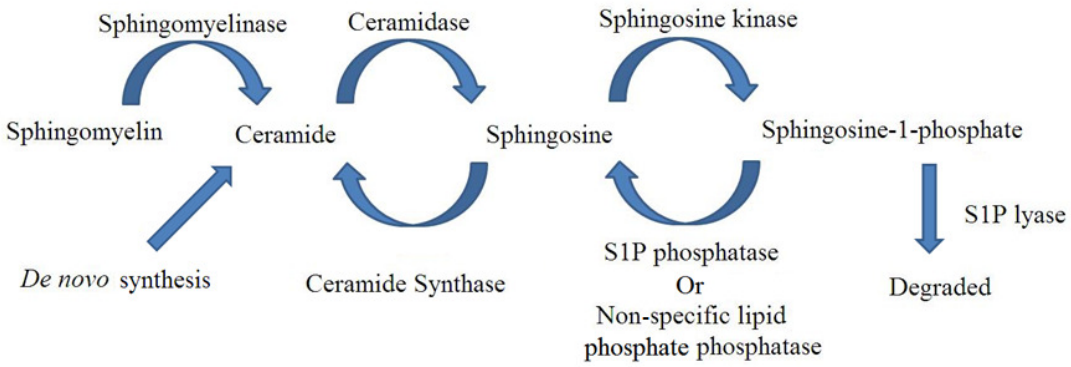


Figure 1.2 Metabolisms of important sphingolipids. *De novo* synthesis or degradation of sphingomyelin produces ceramide which is the precursor of sphingosine. Sphingosine is phosphorylated by sphingosine kinase to form sphingosine-1-phosphate (S1P) which can be recycled back to sphingosine by enzymes like S1P phosphatases. S1P can also be irreversibly cleaved by S1P lyase (Pyne and Pyne, 2000).

Several isoforms of SK have been found in different organisms including yeasts, worms, mice, plants and humans with two distinct isoforms exist in mammalian cells: SK1 and SK2. Murine SK1 (mSK1) was first purified and characterised from rat kidney with an apparent molecular mass of 49 kDa (Olivera *et al*., 1998). Subsequently, human SK1 (hSK1) which has a high cDNA sequence homology and shows approximately 85% similarity in amino acid sequence identity with mice, has also been purified and cloned (Nava *et al*., 2000b; Melendez *et al*., 2000; Pitson *et al*., 2000a). Pitson and colleagues (2000a) found that amino acid residues 16-153 of hSK1 show high sequence similarity with the catalytic domain of diacylglycerol kinase (DGK). DGK catalyses the formation of phosphatidic acid (PA) from diacylglycerol and has at least ten distinct isoforms (Raben and Wattenberg, 2009). Northern analysis has revealed wide hSK1 expression in adult brain, lung, heart, liver, kidney, thymus and skeletal muscles (Nava *et al*., 2000b; Melendez *et al*., 2000) suggesting that SK1 has important regulatory functions not only during developmental processes but also in maintaining normal functions of vital organs. Both human and murine SK1 exhibit typical Michaelis-Menten kinetics with a broad pH optimum (6.8-7.4) (Olivera *et al*., 1998; Pitson *et al*., 2000a). The successful cloning and

characterisation of hSK1 enables the use of recombinant enzyme to screen for potential inhibitors and to investigate intracellular interaction of SK1 with other proteins.

Existence of multiple isoforms of SK has initially been discovered in human platelets (Banno *et al*., 1998). Two splice variants of murine SK1 (named mSK1a and mSK1b) have also been cloned and characterised (Kohama *et al*., 1998). Despite a difference of a few amino acid residues at the N terminus, mSK1b exhibits lower activity than mSK1a. Moreover, mSK1b has a lower stability due to a higher tendency to associate with cell membrane (Kihara *et al*., 2006). In contrast, mSK1a which is more widely expressed also has higher resistance to proteasomal degradation and thus higher stability (Kihara *et al*., 2006). Three spliced variants of hSK1 with similar enzymatic activity have also been cloned (Billich *et al*., 2003). These variants have 384, 398 and 470 amino acids and are denoted as SK1a, SK1a+14 and SK1b respectively (Loveridge *et al*., 2010; Fig. 1.3). These splice variants of hSK1 have different stability and localisation. For example, SK1a+14 shows higher tendency to associate with cell membrane whereas SK1a is predominantly present in the cytoplasm (Venkataraman *et al*., 2006). Further studies on these splice variants in cancer cell lines have revealed that they have different sensitivity to proteasomal degradation induced by SK1 inhibitor (see section 1.3).

Using the mSK1 cDNA sequence together with basic local alignment search tool (BLAST) searches, SK2 has been cloned as an isoform with distinct biochemical and enzymatic properties from SK1 (Liu *et al*., 2000a). SK2 has at least two splice variants (termed SK2-S and SK2-L) which differ in their N-terminal regions by an extension of 36 amino acids in SK2-L (Billich *et al*., 2003; Fig. 1.3). During development, mSK1 is mainly expressed in the lung and spleen with highest expression on day 7 of embryogenesis whereas mSK2 is primarily expressed in liver and heart with peak expression on day 17 of embryogenesis (Liu *et al*., 2000a). Both SK1 and SK2 catalyse the formation of S1P from D-*erythro*-sphingosine yet they differ remarkably in their kinetic properties and subcellular localisations (Liu *et al*., 2000a; Igarashi *et al*., 2003). First, the activity of SK2 can be stimulated by a reaction buffer containing high salt

concentration e.g. 400mM KCl, whereas the activity of SK1 is largely inhibited under this condition. Second, presenting substrate by incorporating sphingosine in Triton X-100 enhances SK1 activity while SK2 activity is inhibited. In addition, these isoforms differ in their substrate specificity. For example, SK1 phosphorylates both D,L-*erythro*-sphingosine and D,L-*erythro*-dihydrosphingosine but not D,L-*threo*-sphingosine, D,L-*threo*-dihydrosphingosine (DHS), N,N-dimethylsphingosine (DMS) and N,N,N-trimethylsphingosine (TMS). In fact, D,L-*threo*-sphingosine, DHS, DMS and TMS are SK1 competitive inhibitors whereas DMS is the most potent compound among these inhibitors (Buehrer and Bell, 1992, Yatomi *et al*., 1996, Edsall *et al*., 1998). Additionally, D-*erythro*-sphingosine is a much better substrate for SK1 than L-*erythro*-sphingosine and D,L-*erythro*-dihydrosphingosine which are phosphorylated to a lesser extent (Buehrer and Bell, 1992). In contrast, SK2 has a broader substrate specificity than SK1 since DHS and phytosphingosine can also be phosphorylated by SK2 (Liu *et al*., 2000a). Moreover, DMS inhibits SK1 competitively whereas SK2 is inhibited by DMS non-competitively (Liu *et al*., 2000a). The subcellular localisation of ectopic expression of SK1 and SK2 differs among cell types. For example, SK1 and SK2 are localised in the cytosol of HEK 293 cells but SK2 is mainly a nuclear protein in HeLa cells due to the presence of a nuclear localization signal (NLS) (Igarashi *et al*., 2003). On the contrary, endogenous SK2 has been found mainly at the plasma membrane of HEK 293 cells (Hait *et al*., 2005). SK2-L is a splice variant of SK2 and is only expressed in human but not mouse. Under serum-deprived condition, its expression is up-regulated and accumulated in the nucleus leading to inhibition of DNA synthesis depending on cell type (Okada *et al*., 2005). Knockout of either SK1 or SK2 in mice does not generate an obvious phenotype suggesting functional redundancy between SK1 and SK2; however, SK1 and SK2 double-knockout are embryonically lethal (Mizugishi *et al*., 2005). The double-knockout mice have severe internal bleeding throughout the body and exhibit cranial neural tube defect, suggesting that SK/S1P signalling is critical for vascular and neural development (Mizugishi *et al*., 2005).

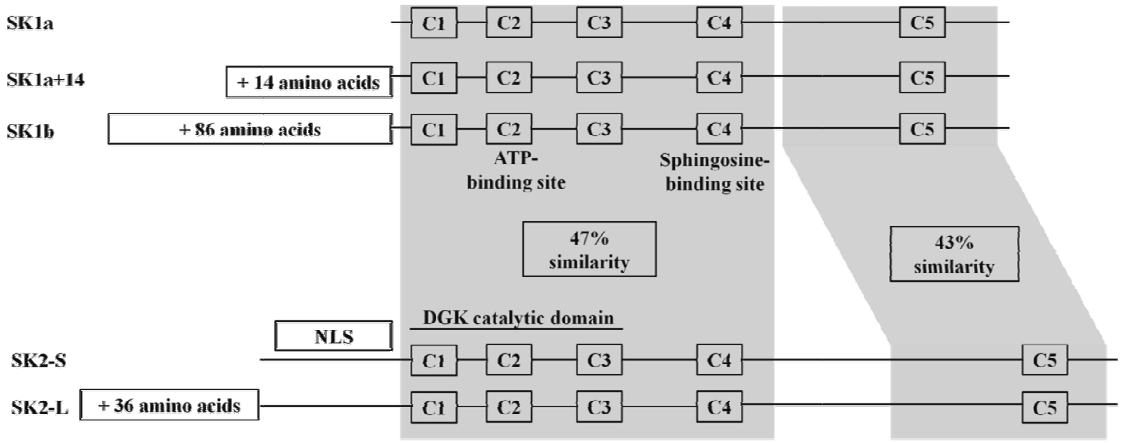


Figure 1.3 Sphingosine kinase isoforms and splice variants. There are three splice variants for SK1: SK1a (GenBank™ number: NM_001142601), SK1a +14 (GenBank™ number: NM_021972) and SK1b (GenBank™ number: NM_182965), and two splice variants for SK2: SK2-S (GenBank™ number: AF245447) and SK2-L (GenBank™ number: NM_020126). A third SK2 splice variant (GenBank™ number: EF107108) may also exist (Alemany *et al*., 2007). More work is needed to validate the existence of this variant. Evolutionarily conserved domains are denoted by C1-C5. The shaded regions indicate similarity in amino acid sequence between these enzyme isoforms. The C1-C3 regions in SK1/SK2 are homologous to the catalytic domain of DGK. The domain containing the binding sites for ATP (C2) and sphingosine (C4) are also shown. The nuclear localisation signal (NLS) of SK2 is located at the N-terminus. Adapted from Taha *et al*., (2006a) and Pitson, (2011).

Despite differences in the biochemical and kinetic properties of these enzyme isoforms, both SK1 and SK2 display significant homology and share five highly conserved domains (C1-C5) in which C1-C3 are highly homologous to the catalytic domain of related lipid kinases including DGK (Kohama *et al*., 1998; Liu *et al*., 2000a; Fig. 1.3). SK2 has additional polypeptide regions in the middle and in the N-terminus which has been shown to contain the NLS responsible for its nuclear localisation (Igarashi *et al*., 2003). A highly conserved ATP binding domain ($SGDGx_{17-21}K$) located at the C2 domain has also been identified (Pitson *et al*., 2002). Mutation of Gly^{82} to Asp in SK1 (Pitson *et al*., 2000b) or Gly^{213} to Glu in SK2 (Maceyka *et al*., 2005) generates catalytically deficient enzymes. The sphingosine binding site is located at the C4 domain which is conserved only among sphingosine kinases and not other closely related lipid

kinases such as DGK. Mutation of Asp^{177} to Asn in mSK1 led to ~10-fold increase in the K_m for sphingosine confirming an essential role for this residue in the binding of sphingosine (Yokota *et al*., 2004). Together, site-directed mutagenesis has revealed important amino acid residues mediating substrate recognition by SK1 and SK2.

SK1 can be activated by extracellular agonists and regulated by intracellular protein-protein and/or lipid-protein interaction (Wattenberg *et al*., 2006; Alemany *et al*., 2007). There are various agonists such as growth factors which stimulate S1P production via activation of SK1. For instance, PDGF and serum activate SK1 leading to ~1.5 fold increase of its catalytic activity (Olivera and Spiegel, 1993). Pyne and colleagues (1996) also demonstrated that PDGF induces S1P formation leading to extracellular signal-regulated kinases 1/2 (ERK1/2) activation in airway smooth muscle cells. Other prominent examples include tumour necrosis factor (TNF-α) which not only activates SK1 but also promotes binding of SK1 to TNF receptor-associated factor 2 (TRAF2) preventing apoptosis through activation of nuclear factor-κB (NF-κB) (Xia *et al*., 1999; Xia *et al*., 2002). In addition, others have demonstrated that activation of SK1 is dependent on its translocation to the plasma membrane after phosphorylation by intracellular proteins including protein kinase C (PKC) and ERK1/2 (Johnson *et al*., 2002; Pitson *et al*., 2003). ERK1/2-dependent phosphorylation of Ser^{225} is necessary to increase SK1 catalytic activity and induce its translocation. Interestingly, translocation of SK1 is independent of its catalytic activity because dominant-negative SK1 (G82D mutant) can be detected at the plasma membrane after phorbol 12-myristate 13-acetate (PMA) treatment (Johnson *et al*., 2002). In contrast to transcriptional up-regulation of SK1 activity by certain agonists such as oestrogen (Sukocheva *et al*., 2003), post-translational (phosphorylation-dependent) activation of SK1 is transient, due to rapid dephosphorylation by protein phosphatase 2A (Barr *et al*., 2008). In addition, SK1 has been shown to be a downstream effector for PA, an acidic phospholipid that enhances SK1 activity and association with the Golgi (Delon *et al*., 2004). Indeed, retention of translocated SK1 at the plasma membrane is facilitated by interaction with other acidic phospholipids such as phosphatidylserine (PS) (Stahelin, *et al*. 2005).

SK2 can also be regulated by extracellular agonists and intracellular effectors. One major finding is that epidermal growth factor (EGF) not only stimulates SK1 but also activates SK2 in HEK 293 cells. However, EGF only increases catalytic activity of SK2 but not its localisation (Hait *et al.*, 2005). Similarly, cross-linking of FcεRI, a high affinity IgE receptor activates both SK1 and SK2 in a Src tyrosine kinase, Fyn-dependent manner in mast cells (Olivera *et al.*, 2006). This suggests that activators of SK1 may also stimulate SK2. Subsequently, SK2 over-expressed in HEK 293 cells has been shown to be activated by both PMA and EGF leading to ERK1/2-dependent phosphorylation of SK2 at Ser^{351} and Thr^{578} in a manner analogous to SK1 activation. Phosphorylation of SK2 at these residues is also essential for EGF-induced cell migration in MDA-MB-453 cells (Hait *et al.*, 2007). PMA can also induce a protein kinase D (PKD)-dependent phosphorylation of Ser^{419} and Ser^{421} at the nuclear export signal (NES) of SK2 leading to its translocation from the nucleus to the cytoplasm (Ding *et al.*, 2007). Therefore, depending on cell type, it appears that PMA not only activates PKD leading to phosphorylation and export of SK2 from the nucleus, but also activates ERK1/2 leading to activation of SK2 in the cytoplasm/plasma membrane.

Calcium is known to regulate the activity of SK. Pitson and colleagues (2000a) identified three calcium/calmodulin binding motifs in hSK1, suggesting that SK acts as a downstream effector of Ca^{2+}. Indeed, Ca^{2+} mobilisation from intracellular stores upon GPCR activation induces translocation of SK1 to the plasma membrane in a calmodulin-dependent manner without stimulating SK1 activity (Young *et al.*, 2003). However, SK can also act as an upstream mediator of Ca^{2+} signalling. For example, activation of muscarinic receptors stimulates SK and increased intracellular S1P levels, mediating Ca^{2+} release from intracellular stores (Meyer zu Heringdorf *et al.*, 1998). Regulation by Ca^{2+} has recently been found to involve the calcium- and integrin-binding protein (CIB1) which is a mediator for SK1 translocation to the plasma membrane following activation by agonists such as PMA (Jarman *et al.*, 2010). High sequence homology between SK1 and SK2 suggests that these enzyme isoforms may be regulated in a similar way.

Using yeast two-hybrid system, several intracellular proteins which interact with SK1 have been identified. These SK1-interacting proteins either inhibit or activate SK1 and mediate its subcellular localisation. For example, a protein related to the family of protein kinase A anchoring protein (AKAP) has been co-immunoprecipitated with SK1 and identified as a sphingosine kinase interacting protein (SKIP) (Lacaná *et al*., 2002). Over-expression of SKIP reduced SK1-dependent ERK1/2 activation and anti-apoptotic action. Platelet endothelial cell adhesion molecule 1 (PECAM-1) has also been shown to associate with SK1 leading to a reduction in SK1 activity (Fukuda *et al*., 2004). In addition, both truncated C-terminal aminoacylase 1 and the full length protein inhibit SK1 catalytic activity but the full length protein potentiates SK1-dependent pro-growth and anti-apoptotic effects and mediates SK1 redistribution into subcellular compartments (Maceyka *et al*., 2004). One member of the four and one-half LIM protein (FHL) family, FHL2 also interacts with SK1 and inhibits its activity in cardiomyocyte (Sun *et al*., 2006). SK1 is a substrate for the chaperonin containing t-complex polypeptide (CCT), also known as TCP-1 ring complex (TRiC), which folds SK1 into its mature form thereby regulating SK1 levels and activity in cells (Zebol *et al*., 2009). Despite the growing number of reported SK1-interacting proteins, detailed mechanisms of how these proteins activate or inhibit SK1 are not clear. Particularly, whether inhibition or activation of SK1 involves a conserved domain or an allosteric site on SK1 has not been demonstrated.

The membrane localization of SK1 has been linked to the autocrine and paracrine signalling of S1P (Alvarez *et al*., 2007). Indeed, there is substantial evidence that localisation of SK1/SK2 to different subcellular compartments dictates S1P signalling in cells (Wattenberg *et al*., 2006). For example, the oncogenic property of SK1 has been linked to its plasma membrane association rather than enhanced catalytic activity *per se* (Pitson *et al*., 2005). Interestingly, full length SK1 fused with an NLS enters the nucleus and inhibits DNA synthesis (Igarashi *et al*., 2003). Similarly, targeting SK1 to the ER leads to apoptosis (Maceyka *et al*., 2005) suggesting localisation of SK may be important for cell fate. More recently, the pro-survival role of SK1 has been linked to its

translocation to plasma membrane lipid raft microdomains (where sphingosine resides) upon agonist stimulation whereas the pro-migratory role of SK1 is linked to its localisation at membrane fractions containing actin (Hengst *et al.*, 2009). S1P formed intracellularly can also be exported outside the cells to act on five different cognate receptors (Fig. 1.4). Therefore, a wide array of biological functions can be regulated by the SK/S1P signalling pathway.

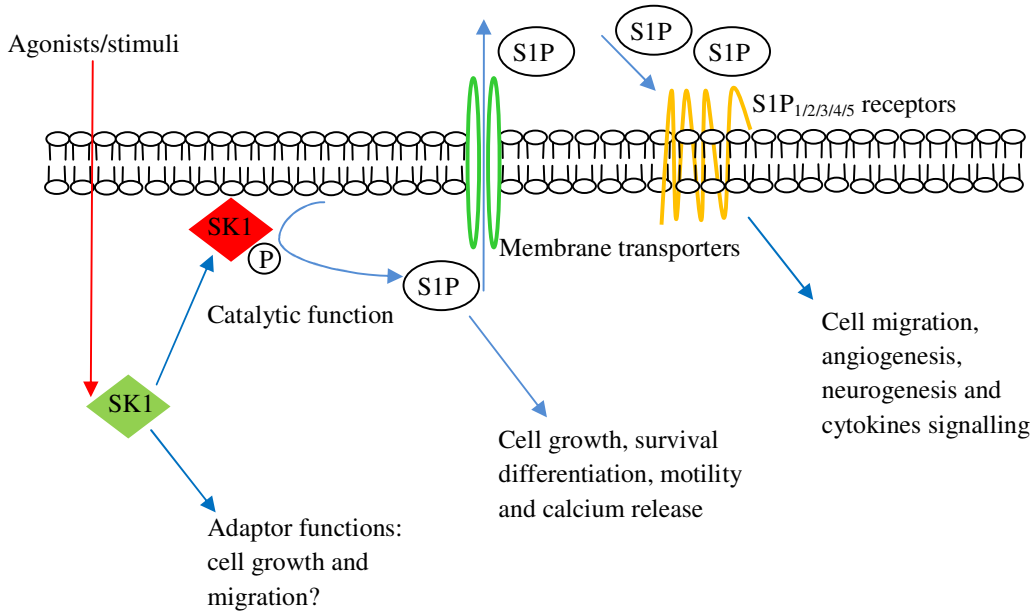


Figure 1.4 SK1/S1P signalling in mammalian cells. Activation of SK1 leads to its translocation to the plasma membrane to catalyse the formation of S1P which either acts as a second messenger intracellularly or exerts its effects extracellularly through binding on five different S1P receptors. These receptors are differentially regulated by distinct G proteins which mediates different cellular processes (Pyne and Pyne, 2000).

1.2.2 Sphingosine-1-phosphate receptors

Acting as a first messenger, S1P binds to its extracellular receptors to elicit various cellular responses. S1P is an endogenous ligand for five heptamembrane-spanning GPCRs ($S1P_{1-5}$). These receptors regulate diverse biological effects depending on the signal transduction pathways mediated by different G-proteins. Each type of receptor is

preferentially coupled to a subset of G proteins which have specific downstream effectors. Therefore, the effects of receptor-mediated S1P signalling depend on relative expression of S1P receptors and cell type. The following will describe the discovery and functional studies of each S1P receptor.

High content of S1P can be found in platelets (Yatomi *et al*., 1996). S1P is also a normal constituent in plasma and serum, mainly produced by erythrocytes (Takabe *et al*., 2008). This suggests that S1P has a functional role in vascular physiology. Indeed, $S1P_1$ as the first S1P receptor to be cloned has initially been identified as an inducible receptor during endothelial cell differentiation (Hla and Maciag, 1990). $S1P_1$ couples exclusively to $G_{i/o}$ which leads to activation of ERK1/2 (Lee *et al*., 1996). Subsequently, S1P has been confirmed as an endogenous ligand for $S1P_1$ and induces morphological changes of HEK 293 cells over-expressing $S1P_1$ through up-regulation of cadherin, which is mediated by activation of the small GTPase, Rho (Lee *et al*., 1998). However, $S1P_1$ mediates cortical actin assembly essential for lamellipodia formation through Rac activation in vascular endothelial cells, which is dependent on the phosphorylation of $S1P_1$ by Akt (Lee *et al*., 1999; Lee *et al*., 2001). Therefore, it appears that the effect on cell motility in response to $S1P_1$ activation can be cell-type specific, which may also be dependent on endogenous expression of other S1P receptors. Direct involvement of $S1P_1$ in vascular maturation has been verified with the generation of $S1P_1^{-/-}$ mice which suffer intrauterine death due to a deficiency of vascular smooth muscle cells and excessive intra-embryonic bleeding (Liu *et al*., 2000b). Apart from enhancing motility in vascular endothelial cells, cell growth is also stimulated by S1P through a $S1P_1$-dependent mechanism thereby confirming a role for $S1P_1$ in angiogenesis (Wang *et al*., 1999a). Besides, $S1P_1$ is essential for the regulation of lymphocytes egress from lymphoid organs; $S1P_1$ down-regulation is associated with the therapeutic efficacy of the immunosuppressant, FTY720 (Matloubian *et al*., 2004). It is now licensed for the treatment of relapsing remitting multiple sclerosis (Brinkmann *et al*., 2010).

In contrast to $S1P_1$, $S1P_2$ couples to multiple G-proteins including $G_{i/o}$, $G_{12/13}$ and G_q to mediate various cellular functions (Windh *et al*., 1999; Okamoto *et al*., 2000). For example, ligation of $S1P_2$ leads to activation of c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK), Ras-dependent ERK1/2 activation and promotion of stress fibre formation through Rho-dependent pathway (Gonda *et al*., 1999). $S1P_2$ also stimulates neurite retraction in PC12 cells and induces cell rounding in HEK 293 cells over-expressing $S1P_2$ (Van Brocklyn *et al*., 1999). Indeed, cell migration and membrane ruffling are inhibited by $S1P_2$ via inhibition of Rac which stimulates Rho-dependent stress fibre formation and focal adhesion (Okamoto *et al*., 2000). Knock-down of $S1P_2$ enhances PDGF and S1P-induced migration in mouse embryonic fibroblasts, as a result of enhanced Rac and p21-activated kinase 1 (PAK1) activation (Goparaju *et al*., 2005). *In vivo* functional studies indicate that $S1P_2$ participates in neuronal development but is not essential for survival since $S1P_2^{-/-}$ mice are born with no obvious physical defects (MacLennan *et al*., 2001, Ishii *et al*., 2002). However, $S1P_2$ plays an important role in maintaining normal auditory and vestibular functions and is responsible for hearing loss in $S1P_2^{-/-}$ mice (Kono *et al*., 2007).

Similar to $S1P_2$, $S1P_3$ also couples to $G_{i/o}$, $G_{12/13}$ and G_q to activate similar downstream effectors (Windh *et al*., 1999; Okamoto *et al*., 2000). Nevertheless, $S1P_3$ is better coupled to Ras-dependent activation of ERK1/2 than $S1P_2$, suggesting similar but distinct signalling characteristics between the two receptors (Okamoto *et al*., 1999). Moreover, in contrast with $S1P_2$, $S1P_3$ stimulates membrane ruffling and promotes cell migration through Rac activation (Sugimoto *et al*., 2003). $S1P_3$ is also not necessary for development since $S1P_3^{-/-}$ mice are phenotypically normal, suggesting that compensatory up-regulation of other S1P receptors may play a role in restoring the normal phenotype (Ishii *et al*., 2001). Though disruption of either $S1P_2$ or $S1P_3$ does not affect survival or development in single mutant mice, double knockout mice have decreased perinatal survival rate (Ishii *et al*., 2002). As expected, $S1P_1$, $S1P_2$, $S1P_3$ triple knockout mice suffer intrauterine death due to massive vascular defects (Kono *et al*., 2004) indicating

an essential role of $S1P_1$ in vascular maturation. $S1P_3$ also mediates regulation of heart rate; hence $S1P_3$ agonism is associated with bradycardia in mice (Sanna *et al*., 2004).

$S1P_4$ shares high sequence homology with $S1P_3$ and is mainly expressed in lymphoid and haematopoietic tissues implying a role in the regulation of immune function (Gräler *et al*., 1998). $S1P_4$ couples to $G_{i/o}$ and activates ERK1/2 and E twenty-six (ETS)-like transcription factor 1 (ELK1) (Van Brocklyn *et al*., 2000). $S1P_4$ may also couple to $G_{12/13}$ and activates Rho to induce stress fibre formation leading to CHO-K1 cell rounding (Gräler *et al*., 2003). However, cell migration may also be promoted by $S1P_4$ through a G_i-dependent activation of cell division control protein 42 (Cdc42) (Kohno *et al*., 2003). Therefore, it appears that $S1P_4$ regulation on cell motility may be influenced by endogenous expressions of other S1P receptor subtypes and is dependent on preferential coupling of the receptor to different subtypes of G proteins. Proliferation of T-lymphocytes is also regulated by $S1P_4$ which inhibits interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) cytokines secretion (Wang *et al*., 2005). Therefore, further investigation is warranted to understand the role of $S1P_4$ in the regulation of immune response.

The last S1P receptor to be cloned is $S1P_5$, primarily expressed in the brain and some peripheral tissues (Im *et al*., 2000; Niedernberg *et al*., 2002). $S1P_5$ couples to $G_{i/o}$ and $G_{12/13}$ but inhibits proliferation of CHO cells over-expressing $S1P_5$ and mediates tyrosine phosphatase-dependent inhibition of ERK1/2 activation (Malek *et al*., 2001). Inhibition of ERK1/2 activation may also be due to constitutive activation of the receptor (Niedernberg *et al*., 2003). It has been shown that natural killer cells express $S1P_5$ which regulates their migration to inflamed organs, suggesting important regulatory role in immune function (Walzer *et al*., 2007). More recently, $S1P_5$ has been shown to colocalise with SK1 and SK2 in the centrosomes, possibly regulating mitosis by acting as a guanine nucleotide exchange factor (Gillies *et al*., 2009). These findings suggest that additional research is warranted to characterise the functional roles of $S1P_5$, especially its expression and localisation in different cell types.

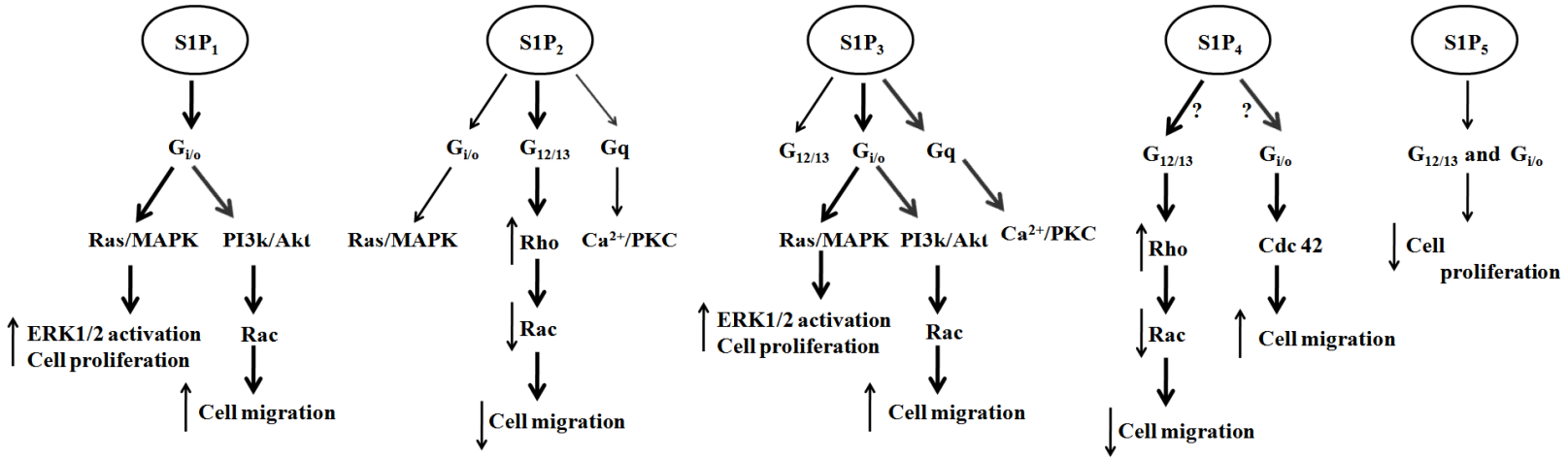


Figure 1.5 S1P receptor subtypes and their differential coupling to G proteins. $S1P_1$ preferentially couples to $G_{i/o}$ inducing Rac activation (to promote cell migration) and activating ERK1/2 (to promote cell growth). $S1P_2$ couples to multiple G proteins, especially $G_{12/13}$ to inhibit cell migration by activating Rho and inhibiting Rac. $S1P_3$ also activates Rac and promotes cell migration. Less is known about $S1P_4$ and $S1P_5$ but these receptors appear to regulate cell proliferation and migration. Of note, depending on signalling gains, different G proteins may be activated leading to diverse downstream responses (thicker arrows indicate predominant signalling pathways). See text for references.

Besides, S1P receptor activation and signal transduction have been shown to interact with other signalling pathways. Cross talk between $S1P_1$ and PDGF-β receptor was first shown in HEK 293 cells (Alderton *et al*., 2001). This finding indicates that tethering of PDGF-β to $S1P_1$ is important for more efficient growth factor receptor signalling, suggesting that other growth factor receptors might utilise tethering to GPCR as a common platform for growth factor signal transduction (Pyne and Pyne, 2002). S1P signalling may also interact with signalling of growth factors such as EGF, vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) and cytokines such as TNF-α and IL-12 as a result of receptor cross-talk and transactivation (Lebman and Spiegel, 2008). With such diverse and intricate interactions with other signalling pathways, extracellular S1P can therefore regulate a plethora of biological functions.

Despite a multitude of studies about S1P receptor-mediated signalling, some biological effects of S1P are mediated independently of extracellular receptors. Being a second messenger, S1P can also act intracellularly to regulate certain physiological functions such as calcium mobilization, mitogenesis and apoptosis suppression. It has been demonstrated that cross-linking of the Fc-gamma receptor 1 (FcγRI) induces intracellular calcium release which is independent of the phospholipase C (PLC)/ inositol triphosphate (IP_3) pathway but requires activation of phospholipase D (PLD) and SK (Melendez *et al*., 1998). This suggests that S1P can mobilize calcium through unknown intracellular targets. Besides, ceramide induces cell death whereas sphingosine also inhibits cell growth. A tipping of the balance by SK1 which produces pro-growth S1P from pro-apoptotic ceramide results in cellular proliferation. Therefore, a rheostat theory has been proposed to explain the differential actions of intracellular sphingolipids in cell growth and survival (Cuvillier *et al*., 1996).

The actions of S1P on cell growth and proliferation may be dependent on both receptor-mediated signalling and intracellular actions of S1P. For example, $S1P_2$ and $S1P_3$ have been shown to mediate part of the mitotic effect of S1P in HTC4 cells through

activation of ERK1/2 leading to up-regulation of c-Fos and c-Jun (An *et al*., 2000). Additionally, autocrine signalling (also referred as "inside-out" or sequential signalling) may operate. Upon agonist stimulation, intracellular S1P can act inside cells or extracellularly through its cognate receptors. By contrast, integrative signalling between growth factor receptor such as PDGF and S1P receptor can enhance co-migratory effect of S1P and PDGF (Waters *et al*., 2006). Not only S1P receptor-mediated effects are responsible for regulation of cell motility, an intracellular role for S1P may also be important. For example, utilising a photolysable (caged) S1P coupled with ultraviolet (UV) irradiation, intracellular S1P is increased leading to suppression of cell motility of MCF-7 and MDA-MB-231 cells, possibly through an effect mediated by Ca^{2+} mobilisation and fluctuation (Wang *et al*., 1999b).

1.2.3 Intracellular targets of sphingosine-1-phosphate

More recently, intracellular targets of S1P have been identified. S1P binds and inhibits histone deacetylases (HDACs) in the nucleus causing an increase in histones acetylation (Hait *et al*., 2009). The direct inhibitory effect of S1P on HDACs can be augmented by PMA which activates SK2 and enhances certain gene transcriptions such as the cyclin-dependent kinase (CDK) inhibitor *p21* and the proto-oncogene c-*fos*. This represents a novel role for S1P in epigenetic regulation of gene transcription (Hait *et al*., 2009). Additionally, TRAF2 has been identified as an intracellular target for S1P in the cytoplasm. S1P increases TRAF2-dependent polyubiquitination of RIP1, preventing receptor interacting protein 1 (RIP1) from mediating pro-apoptotic effects (Alvarez *et al*., 2010). Prohibitin 2 (PHB2) mainly localizes at the inner membrane of mitochondria. Strub and colleagues (2011) showed that SK2 in the mitochondria produces S1P which binds PHB2 and regulates mitochondrial assembly and respiration. S1P has also been linked to the pathogenesis of Alzheimer's disease by interacting with β-site APP cleaving enzyme-1 (BACE1) and enhancing BACE-1 activity, leading to increased amyloid-β peptide production (Takasugi *et al*., 2011). Taken together, these studies shed important light on intracellular S1P signalling in normal and pathological processes and

provide new insights into how S1P regulates cell survival and proliferation independent of its extracellular receptors. This may have important implications in drug discovery targeting S1P signalling. For example, limiting the intracellular bioavailability of S1P through SK1/2 inhibition can be as important as developing antagonists for S1P receptors.

1.3 Role of sphingosine kinase/sphingosine-1-phosphate in cancers

Due to their pivotal roles in cell proliferation and immune functions, deregulation of the SK/S1P signalling pathway has been linked to various hyperproliferative and autoimmune diseases including cancer, atherosclerosis and inflammation. Several functional roles of S1P are involved in cancer progression (Fig. 1.6). First, S1P is linked to cell proliferation, survival and prevention of cell death. Second, S1P regulates blood vessel formation in concert with other growth factors; therefore S1P can promote angiogenesis in tumours. Third, S1P mediates cell migration which is important in the context of cancer metastasis. Overall, the pleiotropic effects of S1P indicate that it promotes tumourigenesis by facilitating the attainment of one or more of the capabilities as described in the hallmarks of cancer (Hanahan and Weinberg, 2011). This section will provide an overview of the role of S1P in cancers with a focus on the most deadly disease in women, breast cancer.

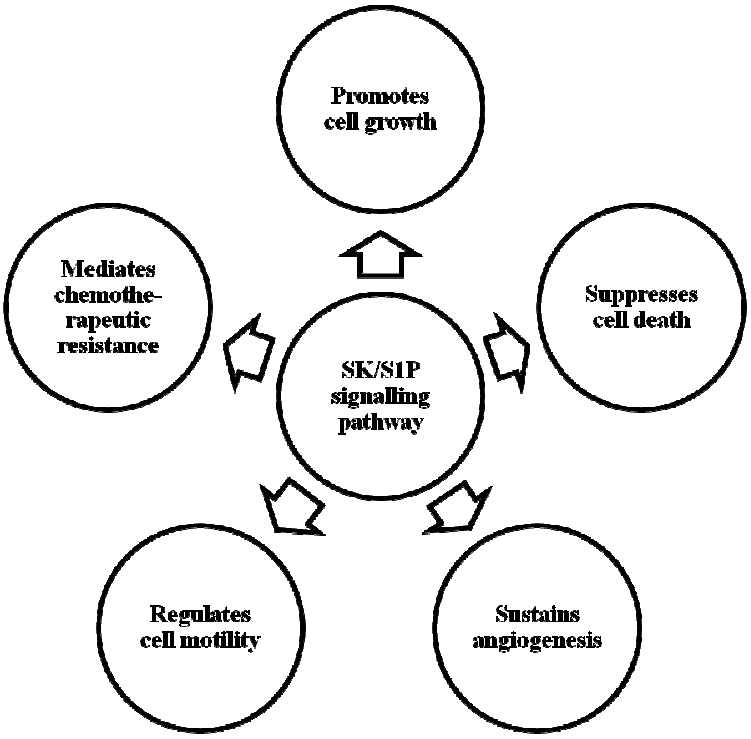


Figure 1.6 Role of SK/S1P signalling pathway in cancer pathogenesis. S1P promotes cell proliferation and suppresses programmed cell death. In addition, S1P is pro-angiogenic and regulates cell motility. Substantial evidence has also demonstrated that SK/S1P signalling pathway mediates resistance to chemotherapy. These features are key characteristics of some of the hallmarks of cancer (Hanahan and Weinberg, 2011).

1.3.1 Pro-growth and anti-apoptotic roles

A large and growing body of literature has substantiated the notion that SK/S1P promotes tumourigenesis. The first clue to an oncogenic role of SK1 comes from a study which shows that overexpressed SK1 leads to tumour formation. Xia *et al*. (2000) proposed that SK1 acts as an oncogenic protein due to its ability to transform normal fibroblasts, NIH3T3 into cancer cells which can form tumour in severe combined immunodeficient mice. Furthermore, localised formation of S1P at certain membrane compartments and/or increased intracellular level of S1P are essential for tumour transformation since phosphorylation of SK1 and its translocation to the plasma membrane are essential for enhancement of proliferation and protection from apoptosis

(Pitson *et al*., 2005). Moreover, the H-*ras* oncogene increased SK1 activity. When H-Ras was co-transfected with dominant-negative G82D SK1, cell transformation was reduced indicating an essential role for SK1 in H-*ras* dependent transformation (Xia *et al*., 2000). Endogenous SK1 is also essential for the maintenance of proliferation and survival of breast cancer cells because knockdown of SK1 induces Bax oligomerisation and ceramide-dependent apoptosis (Taha *et al*., 2006b). Taken together, SK1/S1P promotes tumourigenesis via pro-growth and anti-apoptotic mechanisms.

The anti-apoptotic role of SK/S1P has also been linked to the promotion of macroautophagy (hereafter referred as autophagy, a self-degradation process). The formation of autophagosome and sequestration of cellular components into this compartment is a primary event leading to fusion with lysosomes and subsequent degradation of autophagosomal contents. Autophagy thereby maintains cellular homeostasis by regulating turnover of cellular components, which can be a cell-defence mechanism in response to stress such as nutrient starvation. However, autophagy also plays opposing role in promoting autophagic cell death (Baehrecke, 2005). Lavieu and colleagues (2006) first demonstrated that autophagy-mediated cell survival can be induced by over-expression of SK1 which de-represses autophagy by inhibiting mammalian target of rapamycin (mTOR). Moreover, this is associated with a moderate increase of Beclin1 (a marker for autophagy) level. By contrast, ceramide induces autophagic cell death, in part by stimulating a more robust up-regulation of Beclin1 (Lavieu *et al*., 2006). In this regard, SK inhibitors commonly perturb ceramide/S1P ratio to increase ceramide levels. Interestingly, a newly developed selective SK2 inhibitor induces autophagic cell death (Beljanski *et al*., 2010). Therefore, SK/S1P promotes cell survival, in part through regulation of autophagy.

1.3.2 Pro-angiogenic and pro-migratory roles

Sustained angiogenesis is an important feature of a growing tumour. To survive, tumour cells must find their way to access nutrients and remove metabolic wastes. Therefore,

many tumours are highly vascularised (Hanahan and Weinberg, 2011). Indeed, $S1P_1$ and $S1P_3$ regulate angiogenesis by facilitating morphogenesis of endothelial cells and the formation of adheren junctions (Lee *et al*., 1999). Perhaps, the most important receptor subtype involved is $S1P_1$ due to its essential role in vascular maturation (Liu *et al*., 2000b). Lung carcinoma cells which have been induced to over-express $S1P_1$ promote tumour growth and vascularisation; knockdown of $S1P_1$ with antisense RNA induces tumour regression (Chae *et al*., 2004). Therefore, pharmacological antagonism of $S1P_1$ may reduce tumour vessel growth analogous to angiogenesis inhibitors such as bevacizumab (Avastin®). Notably, a monoclonal antibody directed against S1P acts like a "molecular sponge" to reduce the bioavailability of S1P and inhibit the release of proangiogenic factors such as VEGF (Visentin *et al*., 2006). The anti-S1P antibody also inhibits tumour growth and vascularisation, further strengthening the notion that S1P promotes tumourigenesis through enhanced angiogenesis.

Cancer metastasis is characterised by malignant cells that invade local tissues and migrate to distant organs followed by colonisation—an important feature of end stage cancers. SK/S1P is known to regulate cell migration through receptor-mediated mechanism and/or intracellular actions. For example, EGF-induced MDA-MB-453 cell migration can be blocked by down-regulation of SK1 and SK2 whereas down-regulation of SK1 alone blocks HEK 293 cell migration towards EGF (Hait *et al*., 2005). This suggests that SK1 and SK2 have overlapping roles in regulation of cell motility which may be dependent on subcellular localisation of these isoforms and cell type. Activation of SK1 by agonists such as oestrogen and prolactin also promotes MCF-7 cell migration (Döll *et al*., 2007). However, intracellular S1P has been demonstrated to inhibit cell motility in MCF-7 and MDA-MB-231 cells (Wang *et al*., 1999b). This is in contrast to agonist-induced activation of SK1 and subsequent enhancement of cell migration which may be due to "inside-out" signalling of S1P activating cell surface receptors. In this regard, invasion and cell migration of B16 melanoma cell are blocked by S1P through $S1P_2$-mediated inhibition of Rac and stimulation of Rho (Arikawa *et al*., 2003). By contrast, migration of MCF-7 cells towards S1P is dependent on activation of

$S1P_3$ and subsequent relocalisation of phospho-ERK1/2 and actin into membrane ruffles (Long *et al*., 2010a). In agreement, $S1P_1$ and $S1P_3$ enhance invasion and cell migration of glioma cells whereas $S1P_2$ inhibits cell migration but promotes invasion through enhanced cell adhesion (Young and Van Brocklyn, 2007). Therefore, antagonism of $S1P_1$ and $S1P_3$ but not $S1P_2$ may inhibit invasion and migration of malignant cells. Furthermore, targeting $S1P_4$ in $ER^-/HER2^+$ breast cancer may be useful since expression of $S1P_4$ has recently been found in MDA-MB-453 cells, which mediates HER2 transactivation and ERK1/2 activation (Long *et al*., 2010b). Besides, inhibiting $S1P_1$ and $S1P_3$ may have additional benefits in breast cancer patients. In this regard, over-expression of SK1 confers resistance to tamoxifen-induced cell death in MCF-7 cells (Sukocheva *et al*., 2009). Indeed, high SK1 expression is associated with reduced survival in ER^+ breast cancer patients and the recurrence time during tamoxifen treatment for these patients is reduced by 8 years (Watson *et al*., 2010). However, the major determinant of invasion in cancer cells depends on cell type and the relative expression level of S1P receptors. Hence, although $S1P_{1/3}$ antagonists may retard cell growth, further investigations are needed to evaluate the use of agonist/antagonist of S1P receptors in cancer metastasis.

1.3.3 Chemo-radio therapy resistance

One of the most important outcomes of targeting SK1 in cancer is to restore sensitivity to radio- and chemotherapy. Inhibition of SK1 leads to increased sensitivity to radiotherapy in prostate cancer cells due to enhanced ceramide production (Nava *et al*., 2000a). In addition, blocking SK1 activity in prostate cancer has been associated with improved chemosensitivity. Albeit with different potency, both taxanes and camptothecin induce apoptosis in PC-3 and LNCaP cells respectively by raising ceramide/S1P ratio. Ectopic expression of SK1 abrogates chemosensitivity to both agents, suggesting that SK1 confers chemoresistance in prostate cancers (Pchejetski, *et al*., 2005). Indeed, the expression levels of SK1 mediate chemotherapeutic resistance of prostate cancer cells: a commercially available SK inhibitor, SKi (2-(*p*-

Hydroxyanilino)-4-(*p*-chlorophenyl)thiazole) induces apoptosis only in LNCaP cells but not LNCaP-AI (androgen-independent) cells which have elevated levels of SK1 (Loveridge *et al*., 2010). Upon SKi treatment, both SK1a and SK1b are down-regulated in LNCaP cells whereas only SK1a but not SK1b is down-regulated in LNCaP-AI cells, suggesting that the total expression levels of SK1a and SK1b dictate the sensitivity of these cancer cells to chemotherapeutic agents (Loveridge *et al*., 2010). Similarly, SK1 is implicated in chemoresistance of acute myeloid leukemia cells. Targeting SK1 with a natural inhibitor, F-12509A induces apoptosis in HL-60 leukaemia cells (Bonhoure *et al*., 2006). It has also been suggested that SK1 has an important role in regulating chemotherapeutic sensitivity of MCF-7 cells since down-regulation SK1 by siRNA increased sensitivity of these cells to doxorubicin-induced apoptosis (Sarkar *et al*., 2005). Furthermore, tamoxifen-resistant MCF-7 cells (selected by prolonged culturing in medium containing tamoxifen) show increased expression of SK1 which is correlated with resistance to tamoxifen-induced apoptosis; down-regulation of SK1 but not SK2 by siRNA restores chemosensitivity to tamoxifen (Sukocheva *et al*., 2009). Therefore, the SK1/S1P signalling pathway is involved in mediating resistance to chemotherapy.

French *et al*., (2003) reported a series of non-lipid SK inhibitors capable of inhibiting the growth of several drug-resistant cell lines. One of these is SKi, an orally bioavailable inhibitor of SK, which was selected for *in vivo* studies and showed sensitivity against multidrug-resistant JC mouse mammary adenocarcinoma cells (French *et al*., 2006). SKi is also active against cells over-expressing P-glycoprotein (a multidrug-resistant pump) suggesting a role for SK in drug resistance. In addition, L-*threo*-dihydrosphingosine (safingol) has been shown to increase doxorubicin sensitivity in drug-resistant MCF-7 cells (Sachs *et al*., 1995). The study provides evidence that safingol inhibits PKC activity which reduces phosphorylation of P-glycoprotein and enhances drug sensitivity. However, safingol is also a competitive inhibitor for SK1 (Banno *et al*., 1998). Therefore, reversal of drug resistance may be due to direct inhibition of SK1 activity. It is hoped that with the use of SK inhibitors in tumours which over-express SK1, drug resistance can be reversed.

1.3.4 Oncogenic signalling in breast cancer

Apart from experimental observation of an oncogenic role through ectopic expression of SK1 which may not reflect the functions of endogenous enzyme, compelling evidence has been obtained from studies showing increased expression of SK1 in various human cancers (Pyne and Pyne 2010). Over-expression of SK1 has been observed in many tumours at the transcriptional level. Nearly 2-fold higher expression was noted in breast, lung, colon and other cancers in tissues samples from cancer patients compared with corresponding normal tissues from the same patients (French *et al*., 2003; Johnson *et al*., 2005). However, an increase in mRNA expression may not always correlate with the level of protein expressions. Using microarray and immunohistochemical analysis, poor prognosis has been linked to over-expression of SK1 in both ER^+ and ER^- tumours from breast cancer patients (Ruckhäberle *et al*., 2008), indicating that SK1 is implicated in disease progression of breast cancer patients. More recently, over-expression of SK1, $S1P_1$ and $S1P_3$ have been linked to reduced relapsed time and increased resistance to tamoxifen in ER^+ breast cancer patients (Watson *et al*., 2010). Paradoxically, increased SK1 expression in patients with $ER^+/HER2^+$ tumours improved survival and reduced tamoxifen resistance (Long *et al*., 2010a). This clinical observation has been recapitulated using MCF-7 HER218 cells (stably expressing HER2 oncogene). HER2 increases SK1 expression which in turn suppresses HER2 expression and induces PAK1 degradation leading to inhibition of cell motility—SK1 induces tolerance to HER2 oncogenic activity and thereby is protective for $ER^+/HER2^+$ patients (Long *et al*., 2010a). As a result, the prognosis and treatment of cancer patients need to be assessed in a holistic manner taking consideration of all deregulated pathways.

The molecular mechanism in which SK1 promotes breast cancer progression has also been extensively studied. Forced expression of SK1 in transformed MCF-7 cells reduces activation of signalling molecules related to apoptosis such as caspase 7 and confers a growth advantage which is dependent on ERK1/2 activation with synergistic contribution from oestrogen signalling (Nava *et al*., 2002). Moreover, *in vivo* study indicates that over-expressed SK1 in MCF-7 cells produces a larger tumour with higher

vascularisation compared with controls (Nava *et al*., 2002). Furthermore, oestrogen can activate SK1 in two phases: transient activation and delayed transcriptional up-regulation (Sukocheva *et al*., 2003). Expression of a dominant negative (G82D) SK1 abrogates oestrogen-dependent cell proliferation, calcium mobilisation and ERK1/2 activation (Sukocheva *et al*., 2003). Prolactin also activates SK1 and up-regulates its expression in a biphasic manner to enhance proliferation and migration of MCF-7 cells (Döll *et al*., 2007).

Work from Sarkar *et al*., (2005) indicates that SK1 is required for MCF-7 cells to survive, migrate and proliferate. More importantly, EGF activates SK1 and cell migration induced by EGF is dependent on SK1. In agreement, down-regulation of endogenous SK1 using antisense oligonucleotide for SK1 promotes MCF-7 cell apoptosis through perturbation of "sphingolipid rheostat" by up-regulating ceramide level (Taha *et al*., 2006b). SKi also promotes down-regulation of SK1 and induces apoptosis in MCF-7 cells (Loveridge *et al*., 2010). In addition to EGF, oestrogen can also activate SK1 in MCF-7 cells. Therefore, a potential link in promoting breast cancer progression exists between these agonists. Indeed, cross talk between different receptors such as EGFR, oestrogen receptor (ERα and/or GPR30) and $S1P_3$ mediates oncogenic signalling of the agonists, EGF, oestrogen and S1P respectively. This "criss-cross" signalling depends on initial transactivation of EGFR by oestrogen which may induce extracellular release of S1P. Indeed, extracellular release of S1P has been demonstrated by activation of ERα and subsequent export through ABCC1 and ABCG2 transporters (Takabe *et al*., 2010). Binding of S1P to $S1P_3$ then releases EGF through a metalloproteinase-dependent manner and activates ERK1/2 (Sukocheva *et al*., 2006; Long *et al*., 2010b). Taken together, these findings indicate an oncogenic role for SK1/S1P in breast cancers.

Despite over-expression and up-regulation of activity, no mutation of SK has been detected in cancers. Oncogenic addiction and tumour suppressor gene hypersensitivity are important in characterising different types of cancer (Weinstein and Joe, 2006).

Based on these concepts, some cancers depend heavily on the contribution from an oncogene or the loss of a tumour suppressor gene in their growth and progression. When the corresponding normal gene is restored, tumour growth usually ceases. SK1 regulates diverse cellular functions through promotion of surviving signals, prevention of apoptosis, induction of cellular genetic alterations and regulation of angiogenesis which have been implicated in cancer progression. Therefore, it has been suggested that the consequence of cancer cells relying heavily on the SK/S1P pathway is not due to the activation of oncogenes but rather by an over-dependence on this pathway (termed as "non-oncogene addiction") as there is no obvious direct mutation in SK1 (Vadas *et al*., 2008). Alternatively, epigenetic modulation may play a role by up-regulating the expression of SK1.

1.4 Sphingosine-1-phosphate receptor ligands and sphingosine kinase inhibitors

In view of the plethora of biological effects mediated by S1P, ranging from cell proliferation to lymphocyte trafficking, there has been intense research effort in developing S1P receptor agonists and antagonists. Not only do these compounds serve as valuable pharmacological tools to dissect receptor functions, they may be developed into highly effective therapeutic agents that address unmet clinical needs. This is exemplified by recent development and marketing of FTY720 as a novel oral therapy for multiple sclerosis. Some of the receptor ligands have also been evaluated in preclinical studies of inflammation, cardiovascular function and immune regulation. Similarly, identifying selective SK1 and SK2 inhibitors will allow the use of chemical probes to inform on the biology of these enzyme isoforms. Moreover, SK/S1P has been linked to the development of cancers which has been the focus of intensive research in the past two decades. This section will provide an overview of some commonly used agonists/antagonists for S1P receptors and the development of SK inhibitors.

1.4.1 Sphingosine-1-phosphate receptor ligands

FTY720 (Fig. 1.7) was initially developed from a fungal metabolite, myriocin as an immunosuppressant to regulate lymphocyte trafficking. FTY720 is phosphorylated by SK2 to FTY720 phosphate which is then transported extracellularly, where it can bind to all S1P receptors except $S1P_2$. Down-regulation of $S1P_1$ occurs due to receptor internalisation and proteasomal degradation upon binding of FTY720 phosphate, suggesting that FTY720 phosphate is a functional antagonist. In addition, FTY720 may have other cellular actions depending on the subtypes of receptor or intracellular targets that are affected (see chapter 3, section 3.1.2). For example, FTY720 has also been shown to exhibit anticancer activity against several breast and colon cancer cell lines (Nagaoka *et al*., 2008). However, these findings suggest that anticancer mechanisms of FTY720 may be different from its immunomodulatory effects since inhibition of cell growth is independent of its phosphorylation. Therefore, further investigation into its exact modes of action is necessary.

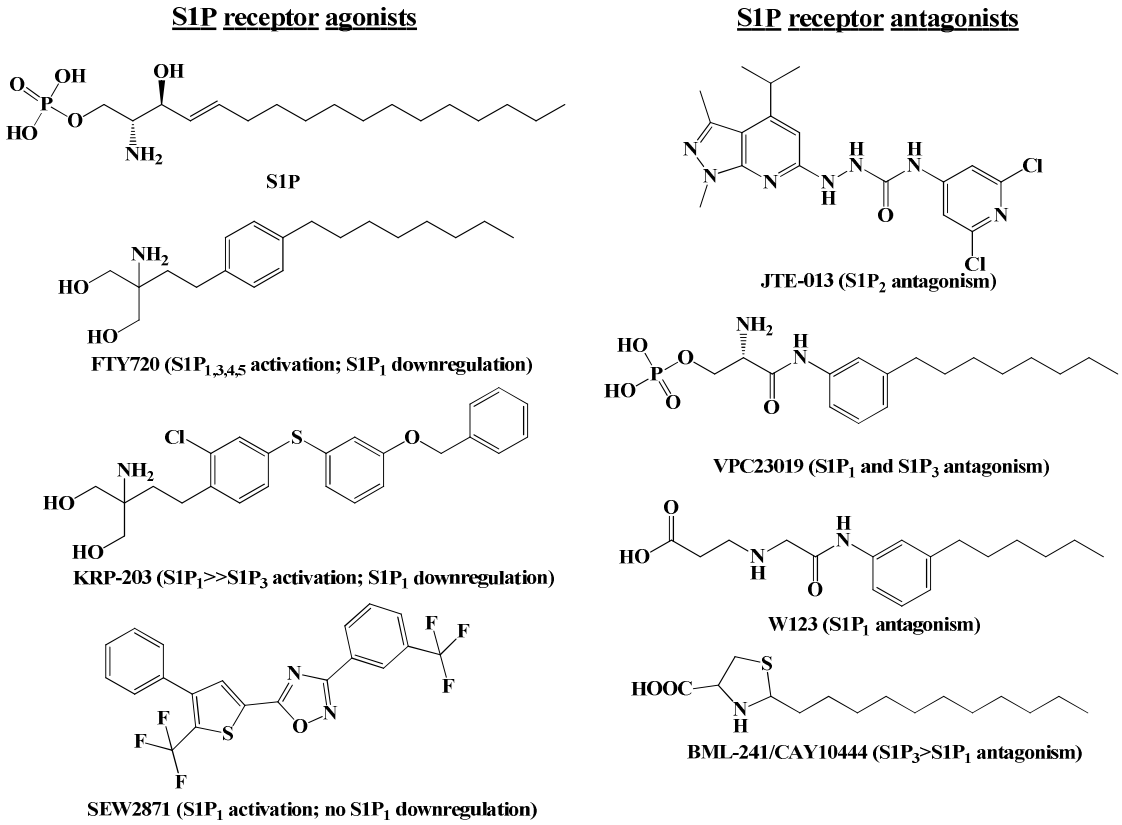


Figure 1.7 Selected structures of S1P receptor ligands. Both S1P receptor agonists and antagonists are shown. These compounds vary in their selectivity for different S1P receptor subtypes.

Several synthetic analogues inspired by FTY720 were synthesised and evaluated by Zhu *et al*., (2007). Asymmetric synthesis of four of the diasteriomers followed by chiral chromatographic separation revealed different activity profiles for the isomers. One of these compounds (3a-P) retains partial agonistic activity at $S1P_1$ but antagonizes $S1P_3$. Therefore, it is important to characterise structure-activity relationship (SAR) of these compounds in order to develop molecules which have high selectivity against specific S1P receptor. In this regard, a highly selective $S1P_1$ agonist, SEW2871 with nanomolar potency has been discovered through high-throughput screening against a large compound library (Jo *et al*., 2005). SEW2871 is a $S1P_1$ agonist which does not induce $S1P_1$ degradation but elicits some downstream responses mediated by S1P including

activation of ERK1/2, Akt and Rac pathways (Jo *et al*., 2005). Furthermore, it has been postulated that the lymphopenic effect of SEW2871 may be due to its agonism on $S1P_1$ rather than receptor down-regulation. This is demonstrated by reversible and supraphysiological $S1P_1$ agonism by SEW2871 which reduces vascular permeability and closes "stromal gates" leading to lymphocyte sequestration (Wei *et al*., 2005). These opposing views on lymphocyte trafficking are not mutually exclusive since they were derived from complementary approaches (chemical *versus* genetics tools) to a complex biological problem (Rosen, 2007). Therefore, lymphocyte trafficking may be affected by agents which induce down-regulation of $S1P_1$ or ultra-high affinity $S1P_1$ agonists, which induce supraphysiological agonism of $S1P_1$.

A structural analogue of FTY720, KRP-203 has also been developed as a potential immunosuppressant. Combination of KRP-203 with sub-therapeutic dose of cyclosporine improves heart allograft survival in mice and reduces cardiotoxicity associated with $S1P_3$ agonism (Shimizu *et al*., 2005). KRP-203 also induces lymphopenia by $S1P_1$ agonism. However, it is not known if $S1P_1$ down-regulation is involved. Despite showing efficacy in rodent models of transplantation, it remains to be determined if KRP-203 will be useful in human organ transplantation since FTY720 fails to provide additional benefit to reduce concomitant administration of cyclosporin in Phase III clinical trials for renal transplantation (Brinkmann *et al*., 2010). A paradigm surrounding receptor agonism and antagonism is that ligands bind to selective conformations of a receptor and activate signalling pathways mediated by a particular conformation. This has been demonstrated with a protean agonist of $S1P_1$, SB649146 which acts as an inverse agonist to reduce constitutive signalling, a competitive antagonist to block S1P-induced ERK 1/2 activation and a partial agonist to activate ERK1/2 (< S1P) (Pyne and Pyne, 2008).

Developing subtype-selective S1P receptor antagonists has proved to be difficult, possibly due to the existence of multiple receptor conformations and/or overlapping binding sites between different receptor subtypes. For example, a structural analogue of

S1P, VPC23019 inhibits both $S1P_1$ and $S1P_3$ whereas VPC22277 is a $S1P_{1/3}$ agonist inducing cell migration in T24 cells over-expressing $S1P_1$ (Davis *et al*., 2005). Both VPC23019 and W123 (a $S1P_1$ antagonist) inhibit SEW2871-induced T cell retention in lymph validating their antagonistic profile; however, the selectivity of W123 is unknown and its K_d value is in the sub-micromolar range (Wei *et al*., 2005). JTE-013 has initially been developed as a $S1P_2$ antagonist that abrogates $S1P_2$-dependent inhibition of cell migration in the vasculature (Osada *et al*., 2002). More recently, JTE-013 has been found to inhibit $S1P_4$ and reduce $S1P_4$-dependent Ca^{2+} mobilisation (K_i=236.9nM) and ERK1/2 activation (Long *et al*., 2010b).

There are continued efforts to develop selective antagonists for S1P receptors. CAY10444, also known as BML-241 has been developed as a $S1P_3$ antagonist through pharmacophore modelling of S1P structure and 3D database searches (Koide *et al*., 2002). Based on cell based calcium mobilisation assay, CAY10444 has been shown to be selective against $S1P_3$ and has no effect on $S1P_2$ and $S1P_4$ (Long *et al*., 2010b). To date, no selective antagonists of $S1P_4$ or $S1P_5$ have been reported. Of note, another FTY720 analogue, (*S*)-FTY720 vinylphosphonate acts as a pan S1P receptor antagonist; it is a partial antagonist against $S1P_2$ and $S1P_5$ but a full antagonist at $S1P_1$, $S1P_3$ and $S1P_4$ with K_i of 384nM, 39nM and 1190nM, respectively (Valentine *et al*., 2010).

Since unexpected protein-ligand interactions exist, development of selective inhibitors is intrinsically difficult (Newman, 2008). However, one should define the purpose of developing highly selective or even specific drugs. For example, will "off-target" effects cause undesirable side effects which reduce patients' tolerance? Targeting multiple proteins involved in different signalling pathways that share similar pathological manifestation could be beneficial. Most drugs are not without side effects and this may simply be due to the dosage (local concentration or bioavailability at the targets). For example, FTY720 is non-selective as it has affinity for $S1P_{1/3/4/5}$. Yet, it is clinically efficacious and with careful patient monitoring, the drug has rekindled hopes for the treatment of multiple sclerosis.

In summary, there is a range of different S1P receptor ligands available in the field; however, the development of subtype-selective inhibitors can be difficult. There is also a need to develop selective $S1P_4$ or $S1P_5$ ligands since evidence has emerged that these receptors may play a role in the regulation of immune functions. Alternative strategies are also needed to improve the diversity and efficacy of new agents; most S1P receptor ligands have been developed based on structural modification of S1P or ligand binding sites on receptors (i.e. they are S1P mimetics). In this regard, identifying potential allosteric binding sites on S1P receptors may improve selectivity.

1.4.2 Sphingosine kinase inhibitors

Apart from S1P receptor ligands, several SK inhibitors have been discovered and developed (Fig. 1.8). Despite having a collection of these compounds, there is a lack of selective and potent inhibitors in the field. For example, early studies on intracellular actions of S1P depend, in part, on the use of DMS or DHS as SK inhibitor. However, DMS is not a specific SK inhibitor as it also inhibits protein kinase C (PKC) (Kim *et al*., 2005). DHS is also a substrate for SK2. Therefore, DHS and DMS are considered as pan-competitive inhibitors of SK due to their non-selectivity against a vast range of kinases (Pyne and Pyne, 2000). They are only used as experimental tools given that drug delivery of these compounds could be difficult due to their lipophilic properties. However, safingol, an enantiomer of DHS, has been shown to potentiate the pro-apoptotic effect of mitomycin C in gastric cancer cells (Schwartz *et al*., 1995) and can be administered as an emulsion which enhanced the efficacy of co-administered doxorubicin in Phase I clinical trial (Schwartz *et al*., 1997). Safingol has also been co-administered with cisplatin in patients with advanced solid tumours in a recent phase I clinical trial showing good tolerability (Dickson *et al*., 2011).

Several synthetic SK inhibitors (SKI I-V including SKI II, which is termed SKi in this project; Fig. 1.8) have also been discovered by French *et al*. (2003). This was a successful attempt from a screen of over 16000 chemical compounds. Due to ease of

synthesis and desirable physicochemical properties, SKi has been studied in murine xenograft model and shown to be orally bioavailable (French *et al*., 2006). Though effective, these inhibitors are not selective against different SK isoforms. To address the non-selectivity of some of these compounds, Hengst and colleagues (2010b) have investigated the SAR of SKI-I and optimised its structure leading to improved potency and selectivity against SK1. This highlights the importance of continuing medicinal chemistry efforts to optimise the activity of lead compounds in drug discovery. Alternatively, others have synthesised selective SK1 inhibitor based on SAR of sphingoid-based inhibitors. For example, SK1-I (BML-258) is a selective SK1 inhibitor (K_i ~10μM) which is active against leukaemia and xenograft tumour growth (Paugh *et al*., 2008). Development of these synthetic SK inhibitors which are active both *in vitro* and *in vivo* has driven the development of novel inhibitors with improved selectivity and potency.

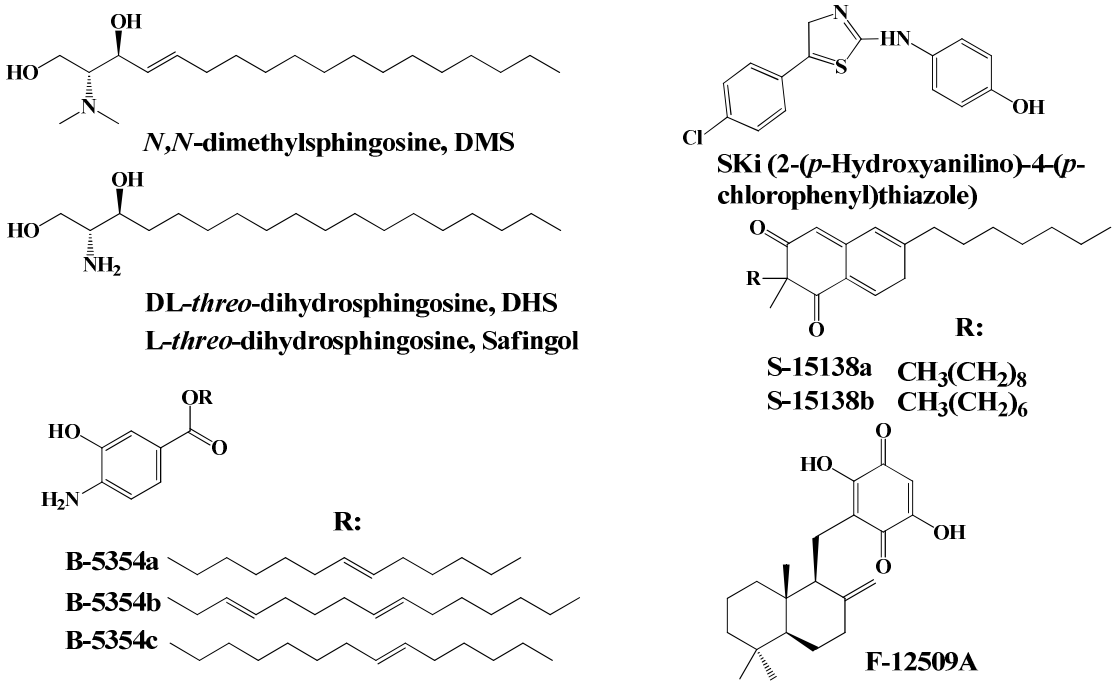


Figure 1.8 Selected structures of SK inhibitors. DMS and DHS are present endogenously whereas F-12509, B-5354 and S-15138 were isolated from fungal and bacterial source. SKi is a synthetic compound available commercially.

Some subtype selective SK2 inhibitors are also available. The first report of this are the SG compounds, which show high selectivity against SK2 but not SK1 or PKC (Kim *et al*., 2005). Interestingly, SG12 and SG14 are also cytotoxic against CHO-K1 cells, indicating that SK2 inhibitors might be effective anticancer compounds. However, more work is needed to investigate the cytotoxic effect of these compounds, particularly whether SK2 alone or other targets are involved. More recently, ABC29460 which retards tumour cell growth and migration, has been developed as a selective SK2 inhibitor with good oral bioavailability (Ki~10μM; competitive with sphingosine) (French *et al*., 2010). ABC29460 might promote autophagic death associated with increased level of two autophagic markers, LC3-II and Beclin1 (Beljanski *et al*., 2010). Formal investigation is needed to address whether inhibition of SK2 and/or SK1 can induce autophagic cell death and if this is a cell-type specific response.

Recent medicinal chemical efforts have yielded several small molecule SK inhibitors active at low micromolar range. Of note, two of these amidine-based lipids, Compound 23 (a dual SK1 and SK2 inhibitor) and Compound 28 (40-fold increase in selectivity for SK1 over SK2) have submicromolar potency (Mathews *et al*., 2010). Further optimisation of these amidine-based inhibitors has led to the development of highly potent SK subtype selective inhibitors with low nanomolar potency (Kennedy *et al*., 2011). Moreover, these compounds are more potent than SKi in lowering intracellular S1P content of U937 cells. Amidine-based inhibitors also act as competitive inhibitors against sphingosine as they are structural analogues of an amino-alcohol substrate, VPC45129 (Mathews *et al*., 2010). The development of these potent compounds with nanomolar efficacy shows promising outlook for SK1 as a target in cancer drug discovery.

Several inhibitors have been discovered from natural products which are now used as pharmacological tools or therapeutic agents that perturb SK/S1P signalling. One example is phenoxodiol, a plant-derived antitumour compound, which has been classed as a multiple signal transduction regulator with potent effects against angiogenesis

(Gamble *et al*., 2006). It is now in Phase III clinical trial for recurrent ovarian cancers. Since S1P is implicated in blood vessel formation and maturation, phenoxodiol was initially tested for activity against SK. Indirect evidence of SK inhibition was obtained from phenoxodiol attenuating the activation of SK by TNF-α (Gamble *et al*., 2006). Further investigation is needed to assess whether phenoxodiol can directly inhibit SK by performing *in vitro* enzymatic assays. In addition, phenoxodiol is non-selective as it is also active against reduced nicotinamide adenine dinucleotide (NADH) oxidase and DNA topoisomerase II, which induces down-regulation of Akt and anti-apoptotic proteins (Pyne and Pyne 2010). Other Nature-derived SK inhibitors isolated from fungi include F-12509A, S-15183a and b (Kono *et al*., 2000b; Kono *et al*., 2001) (Fig. 1.8). Being classified as azaphilones, these inhibitors are selective for SK at mid-micromolar range. Besides, several natural SK inhibitors have also been isolated from marine bacteria including B-5354 which shows higher selectivity albeit with lower potency than DMS (Kono *et al*., 2000a; Kono *et al*., 2000c). Further characterisation of these inhibitors revealed that F-12509A and B-5354 inhibit both SK1 and SK2 with different kinetic properties (Kono *et al*., 2002). Isolation of SK inhibitors from microorganisms suggests that these compounds are evolutionarily produced for a specific function which is yet unknown but may involve the regulation of host immune function since S1P also regulates the egress of lymphocytes in mammals (Goetzl and Rosen, 2004).

RNA interference technology has also been used to inhibit SK1 by down-regulating its expression with antisense RNA and siRNA. Though highly specific and effective, RNA interference is only used experimentally due to drug delivery obstacles as well as unknown side effects. Another selective technique is the use of monoclonal antibody against S1P. With the use of this antibody, tumour regression and growth inhibition have been shown in xenograft model and *in vitro* studies. Of note, the antibody is not specific since it can also bind to closely related lipid molecules such as dihydro-S1P and sphingosyl-phosphorylcholine (Visentin *et al*., 2006). A humanized monoclonal anti-S1P antibody, LT1009 (Sonepcizumab^{TM}) has been developed; it has entered Phase I clinical trial for cancer and age-related macular degeneration (O'Brien, 2009). Therefore,

in light of the evidence of SK/S1P signalling in cancer and efficacy of currently available inhibitors acting on this pathway, drug discovery of more of these agents/compounds could serve as important pharmacological tools or novel drugs.

1.5 Plants as a natural product for drug discovery

One of the best sources to identify hits and develop leads for drug discovery is products from natural origin. However, drug discovery in natural products is a lengthy process coupled with difficulties in obtaining sufficient samples for isolation and low-yields of active compounds. Therefore, natural products were slowly abandoned while pharmaceutical companies endeavoured to speed up the development of new drugs with synthetic chemical compounds in the past. Nevertheless, due to vast biodiversity, there has been a recent resurgence of interest in natural products. For instance, from 1981 to 2002, around 48% of new chemical identities were derived from natural products. Between 1940 and 2002, 40% of currently used anticancer drugs were natural products or derivatives from natural leads with another 8% being natural mimics (Balunas and Kinghorn, 2005). Surprisingly, only one *de novo* synthetic compound was approved from 1981 to 2006 (Newman, 2008). This exemplifies the pitfall of relying solely on synthetic compounds and further reinforces the importance of drug discovery in natural products. Arguably, natural compounds have a lower attrition rate than synthetic compounds in drug development. This suggests that natural compounds are more likely to complete clinical trials and be approved for clinical use (Harvey, 2008). Even compounds isolated and studied previously but abandoned due to negative results can still be useful for future screening of new targets because the structures of these compounds are diverse and complex.

Similar to other natural products, plant-derived compounds are bioactive and varied in their structural complexity. Generally, these compounds have several chiral centres (being optically active) and heterocyclic and polycyclic aromatic rings. This has been suggested to closely resemble drug-like compounds since the complex scaffolds offered

by these compounds can bind better with the dynamic structural domains of the targets—reviewed by Newman (2008). Accordingly, concepts like 'diversity-oriented synthesis' and 'privileged structures' have been explored. These concepts have been extended by different groups of researchers who have exploited the structural diversity of the compounds to produce the most promising drug lead. As a result, several compounds have been developed and are now in different phases of clinical trials. For instance, seliciclib, a novel CDK inhibitor developed from the plant secondary metabolite olomucine , completed phase I trial to assess the optimum dosing regimen (Benson *et al*., 2007). The drug appears to be well tolerated with nausea being the most frequent complaint. Like most plant-derived anticancer compounds, it may be added to current anticancer armamentarium. Therefore, there have been great interests in research on structural diversity of plant-derived compounds.

Due to their complex scaffolds, bioactive metabolites produced by plants have been extensively characterised. Plants are known to produce both primary and secondary metabolites. It is the secondary metabolite that has been shown to be important for leads discovery (Chin, 2006). Some examples of these compounds include alkaloids, flavonoids and terpenoids. It has been suggested that a number of flavonoids such as flavones and flavanones are potential agents for cancer chemoprevention, i.e. the use of non-toxic phytochemicals in preventing cancer progression (Kinghorn *et al*., 2004). Chemopreventive agents either block cancer initiation (blocking agent) or inhibit cancer progression (suppressing agents). The former involves detoxification whereas the latter may be an antagonist acting at growth factor receptors. For example, natural withanolides isolated from *Solanaceous* plants possess significant inhibitory activity against quinone reductase which results in induction of phase II detoxifying enzymes (Kinghorn *et al*., 2004). Thus, it is possible that compounds isolated from plants can have one or more of these chemopreventive effects which contribute to their efficacy in cancer treatment. Furthermore, four different classes of anticancer drugs have been discovered from plants for routine clinical use today. These are epipodophyllotoxin, camptothecin, taxanes and vinca alkaloids. Most of these were derived from plant leads

through semi-synthesis (Kinghorn, 1999). These drugs have been the mainstay in current anticancer treatment due to their proven efficacy.

Even though plant-derived compounds offer exciting opportunity for effective drug treatment, the needs for plants to produce these compounds have intrigued scientists for many years. It is now widely understood that these metabolites not only serve normal physiological roles in growth and reproduction (pollination) but also have an important role in defence mechanisms of plants. As with other living systems, plants evolve several ways to defend themselves against the attack from predators which include herbivores, insects and microorganisms. For example, physical protection arises from structures such as thorns and waxy skin; production of secondary metabolites which can be used to lure the enemies of predating herbivores such as parasites (Arimura *et al*., 2005). Some metabolites could also be toxic or exhibit antimicrobial properties for defence purposes. With these toxic metabolites, plants effectively ward off their predators by killing them or making them sick. The production of these metabolites is surprisingly conserved in certain plant families (Agrawal, 2007) raising the possibilities of finding similar compounds in plants from the same family.

Isolating bioactive compounds from plant is, however, no easy task. Unlike microorganisms, plants do not produce bioactive secondary metabolites in large amounts sufficient for immediate drug development. In contrast, due to the advent of biotechnology, the production of bioactive compounds isolated from microorganisms can be scaled up. Obtaining sufficient samples from plants for compound isolation can also be difficult due to the issues of intellectual property rights (Balunas and Kinghorn, 2005). Nevertheless, advances in phytochemical analysis and biotechnology have accelerated drug discovery in plants significantly. With the use of nuclear magnetic resonance spectroscopy and mass spectrometry in phytochemistry, structural elucidation of very complex natural compounds can be achieved. In addition, plant cell culture is a novel technique where low yield secondary metabolites are produced by culturing plant cells *in vitro* to ease the production of these metabolites. Besides, this technique has also

been used to establish biosynthetic pathways of the metabolites (Phillipson, 2007). Importantly, procedures which allow identification of known compounds that have been reported previously are necessary to avoid replication. These de-replication procedures such as the bioautographic procedures have been employed by Kinghorn *et al*., (1999). In summary, there has been renewed interest in drug discovery of natural products, especially plants because of their natural biodiversity.

1.6 Research aims

Sphingolipid metabolites have been the focus of intense research in recent years due to their implications in the development and progression of various diseases, especially cancer. Therefore, there has been an increasing interest in identifying novel ligands which can act on this pathway. These compounds can provide insights into disease processes and new leads for drug development. For instance, ligands acting on different subtypes of S1P receptors can serve as important pharmacological tools to dissect the effects of these receptors on cellular functions. More importantly, inhibitors that target the enzymes, SK1/SK2 which catalyse S1P formation may represent a novel approach to cancer treatment.

Until today, there are no reported selective plant-derived SK inhibitors in the literature. Nevertheless, SK inhibitors have been isolated from fungi and bacteria. In addition, sphingosine and phytosphingosine can be phosphorylated respectively in plants to form S1P and phyto-S1P which are involved in regulation of stomata opening and seed germination (Worrall *et al*., 2008). It is likely that if SK/S1P signalling is evolutionary conserved, plants should contain metabolites that can interfere with the signal transduction of this pathway. To facilitate inhibition, the metabolites could be acting as inhibitors against SK or antagonists at S1P receptors. Besides, plants are reputable for discovery of anticancer drugs and may produce these compounds because of the diversity of their metabolites. Therefore, the aims of this project were to isolate and characterise cytotoxic compounds which can act as potential SK inhibitors from plants.

Meanwhile, despite widespread use of SKi as the prototypical SK inhibitor, the molecular mechanism and kinetic inhibition are not fully understood. Therefore, this project also explores how SKi inhibits SK and cancer cell growth. In addition, FTY720 has been shown to retard tumour growth apart from limiting lymphocyte trafficking. Several new chemical entities and synthetic analogues of FTY720 are available through collaboration with Prof. Robert Bittman. These compounds will be assessed for their activities against SK in this project. Kinetic characterisation and downstream effects of SK inhibition will also be investigated. It is hoped that novel compounds acting on SK can be identified, aiding anticancer drug discovery.

Chapter 2 General Materials and Methods

2.1 Materials

2.1.1 General materials

All materials such as chemicals and reagents were of highest quality available commercially. Unless otherwise specified, they were purchased from Sigma-Aldrich Co. Ltd. (UK). A list of commonly used materials purchased from other companies is given below:

Company	**Material**
Bio- Rad (UK)	BIO-RAD protein assay reagent
Fisher Scientific (UK)	Scintillation cocktail (ScintiSafe 3)
GE Healthcare (UK)	Hybond^{TM} ECL^{TM} nitrocellulose membrane
Invitrogen (UK)	Lipofectamine^{TM} 2000
Invitrogen (UK)	PureLink™ HiPure Plasmid Maxiprep Kit
Invitrogen (UK)	PureLink™ Quick plasmid miniprep kit
Invitrogen (UK)	One Shot® TOP10 chemically competent *E. coli*
MP Biomedicals (UK)	3-Aminophthalhydrazide (Luminol)
Sigma-Aldrich Co. Ltd. (UK)	Prestained molecular weight marker (SDS7B2)
Technical Photo Systems (UK)	Kodak LX24 developer, Kodak Industrex fixer
Vector labs (UK)	Vectashield hardset mounting medium with DAPI

2.1.2 Cell culture

Cell culture reagents such as Dulbecco's Modified Eagle Medium (DMEM), Modified Eagle Medium (MEM), Opti-MEM Reduced-Serum Medium with GlutaMAX I (Opti-MEM), European Foetal Calf Serum (EFCS), penicillin/streptomycin (10,000 units/ml penicillin; 10,000 µg/ml streptomycin) and trypsin (0.25% with EDTA 4Na) were purchased from Invitrogen Ltd. (UK). G418 Sulfate was purchased from Merck Biosciences (UK). HEK 293 cells stably over-expressing GFP-tagged SK1 were kind gifts of Dr. Nicholas Ktistakis (Babraham Institute, Cambridge, UK). MCF-7 parental and MCF-7 expressing the Neo vector or HER218 oncogene (MCF-7/Neo; MCF-7/Her2) cells were given by Dr. Rachel Schiff (Baylor College of Medicine, Houston, USA).

2.1.3 Antibodies

Company	**Antibody**
BD Biosciences (UK)	Anti-ERK 2 (Cat. # 610104)
Cell Signalling Technology (UK)	Anti-PARP (Cat. # 9542)
Santa Cruz Biotechnology (USA)	Anti P-ERK1/2 (Cat. # sc-7383)
Santa Cruz Biotechnology (USA)	Anti-c-Myc (Cat. # sc-40)
Sigma-Aldrich Co. Ltd. (UK)	Anti-actin (Cat. # A2066)
Sigma-Aldrich Co. Ltd. (UK)	Anti-mouse horseradish peroxidise (HRP)-linked IgG, (Cat. # A9044)
Sigma-Aldrich Co. Ltd. (UK)	Anti-rabbit HRP-linked IgG (Cat. # A0545)
Sigma-Aldrich Co. Ltd. (UK)	Protein G Sepharose (Cat. # P9424)
Stratagene (USA)	Anti-FLAG M2 (Cat. # 200472-21)

Polyclonal anti-SK1 antibodies (Ab-64) were given by Dr. Andrea Huwiler (University of Bern, Bern, Switzerland) (Huwiler *et al*., 2006).

2.1.4 Lipids and growth factors

Company	Compound
Avanti Polar Lipids (USA)	DMS
Avanti Polar Lipids (USA)	D-*erythro*-sphingosine
Avanti Polar Lipids (USA)	D-*erythro*-sphingosine-1-phosphate
Merck Biosciences (UK)	EGF

2.1.5 Inhibitors

Company	Inhibitor
Cayman (Estonia)	FTY720
Cayman (Estonia)	*cis*-resveratrol
Enzo Life Sciences (UK)	*trans*-resveratrol
Enzo Life Sciences (UK)	Ac-DEVD-CHO
Enzo Life Sciences (UK)	Proteasome inhibitor (MG-132)
Merck Biosciences (UK)	Sphingosine kinase inhibitor (SKi)
Merck Biosciences (UK)	Cathepsin B inhibitor IV (CA-074 Me)

2.1.6 Radioisotopes

Company	Radioisotope
GE Healthcare (UK)	[^{3}H] Thymidine (25 Ci/mmol)
PerkinElmer	[^{32}P] ATP (3000 Ci/mmol)

2.1.7 Solvents

All solvents used in the extraction and purification of active compounds were of HPLC grade and were purchased from Fisher Scientific (UK). These include *n*-pentane, *n*-hexane, chloroform, ethyl acetate, acetone, DMSO, methanol and acetic acid. Deuterated solvents (99.9 atom % D) including dimethylsulfoxide-d_6, acetone-d_6, pyridine-d_5 and chloroform-d were obtained from Sigma-Aldrich Co. Ltd. (UK)

2.1.8 Other reagents and chemicals used in plant extraction

Thin layer chromatography (TLC) grade silica gel coated aluminium sheet, TLC grade silica gel 60 H and column grade silica gel particle size 40-63 µm were obtained from Merck (Germany).

2.1.9 Plant materials and novel synthetic compounds

In preliminary screening, the first batch of dried and ground leaves of *Hopea dryobalanoides* were originally collected by the Forest Institute of Malaysia (FRIM) and stored/extracted at Strathclyde Innovations in Drug Research (SIDR). Due to limited quantity of plant materials obtained for preliminary screening, a new batch of *Hopea dryobalanoides* was requested from FRIM. Around 1-2kg of dried and ground leaf, bark and twig of *Hopea dryobalanoides* were obtained.

QAB compounds were obtained from the Drug Discovery Portal at Strathclyde whereas FTY720 synthetic analogues and other novel synthetic compounds were kind gifts of Prof. Robert Bittman (Queens College of The City University of New York, New York, USA).

2.2 Method

2.2.1 Cell culture maintenance

Aseptic technique was maintained in all cell culture work which was carried out in a laminar flow hood. All incubations were performed in a humidified 5% CO_2, 37°C incubator. DMEM was used as a basic medium to maintain cell culture. Depending on different cell types, the medium was added with supplements and antimicrobials to promote cell growth and reduce risk of contamination. HEK 293 cells were grown in MEM containing 10% (v/v) EFCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids and in the presence of DMEM (instead of MEM) and 800 µg/ml G418 Sulfate for HEK 293 cells stably over-expressing recombinant SK1. MCF-7 cells were supplemented with DMEM containing 10% (v/v) EFCS, 15 µg/ml insulin, 100 U/ml penicillin, 100 µg/ml streptomycin whereas 400 µg/ml G418 Sulfate was also added to supplement the growth of MCF-7 cells (either stably expressing vector (Neo) or human epidermal growth factor receptor 2 (HER 2)).

2.2.2 Trypsinizing and passaging cells

Primary cell culture was passaged when cells were grown to a confluent monolayer. Briefly, adhering cell monolayer was washed with 37°C DMEM to remove any residual EFCS which may inhibit the action of trypsin. Then, 2 ml of trypsin was added to culture grown in a 75cm^2 flask to cover the adhering cells before leaving at 37°C in the incubator. After 2 mins, the flask was tapped gently to dislodge cells. Next, 8 ml of complete medium was added to the flask to inactivate any trypsin activity. To each new culture in a 75 cm^2 flask, 1 ml of this cell suspension was added with 9 ml complete medium. After 3 or 4 days, old medium was removed and subconfluent cultures were fed with 10 ml fresh complete medium. Confluent secondary culture was passaged by repeating all steps described above.

2.2.3 Freezing cells

Confluent cell lines were frozen for long term storage to preserve cells. Freezing medium for MCF-7 cells was made of DMEM supplemented with 20% (v/v) EFCS and 10% (v/v) DMSO whereas 80% (v/v) EFCS and 10% (v/v) DMSO were added to DMEM to prepare the freezing medium for HEK 293 cells stably over-expressing recombinant SK1. Then, confluent cells were trypsinised as described in Section 2.2.2. Next, the cell suspension was centrifuged for 3 mins at 800 rpm. Supernatant was removed and the cell pellet was resuspended in 4 ml 4°C freezing medium. 0.5 ml aliquots of cell suspension were added into 2 ml cryogenic vials. The vials were placed at -80°C freezer overnight before being transferred to liquid nitrogen freezer for long term storage.

2.2.4 Thawing and recovering cells

Cryogenic vials containing frozen cells were removed from liquid nitrogen freezer and placed in 37°C water bath immediately for rapid thawing. Thawed cells were drawn into a Pasteur pipette to break up cell clumps before being transferred into a new flask with the addition of 10 ml complete medium. After 24 hrs, cultures were checked to ensure cell attachment to the flask and old medium was replaced with fresh complete medium.

2.2.5 Cloning and purification of plasmid DNA

The wild type and the G81D mouse SK1 and wild type mouse SK2 were cloned into pcDNA4/myc-His by Dr. Dawn Thompson (University of Strathclyde, UK). The FLAG-tagged wild type human SK1 and FLAG-tagged G113A human SK1 plasmid constructs (pcDNA 3.0) were kind gifts of Dr. Stuart Pitson (University of Adelaide, Australia). FLAG-tagged D178N human SK1 was generated by Dr. Carolyn Loveridge (University of Strathclyde, UK).

Plasmid DNA was usually isolated by transforming competent cells or inoculating new culture from glycerol stocks aseptically. To culture bacteria on agar plates, 1.5 g Bacto-agar was dissolved in 100 ml of LB medium containing 1% (w/v) Bacto-tryptone, 0.5 % (w/v) Bacto-yeast extract and 1% (w/v) NaCl and autoclaved before use. After autoclaving and cooling to 55°C, the mixture was supplemented with ampicillin (final concentration: 50 ng/ml) and poured into 9 cm petri dishes. After solidification, the plates were inverted and stored in the dark at 4°C if not used immediately. Chemically competent *E. coli* was transformed according to manufacturer's instruction. Briefly, one vial of cells was thawed on ice. Then, 1 to 5 µl of the DNA (10 pg to 100 ng) was added into the vial and mixed gently. The vial was then incubated on ice for 30 mins prior to heat shocking the cells at 42°C for 30 secs. The vial was placed on ice immediately for 2 mins. Pre-warmed Super Optimal broth with Catabolite repression (SOC) medium (supplied with the kit) was then added to the vial which was then shaken horizontally at 37°C for 1 hr at 225 rpm. Later, 20-200 µl of transformed cell mixture was spread onto pre-warmed LB agar plates and left to incubate overnight at 37°C. Several colonies were selected from each plate and analysed by plasmid isolation.

Usually, miniprep cultures were prepared to assess plasmid quality or to isolate high quality plasmid (up to 30 µg) using PureLink™ Quick plasmid miniprep kit. Briefly, a 1-5 ml of an overnight culture (transformed *E. coli* grown in 5 ml LB medium containing 50 ng/ml ampicillin for 12-15 hrs at 37°C in a shaking incubator) was pelleted. The pellet was then processed according to manufacturer's instruction which is similar to the Maxiprep protocol to be described below. Miniprep cultures showing the highest concentration and best plasmid quality ($Abs_{260}/Abs_{280} > 1.8$) were chosen for preparation of Maxiprep culture and glycerol stock. For example, 700 µl of miniprep culture was mixed with 300 µl of 50% (v/v) sterile glycerol. The glycerol stock was kept at -80°C.

To obtain more plasmid DNA (e.g. 500-850 µg) for downstream application including cell transfection, plasmid isolation was performed using PureLink™ HiPure plasmid

maxiprep kit. Briefly, cells were harvested from 250-500 ml of an overnight LB culture by centrifuging at 4000 g for 10 mins. The pellet was then resuspended in Resuspension Buffer (R3) containing pre-added RNase A. Next, cell suspension was lysed with Lysis Buffer (L7) for 5 mins at room temperature. Immediately, Precipitation Buffer (N3) was added to neutralise the lysate and precipitate unwanted cellular debris. The mixture was centrifuged at 12000 g for 10 mins and the supernatant (clear lysate) was then loaded onto a pre-equilibrated column to drain by gravity flow. Once lysate filtration was complete, the column was washed with Wash Buffer (W8). Plasmid DNA was then eluted from the column by addition of Elution Buffer (E4) prior to isopropanol precipitation at room temperature to concentrate and desalt the plasmid DNA. After centrifugation at 15000 g for 30 mins at 4°C, the DNA pellet was washed with 70% ethanol to remove isopropanol and residual salt. Again, the mixture was centrifuged at 15000 g for 5 mins at 4°C. Supernatant was carefully removed and the DNA pellet was briefly air-dried and re-dissolved in appropriate volume of TE buffer (e.g. 200-500 µl). The concentration and quality of the plamid DNA can be assessed by UV spectrometry where $Abs_{260}/Abs_{280} > 1.8$ and $Abs_{260}/Abs_{230} > 2.0$ indicate high DNA purity.

2.2.6 Cell transfection

HEK 293 cells were transfected with plasmid DNA using Lipofectamine™ 2000 according to manufacturer's instruction. Briefly, 1-5 x 10^5 cells/ml of cells in complete medium were plated one day before transfection so that cells would be 90-95% confluent on the day of transfection. For transfection in a 12-well plate, the media in plates containing the cells to be transfected were replaced with 800 µl of Opti-MEM (containing 1% serum). Typically, for each transfected sample, DNA complex was prepared by incubating 1 µg of DNA with 1.5 µl of Lipofectamine™ 2000 in 200µl Opti-MEM (without antibiotics) and incubated for 20 mins at room temperature. In some cases where varying amount of DNA was used, the DNA (µg): Lipofectamine™ 2000 (µl) ratio was kept at 1:1.5. Transfection was carried out by adding DNA complex to each well containing the cells and medium. Then, cells were incubated in a

humidified 5% CO_2, 37°C incubator. Depending on the type of experiments, cells were generally maintained for 24 hrs before experimentation.

2.2.7 Determination of protein concentration

The Bradford protein assay was used to determine protein concentration (Bradford, 1976). Briefly, one part of dye reagent was diluted with four parts of distilled water. Several dilutions of BSA were prepared (final concentration: 0.2-0.9 mg/ml) to generate a standard curve. Usually, 5 to10 µl of a sample of a protein extract was added to 1 ml of diluted dye reagent and left to incubate for 5 mins at room temperature prior to the measurement of absorbance at 595 nm. The protein concentration of the sample was then determined from a graph of [BSA] against Abs_{595}.

2.2.8 Immunoprecipitation

To study oligomerisation of SK1, HEK 293 cells were transiently transfected with myc-tagged G81D SK1, FLAG-tagged D178N SK1, myc-tagged WT SK1, and/or FLAG-tagged WT SK1. The medium was removed after transfection with the indicated plasmid constructs for 48 hrs. Cells were lysed in ice-cold immunoprecipitation (IP) buffer (500 µl) containing 20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mg/ml bovine serum albumin, 0.5 mM $Na_4P_2O_7$, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), leupeptin, and aprotinin (all protease inhibitors were at 10 µg/ml, pH 8.0) for 20 mins at 4°C. The material was harvested and transferred to microfuge tubes, which were further mixed for 1 hr at 4°C, and then centrifuged at 14000 *g* for 10 mins at 4°C to remove cellular debris. After preclearing with protein G Sepharose beads (20 µl of 1 part IP buffer and 1 part protein G Sepharose beads), equal amounts of supernatant from each sample were taken for immunoprecipitation using a protein G Sepharose beads (µl) to antibody (µl) ratio of 10:1. After 2 hrs or overnight agitation at 4°C, the supernatant was removed by centrifugation at 14000 *g* for 15 secs at 4°C. Immunoprecipitates were washed twice

with 1 ml of buffer A containing 10 mM HEPES, pH 7.0, 100 mM NaCl, and 0.5% (v/v) NP-40 and once in 1 ml of buffer A without NP-40. Immunoprecipitates were collected by centrifugation at 14000 *g* for 15 secs at 4°C and combined with boiling sample buffer for SDS-PAGE or combined with SK1 complete buffer to assay for SK1 activity.

2.2.9 Preparation of cell lysate for SDS gel electrophoresis

After appropriate treatment, cell lysates from MCF-7 parental, MCF-7/Neo, MCF-7 HER218 and HEK 293 were prepared by removing old medium and adding 150 µl to 200 µl boiling sample buffer containing 0.125 M Tris Base (pH 6.7), 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 1.25% (v/v) glycerol, 0.5% (w/v) sodium dodecyl sulfate (SDS), 50 mM dithiothreitol (DTT) and 1% (w/v) bromophenol blue. Prepared cell lysates were then stored at -20°C.

2.2.10 One dimensional SDS gel electrophoresis

Polyacrylamide gel was cast as a separating gel topped by a stacking gel made from acrylamide (Table 2.1) and secured in an electrophoresis apparatus filled with a running buffer containing 25 mM Tris Base, 0.21 M glycine and 0.1% (w/v) SDS.

Table 2.1 Ingredients required for preparing two stacking and separating gels

Stock solution	Separating gel	Stacking gel
30% w/v acrylamide:bis-acrylamide (29:1)	5 ml	0.75 ml
1.5M Tris base + 0.4% (w/v) SDS; pH 8.8	3.75 ml	-
0.5M Tris base + 0.4% (w/v) SDS; pH6.7	-	1.25 ml
Distilled water	6.05 ml	3.15 ml
Ammonium persulfate	75 µl	75 µl
Tetramethylethylenediamine	25 µl	10 µl

Equal concentrations of protein samples were loaded into one or more wells together with a prestained molecular weight standard in one or more gel lanes. Gels were then run at 100-120 V until the bromophenol blue tracking dye has reached the bottom of the separating gel.

2.2.11 Immunoblotting

Proteins were electrophoretically transferred to a nitrocellulose membrane at 100 V with cooling in a tank transfer system (Bio-Rad Mini Trans-Blot kit) filled with blotting buffer containing 25 mM Tris base and 0.21 M glycine in 20% (v/v) methanol for 1 hr. Immunoprobing of membranes was carried out by first blocking the membrane with 20 ml blocking buffer containing either 3% (w/v) skimmed milk or bovine serum albumin (BSA) in TBST made with 10 mM Tris base (pH 7.4), 100 mM NaCl and 0.1% (v/v) Tween-20 for 1 hr at room temperature with agitation on a rocking platform. The membranes were then incubated with primary antibodies diluted 1000 times in 1% (w/v) BSA in TBST. After overnight incubation, membranes were washed 3 times with TBST for 7 mins and incubated with diluted (1:80000 in 1% (w/v) BSA in TBST) horseradish peroxidase-anti-Ig conjugates for 1 hr at room temperature with constant agitation. Then, membranes were washed 3 times with TBST for 7 mins before soaking for 3 mins in a 10 ml luminescent substrate buffer which was made by mixing reagent 1 containing 0.04 % (w/v) luminol, 0.1 M Tris base (pH 8.5) and 0.016% (w/v) *p*-coumaric acid and reagent 2 containing 2% (v/v) H_2O_2 and 0.1 M Tris-Base (pH 8.5). In a darkroom, membranes were faced down onto film and autoradiographed for a few seconds to 5 mins. Membranes were then stripped for reprobing with other antibodies in a stripping buffer containing 62.5 mM Tris base (pH 6.7), 2% (w/v) SDS and 100 mM β-mercaptoethanol in an oven for 1 hr at 70°C. Finally, membranes were washed 3 times with TBST for 10 mins before incubation with other primary antibodies.

2.2.12 Densitometry

After immunobloting and film development, protein levels were evaluated by densitometric analysis. Briefly, blots were scanned and protein band densities were analysed using ScnImage program (Scion Corporation, Frederick, MD).

2.2.13 Fluorescence microscopy

To assess morphological changes in MCF-7 cells, fluorescence microscopy was carried out using phalloidin red which binds actin. Briefly, cells were plated onto autoclaved 13-mm glass coverslips in 12-well plates. Cells were grown to 60% confluence before serum-starvation for 48 hrs prior to stimulation with 1 μM S1P for 5 mins. Cells were then fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 mins, and permeabilised with 0.1% Triton X-100 in PBS for 1 min before incubation in blocking solution (5% serum and 1% BSA in PBS) for 1 hr at room temperature. Coverslips were then incubated with phalloidin red (1:100 dilution in blocking solution) for 1 hr at room temperature. Coverslips were washed with PBS and mounted on glass slides using Vectashield® hard set mounting medium with 4',6- diamidoino-2-phenylindole (DAPI), a fluorescent nuclear stain. Actin rearrangement was assessed using Phalloidin red staining. Fluorescence was visualised using a Nikon (Surrey, UK) E600 epifluorescence microscope.

2.2.14 [^{3}H]-thymidine incorporation assay

The ability of plant extracts or inhibitors to inhibit DNA synthesis was assessed by the uptake of [^{3}H]-thymidine by MCF-7 cells. In some experiments where cells were not stimulated, MCF-7 cells were plated at 2.0-4.0 × 10^4 cells/well in 24-well plates and maintained overnight in complete medium. Subsequently, cells were treated with varying concentrations of the inhibitor dissolved in DMSO or vehicle control (0.1% DMSO). Cells were then incubated for another 24 hrs before pulsing with [^{3}H]-thymidine. When cells were stimulated with growth factors in other experiments, cells

were grown in a 24-well plate with complete medium for at least 24 hrs. When cells were grown to 60-70% confluence, old medium was removed and cells were cultured in serum-free DMEM for another 48 hrs. Then, cells were treated with test compounds (inhibitors or plant extracts) at specified concentrations for 15 mins before agonist (S1P 1 μM or EGF 25 ng/ml) was added. Cells were then incubated for another 15 hrs and pulsed with [^{3}H]-thymidine (0.5 μCi/ml) for 5 hrs before termination by washing 3 times with 1ml ice cold 10% (w/v) trichloroacetic acid. Radionucleotides incorporated into DNA were harvested with 0.25 ml 0.1% (w/v) SDS and 0.3 M NaOH and quantified by liquid scintillation counting with 2ml scintillation cocktail. Each test compound was tested in triplicate.

2.2.15 Sphingosine kinase and YegS activity assays

SK1 activity was assayed as described previously (Delon, *et al*., 2004). Briefly, sphingosine was solubilised in Triton X-100 (final concentration 0.063% w/v) and combined with buffer 1 containing 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM Na_3VO_4, 40 mM β-glycerophosphate, 1 mM NaF, 0.007% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine. For SK2, the kinase activity was optimised according to Liu *et al*., (2000a) where sphingosine was complexed with bovine serum albumin (final concentration 0.2 mg/ml) in buffer 1 supplemented with 400 mM KCl. Inhibition of SK1/2 activity was determined by incubating 15 ng purified SK1 or SK2 (Enzo Life Sciences, UK) or 15 μg HEK 293 cell lysates containing stably over-expressed recombinant SK1 for 15-20 min at 30°C, in the presence of 0.5 to 20 μM sphingosine, 250 μM of [^{32}P]ATP (4.4×10^{4} cpm/nmol, 10 mM $MgCl_2$), and varying concentrations of inhibitors dissolved in DMSO or control (5% DMSO). Reactions were terminated by the addition of 500 μl butanol and mixed with 1 ml 2 M KCl. The organic phase containing [^{32}P]-S1P was then extracted by washing twice with 1 ml 2 M KCl before quantification of radioactivity. The kinetic parameters were calculated using the graph plotting and curve fitting programs Biograph (University of Strathclyde, Glasgow UK)

and Prism 4.03 (GraphPad). Substrate kinetics were analysed according to the Michaelis-Menten equation and the inhibition constants (K_{ic} and K_{iu}) were determined using Dixon and Cornish–Bowden plots (Cortés, *et al*., 2001).

YegS partially purified from *Salmonella typhimurium* was obtained from Prof. Alastair Hawkins (University of Newcastle upon Tyne, UK). The measurement of YegS catalytic activity was essentially identical to SK1 activity assay with the exception of using phosphatidylglycerol as the substrate instead of sphingosine.

2.2.16 Plant extraction and compound isolation

Plant extracts obtained from SIDR had already been detannified. Briefly, around 50 g of the plant material was placed in a 1 L brown glass jar where 500 mL of methanol was added. 5 g of polyvinylpyrrolidone (PVP) was added to concurrently detannify the sample. The jar was then sealed and left at room temperature for a minimum of 48-hour extraction. The jars were shaken at least twice daily for 1 to 2 mins. After extraction, the extract was filtered and dried under rotator evaporation to a volume of approximately 20 mL. Further evaporation to dryness was done in a TurboVap apparatus. The extract was then frozen and then freeze-dried for 2-3 days before being stored at -20º C. The above procedures were performed by SIDR. For example, 50 g of the ground *Hopea dryobalanoides* leaves (first batch) had been extracted and subjected to a detannification process to give a gummy dark greenish extract. Then, 0.5 g of this extract was fractionated according to Methods (section 2.2.17) to give 10 major fractions (F1-F10) together with two sub-fractions (F4A and F5B) which were separated manually by combining elution of similar colours. Ampelopsin A (5 mg) was obtained from Fraction 5. The percentage yield of ampelopsin A is 0.01% (based on dried weight of plant material). More active compounds were isolated using the second supply of *Hopea* dryobalanoides. Samples (30 g) of each part of the plant (leaf, bark and twig) were extracted with methanol to obtain three extracts for NMR characterisation. 700 g of the stem bark of *Hopea dryobalanoides* was then used for successive extraction with

hexane, ethyl acetate, and methanol. The ethyl acetate extract (5 g) was further fractionated according to Methods (section 2.2.18) because it appeared to contain the target compounds. Active fractions were then purified by Sephadex LH-20 using methanol as an eluent to isolate balanocarpol (300 mg). The percentage yield of balanocarpol is 0.043% (based on dried weight of plant material).

2.2.17 Flash chromatography

Plant extract was fractionated using Flash chromatography (FC) with a 20 g ISOLUTE® Flash Si II cartridge in Flash Master (Biotage). Briefly, the extract was first adsorbed onto some heptamethylnonane (HMN) granules (approximately double the volume of the extract) and dried under nitrogen flow before being added into the cartridge. The flow rate was set at 20 ml/min for gradient elution using solvents of increasing polarity: hexane, dichloromethane, butan-2-ol and methanol. The volumes of the fractions were then reduced with rotary evaporation. Subsequently, all fractions were freeze-dried and stored at -20ºC.

2.2.18 Vacuum liquid chromatography

TLC grade silica gel was dry-packed under vacuum in a Büchner funnel with a sintered glass disc (Coll and Bowden, 1986). The plant extract was then adsorbed onto a minimum amount of column grade silica gel before being applied on top of the packed vacuum liquid chromatography (VLC) column (the height of the packed layer should be ~5 cm). Similar to flash chromatography, gradient elution with solvent systems of increasing polarity was performed. Collected fractions were then dried (freeze-dried if necessary) and stored at -20ºC.

2.1.19 Low pressure column chromatography

Glass column of appropriate size packed with suitable adsorbents was used to purify fractions obtained from FC/VLC. Briefly, sufficient amount of adsorbents (typically column grade silica gels or Sephadex LH-20) was added with the solvent system to produce a thick slurry which was then packed immediately into the column. To ensure homogenous packing, the column was run freely with the mobile phase and left overnight if necessary. At the same time, sample was adsorbed onto some column grade silica gels with the addition of appropriate solvents. The mixtures were then left in a fume cupboard to dry so that a free-flowing powder can be loaded onto the column. Similarly, elution was done with the use of step gradients by varying the ratio of hexane, ethyl acetate and methanol in the mobile phase. The solvent strength was changed after 2 to 3 column volumes. Only one solvent system was used (usually methanol) when Sephadex LH-20 was packed in the column. Sample was added directly onto the column. Typically 10-20 fractions were collected per column bed volumes to ensure good separation.

2.1.20 Thin layer chromatography

TLC stain anisaldehyde-H_2SO_4 was prepared by mixing 0.5 ml of *p*-anisaldehyde with 10 ml glacial acetic acid, 85 ml methanol and 5 ml of concentrated sulphuric acid. This stain is multi-purpose and enables the detection of most functional groups with different colouration. For example, phenols will give violet spots. However, it is insensitive to alkenes and alkynes. Therefore, TLC was done as a preliminary step for compound detection. Spotted TLC plate was placed in a TLC tank for development as suitable solvent system migrates up the plate. Non-destructive detection of natural compounds was achieved by placing the developed TLC plate under a UV lamp using short (254 nm) and long (366 nm) wavelengths. If recovery of the compounds was not necessary, the TLC plate would be spayed with anisaldehyde-H_2SO_4 stain.

2.1.21 Structure elucidation with NMR and MS

All NMR experiments were performed with a JEOL (JNM LA400) operating at 400 (^{1}H) and 100(^{13}C) MHz using deuterated and residual solvent peaks as internal reference. Typically 10-20 mg and 20-50 mg of samples were dissolved in deuterated solvents for ^{1}H and ^{13}C NMR respectively. NMR sample tubes made of borosilicate glass were used for the measurement of all spectra except when the compound was less than 10 mg which would require the use of a Shigemi tube.

^{1}H-NMR was performed on all samples to establish an initial impression of the type and amount of compounds present in the sample. Further structural analysis was assisted with two-dimensional NMR experiments such as COSY (COrrelation SpectroscopY), HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence). ^{13}C and DEPT (Distortionless Enhancement through Polarisation Transfer) NMR experiments were performed when samples were sufficiently pure and enough to obtain a good signal to noise ratio. Spatial structural information was obtained with NOESY (Nuclear Overhauser Enhancement SpectroscopY).

Mass spectrometry (MS) was used to establish the molecular weights and molecular formulae of selected samples. Around 1 mg of samples was dissolved in appropriate solvents (usually methanol) and separated by High Pressure Liquid Chromatography (HPLC) before being ionised in a ThermoFinnigan LCQ-Decaiontrap or Orbitrap HRESI mass spectrometer. Depending on the nature of the compounds of interest, negative or positive mode ElectroSpray Ionisation (ESI) experiments were chosen. Sample was also run in Agilent Technology 6130, Quadrupole LC/MS using atmospheric pressure chemical ionization (APCI). Samples were submitted to the mass spectroscopy service at Strathclyde Institute of Pharmacy and Biomedical Sciences or Chemistry department. LC-MS was run by MS technicians.

2.1.22 Statistics

All numerical data are presented as means +/- standard deviations for at least three independent measurements unless otherwise stated. The data were usually normalized for comparison of different experiments and analysed statistically by student t-test or One Way ANOVA depending on the experimental setting. Differences were considered statistically significant at $p<0.05$.

Chapter 3 Preliminary screening of anticancer compounds from natural and synthetic origins

3.1 Introduction

One of the first steps in drug discovery is to identify reliable hits for drug optimisation efforts. A hit should demonstrate activity in excess of a defined cut-off value whereas a lead molecule not only should exhibit reproducible activity but also fulfil additional criteria such as desirable physicochemical properties and tractable structures for chemical lead optimisation efforts (Copeland, 2003). There are several ways to identify hits. First, high throughput screening (HTS) is routinely used by pharmaceutical companies to identify potential hits from a large chemical library. This method allows the discovery of novel hits without prior knowledge of compound structures. While this method is useful, the outcome depends on chemical diversity of the compound library. This is exemplified by the discovery of the first non-lipid SK inhibitors by French and colleagues (2003) who screened a library of 16000 chemical compounds and identified four hits. Second, useful drug leads targeting enzymes can be developed through structural modification of enzyme substrates or known inhibitors. For example, sphingoid analogues have been developed into effective and selective SK1 inhibitor (Paugh *et al*., 2008). Using the same approach, selective SK2 inhibitor has also been developed (Kim *et al*., 2005). The main advantage of the second approach is that a higher success rate can be achieved when leads are optimised from known inhibitor structure. A significant disadvantage for HTS is that it is not universally feasible because more resources are needed. On the other hand, analogue inhibitors or "me-too" compounds tend to have limited structural diversity which reduces the potential for further drug development. Therefore, this study sought to identify novel scaffolds by screening plants for activity. Not only could this provide structural novelty, there is also a higher success rate in drug discovery with natural products. Besides, this study also explored the possibility of identifying novel inhibitors by screening synthetic analogues of FTY720. Other synthetic thiazole analogues were also available for screening. This introduction will discuss the process of initial drug discovery efforts in identifying novel SK inhibitors from both natural and synthetic sources. The rationale for choosing FTY720 and its analogues as potential SK inhibitors will also be presented.

3.1.1 Tropical plants with cytotoxic properties

A lot of plants have been found to produce anticancer compounds. The structures for most of these compounds have been elucidated. However, further investigations on the molecular targets of these active compounds have not been pursued. Therefore, to select plants for initial investigation, a large number of tropical plants which show anticancer properties have been identified from the literature. After confirming the availability of these plants in the repository at SIDR, twenty-three plant extracts were selected for preliminary screening for activities against sphingosine kinase and cancer cell growth. Almost all of the selected plants have been shown to display interesting biological activities including anticancer effects. For example, the presence of cytotoxic agents from resins produced by *Aquilaria malaccensis*, also known as agarwood, suggests that these compounds may play an important role against fungal infection since the resin is only produced after an insult to the tree (Gunasekera *et al*., 1981). In addition, cytotoxic saponins such as elliptosides A isolated from *Archidendron ellipticum* are effective anticancer compounds that inhibit human melanoma xenograft tumour growth (Beutler *et al*., 1997). *Ochanostachys amentacea* produces polyacetylenes such as minquartynoic acid which is cytotoxic to a number of cancer cell lines including human breast cancer (Ito *et al*., 2001a). Several alkaloids isolated from *Alangium longiflorum* also inhibit MCF-7 cell growth with submicromolar efficacy (Sakurai *et al*., 2006). Mainly found in primary rainforests of tropical countries, *Monocarpia marginalis* is the only species in its genus; it has been found to produce a cytotoxic tetracyclic lactam (Lim *et al*., 2008). Taken together, these plants produce cytotoxic metabolites with novel structures which warrant formal investigations on their anticancer effects.

Some of the metabolites produced by plants are well known bioactive compounds belonging to different chemical classes. One major class of these compounds is stilbenoids (polyphenolics) which act as phytoalexins to inhibit microbial growth. These polyphenolics have been shown to produce a myriad of biological effects including tumour growth suppression (Jang *et al*., 1997). Trees belonging to the *Hopea* genus are known to produce polyphenolic compounds (Sahidin *et al*., 2005). Other tropical trees

such as *Gnetum gnemon* also produces gnetin I and other related stilbenoids which induce apoptosis in human leukaemia cells (Iliya *et al*., 2006). Similarly, *Phyllanthus emblica* produces a variety of phenolic compounds, several of which are active against various human cancer cell lines (Zhang *et al*., 2004). Another interesting class of compounds is flavonoids which have also been shown to inhibit cancer cell growth. This is exemplified by phenoxodiol, a cytotoxic isoflavone which also inhibits SK activation (Gamble *et al*., 2006). Other cytotoxic flavonoids including epicatechin and procyanidin B_2 are produced by *Litchi chinensis* (Zhao *et al*., 2007). Despite successful isolation of these bioactive compounds and preliminary testing of their cytotoxic effects, their mechanisms of action are poorly understood. Some of these cytotoxic compounds produced by plants may inhibit SK leading to growth suppression. Therefore, this study explores the possibility of uncovering novel SK inhibitors by screening this subset of tropical plants which are known to produce cytotoxic compounds.

3.1.2 FTY720 and synthetic analogues of FTY720, SKi and other compounds

FTY720 (now licensed as Gilenya®) has been developed from a fungal metabolite, myriocin. Acting as an immunosuppressant, it is now a first-line oral treatment for relapsing-remitting multiple sclerosis. Remarkably, FTY720 causes lymphopenia without affecting T cell activation thereby maintaining host response to viral infection (Brinkmann *et al*., 2002). Homing or recirculation of lymphocytes back to secondary lymphoid organs is mediated by (*S*)-FTY720 phosphate, the active product of FTY720 phosphorylated by SK2 (Billich *et al*., 2003). FTY720 phosphate is a promiscuous ligand binding to all S1P receptors, except $S1P_2$. Binding to $S1P_1$ and subsequent down-regulation of the receptor is responsible for its immunosuppressant activity since $S1P_1$ regulates lymphocyte trafficking (Mandala *et al*., 2002; Gräler and Goetzl, 2004). Even though both S1P and FTY720 phosphate cause $S1P_1$ internalisation, FTY720 phosphate induces proteasomal degradation of the receptor leading to loss of $S1P_1$ signalling (Oo *et al*., 2007). Therefore, FTY720 represents a new class of immunosuppressant with unique mechanisms in regulating immune functions.

The similarity between FTY720 and sphingosine raises the possibility that it has alternative binding targets or cellular functions. In fact, FTY720 has been proposed as a substrate or an inhibitor for sphingosine—metabolizing enzymes (Mandala *et al*., 2002). In this regard, FTY720 is a known substrate for SK2 (Billich *et al*., 2003) and a moderate inhibitor of Sphingosine-1-lyase (Bandhuvula *et al*., 2005). In addition, FTY720 inhibits partially purified SK1 activity isolated from rat heart (Vessey *et al*., 2007), suggesting that FTY720 may be used as an SK1 inhibitor to treat cancers. Moreover, FTY720 might inhibit phospholipase $cPLA_2$ directly, which may provide additional benefit by inhibiting the inflammatory response in multiple sclerosis (Payne *et al*., 2007). FTY720 also inhibits ceramide synthases competitively (against dihydrosphingosine) with a Ki of 2.15 μM as measured in total human pulmonary artery endothelial cell lysate (Berdyshev *et al*., 2009). Besides, FTY720 but not FTY720 phosphate, acts as a competitive antagonist of the cannabinoid (CB_1) receptor (Paugh *et al*., 2006). Taken together, FTY720 is a promiscuous ligand acting on multiple targets.

Ligand binding to receptor or enzyme is known to be stereospecific necessitating the ligand to be in a specific configuration. Therefore, study of the effects of different analogues of FTY720 (Fig.3.1) will provide useful information on SAR which can facilitate the lead selection process. FTY720 was initially synthesised to remove a chiral centre from myriocin but phosphorylation of FTY720 unexpectedly reforms the chiral centre. This prompted asymmetric synthesis and evaluation of the enantiomers of FTY720 phosphate. (*S*)-FTY720 phosphate is an agonist for S1P receptors with K_i of 2.1, 5.9, 23, and 2.2 nM for $S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$ receptors respectively (Kiuchi *et al*., 2005). Two enantiomers of a synthetic analogue of FTY720, (named AAL(*R*) and AAL(*S*)) have also been investigated by Brinkmann and colleagues (2002) where the (*R*)-isomer is selectively phosphorylated by SK2. More recently, FTY720 phosphonate and vinylphosphonate analogues have also been synthesised by Lu and colleagues (2009) to improve the cellular stability of FTY720 phosphates. Phosphonate analogues are resistant to dephosphorylation by endogenous lipid phosphate phosphatases. Hence, to further investigate the molecular mechanisms of FTY720, this preliminary study will

examine the effects of different FTY720 analogues on sphingosine kinase 1 and 2 activity.

SKi contains a thiazole moiety which might be important for its activity. Therefore, thiazole analogues have also been synthesised through medicinal chemistry efforts at Strathclyde (Fig. 3.2). The activities of these compounds against SK should allow optimisation of SAR for lead compounds. Additionally, other compounds (Fig.3.3; 3.4; 3.5) have also been put through in the preliminary screening in order to identify more hits for the development of effective SK inhibitors.

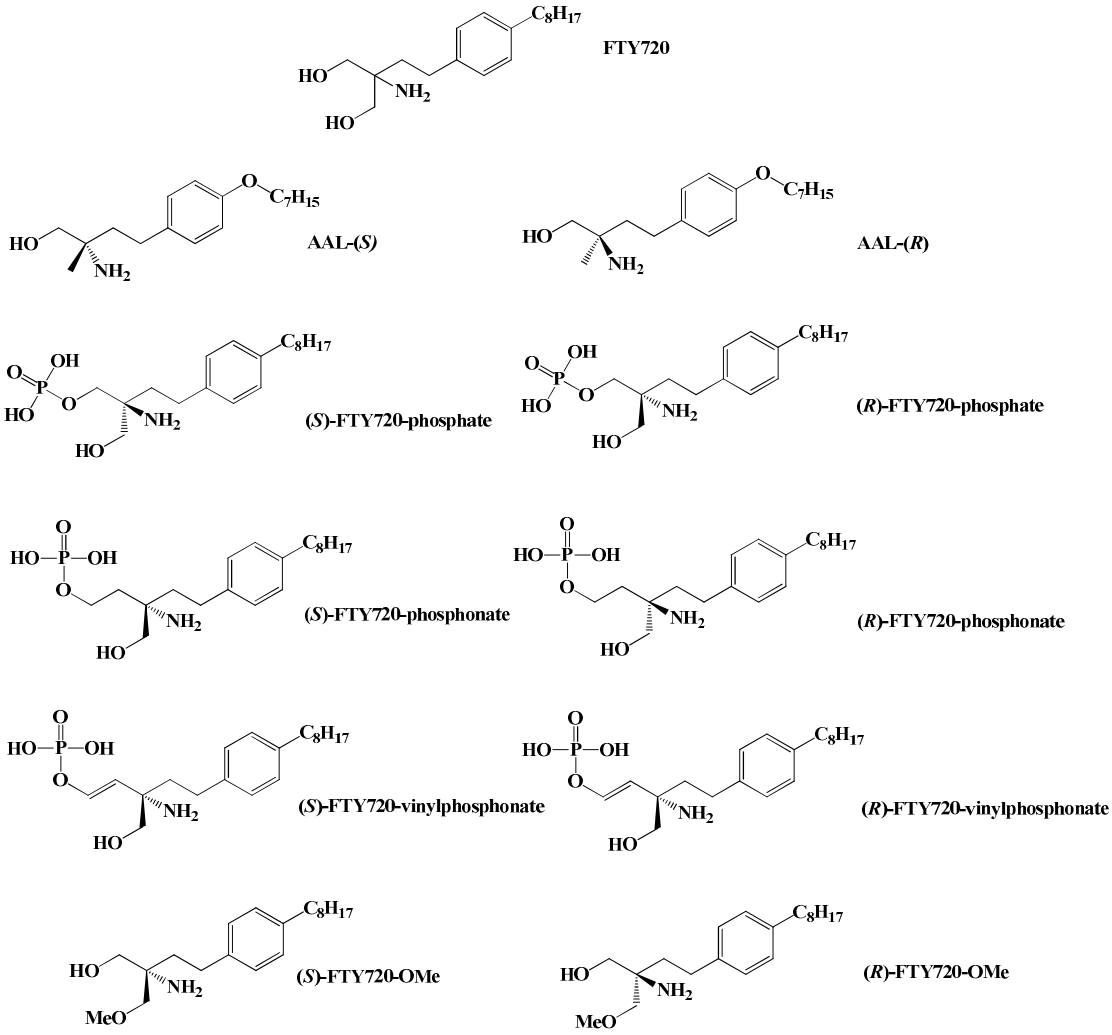


Figure 3.1 Structures of FTY720 analogues. Structures shown are AAL analogues, FTY720 phosphate, FTY720 phosphonate, FTY720 vinyl phosphonate and FTY720 methyl ether.

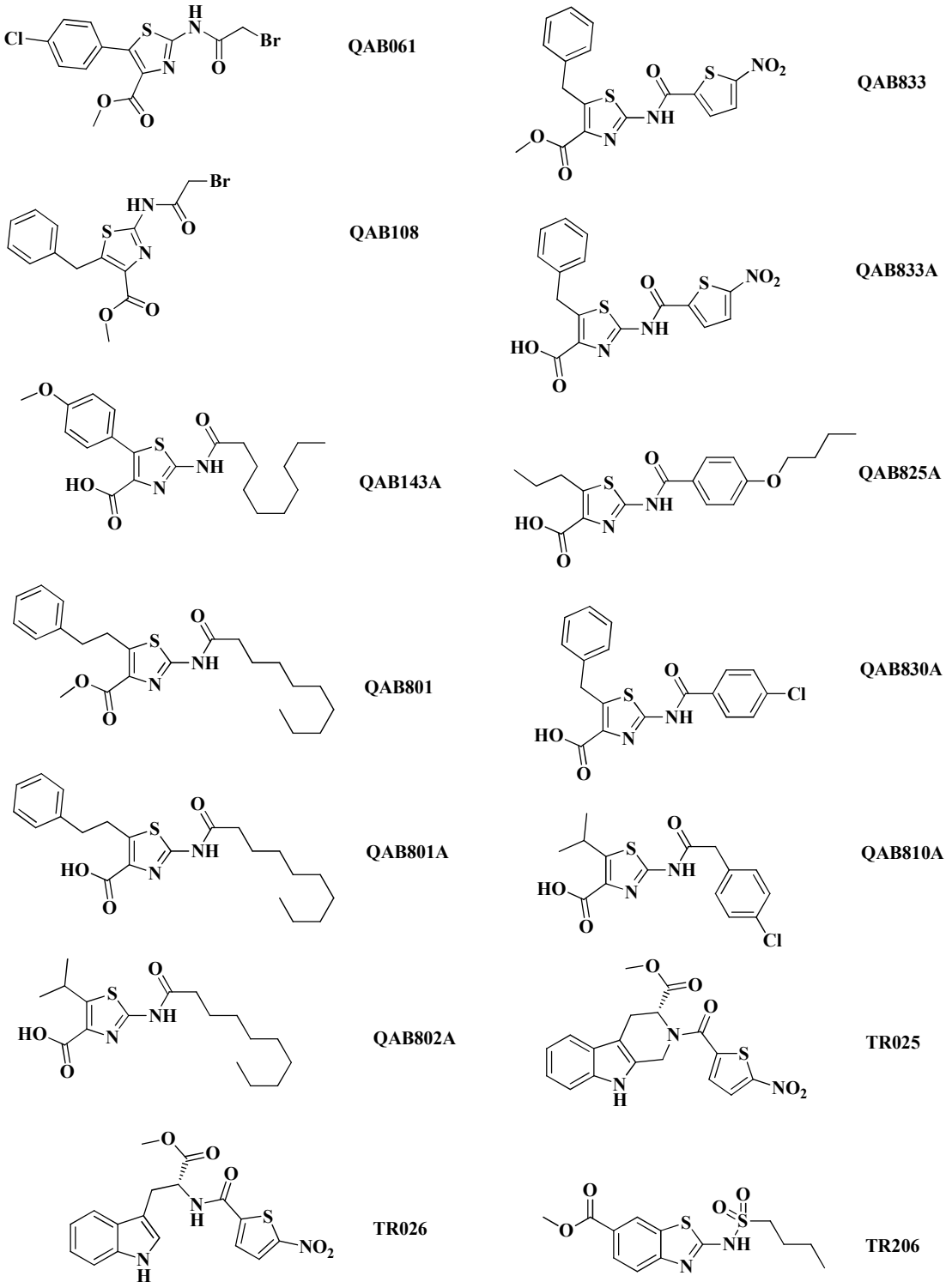


Figure 3.2 Structures of compounds containing thiazole moiety. Structures shown are QAB and TR compounds.

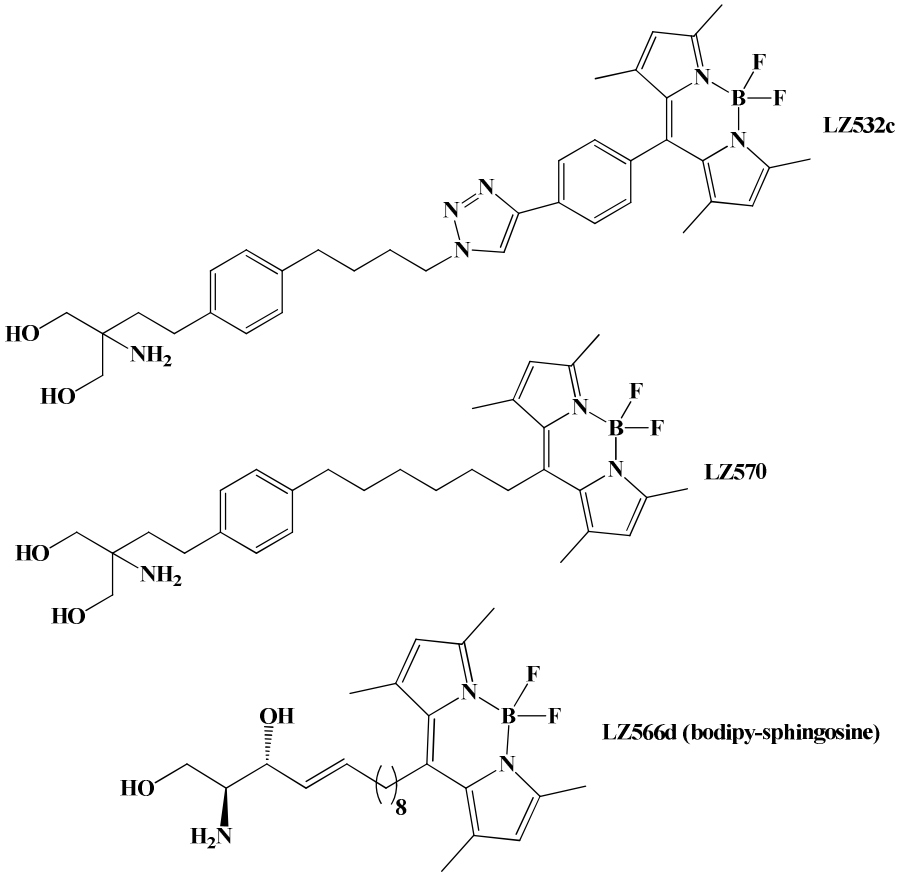
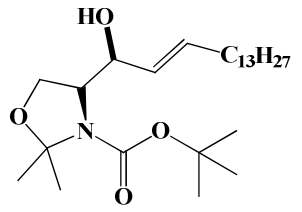
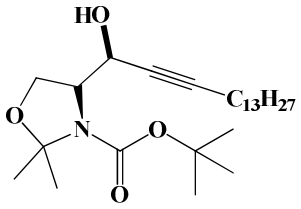


Figure 3.3 Structures of bodipy-tagged compounds



LR232



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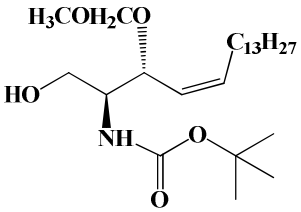
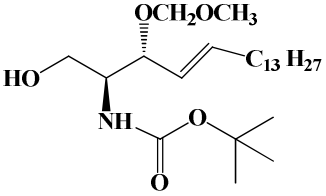


LR292-1



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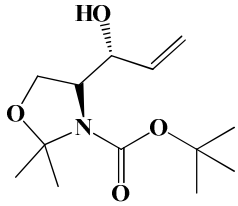
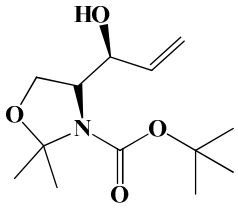
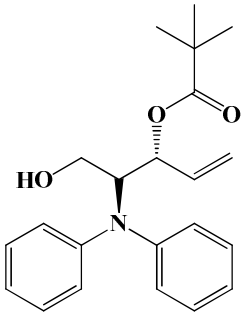


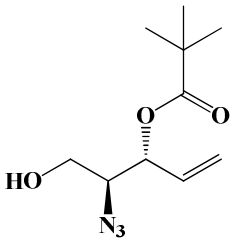
LZ547



LR337-3



LR345



LR353-0

Figure 3.4 Structures of LR and LZ compounds.

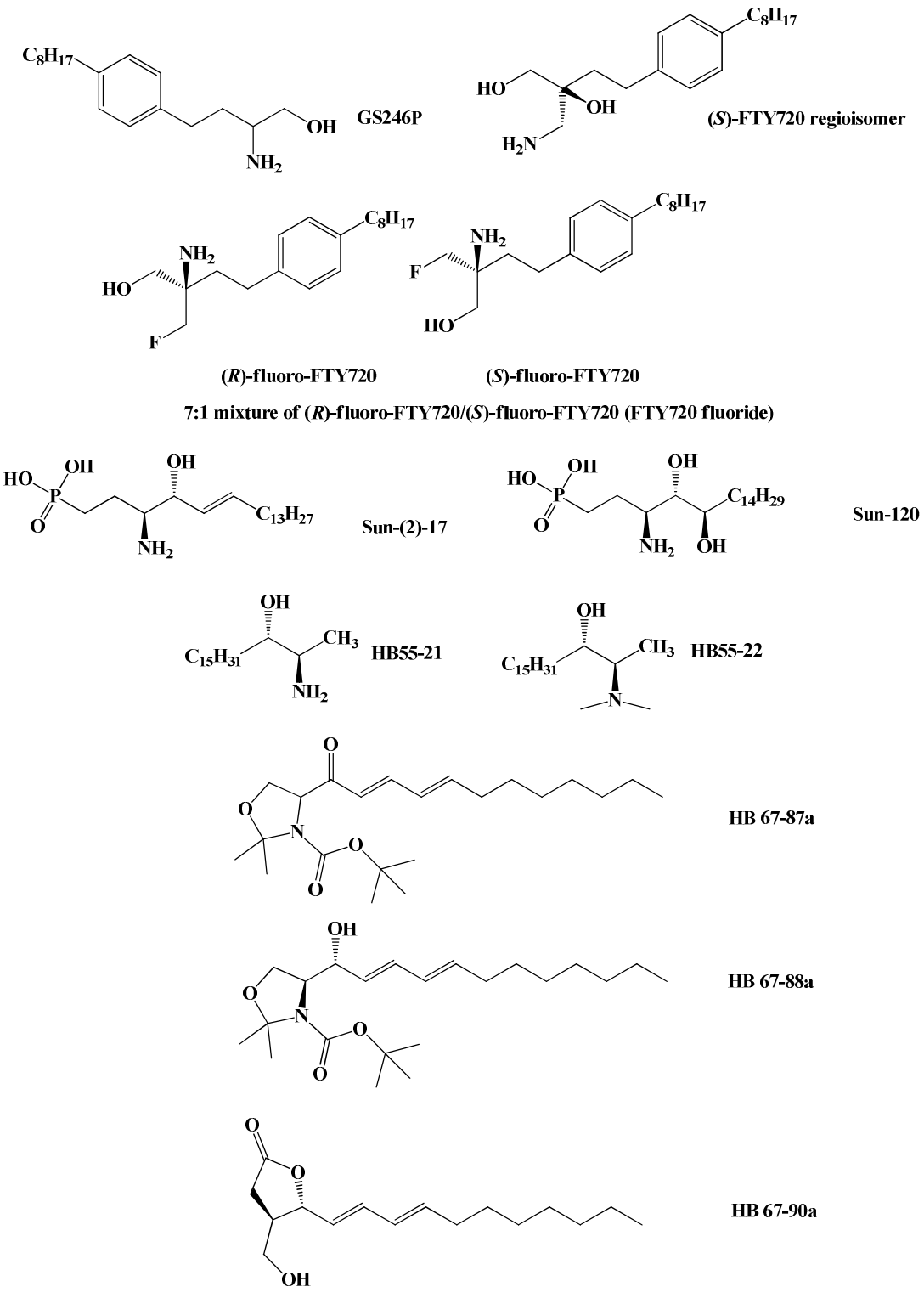


Figure 3.5 Structures of FTY720 regioisomer, FTY720 fluoride, Sun and HB compounds.

3.2 Results

3.2.1 Plant extracts inhibit DNA synthesis in MCF-7 cells

There are various ways to assess cell growth induced by exogenously added agents on a cell line. To confirm the cytotoxicity of selected plant extracts, [^{3}H]-thymidine incorporation assay was used to assess the proliferation of MCF-7 Neo cells in the presence and absence of plant extracts. The effects of SK1 inhibitors on SK1 expression was also assessed in MCF-7 HER218 cells which have higher SK1 expression (see chapter 4; Long *et al*., 2010a). MCF-7 Neo cells were stably transfected with Neo vector only, encoding a neomycin resistance gene whereas MCF-7 HER218 cells were stably transfected with HER2 oncogene. Of note, MCF-7 cells only express one SK1 splice variant, SK1a (hereafter referred as SK1) (Loveridge *et al*., 2010). EGF and S1P were used to stimulate cell proliferation because they are established mitogens in this cell line (Sarkar *et al*., 2005; Long *et al*., 2010a). EGF stimulated cell proliferation more strongly than S1P, as evidenced by higher stimulation of DNA synthesis (Fig. 3.6). Therefore, EGF was used to assess inhibition of agonist-induced DNA synthesis by different plant extracts. All extracts effectively inhibited both basal and EGF-induced DNA synthesis at a concentration of 5 µg/ml (Fig. 3.7A, B). *Ochanostachys amentacea* leaf extract was the most active extract. Inhibition was reduced when plant extracts were diluted to 1 µg/ml (Fig. 3.8 A, B). These findings suggest that cytotoxic compounds might be produced by the plants, some of which have been reported in the literature (see section 3.1.1).

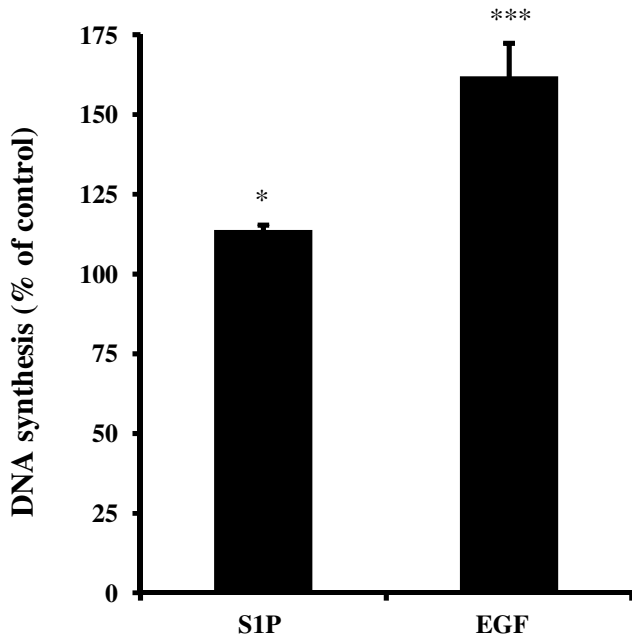


Figure 3.6 Stimulation of DNA synthesis by S1P and EGF. Quiescent cultures of MCF-7 Neo cells were treated with 1 μM S1P or 25 ng/ml EGF for 15 hrs and then with [^{3}H]-thymidine (0.5 μCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, *$p<0.01$ *versus* control. Similar results were observed in three independent experiments.

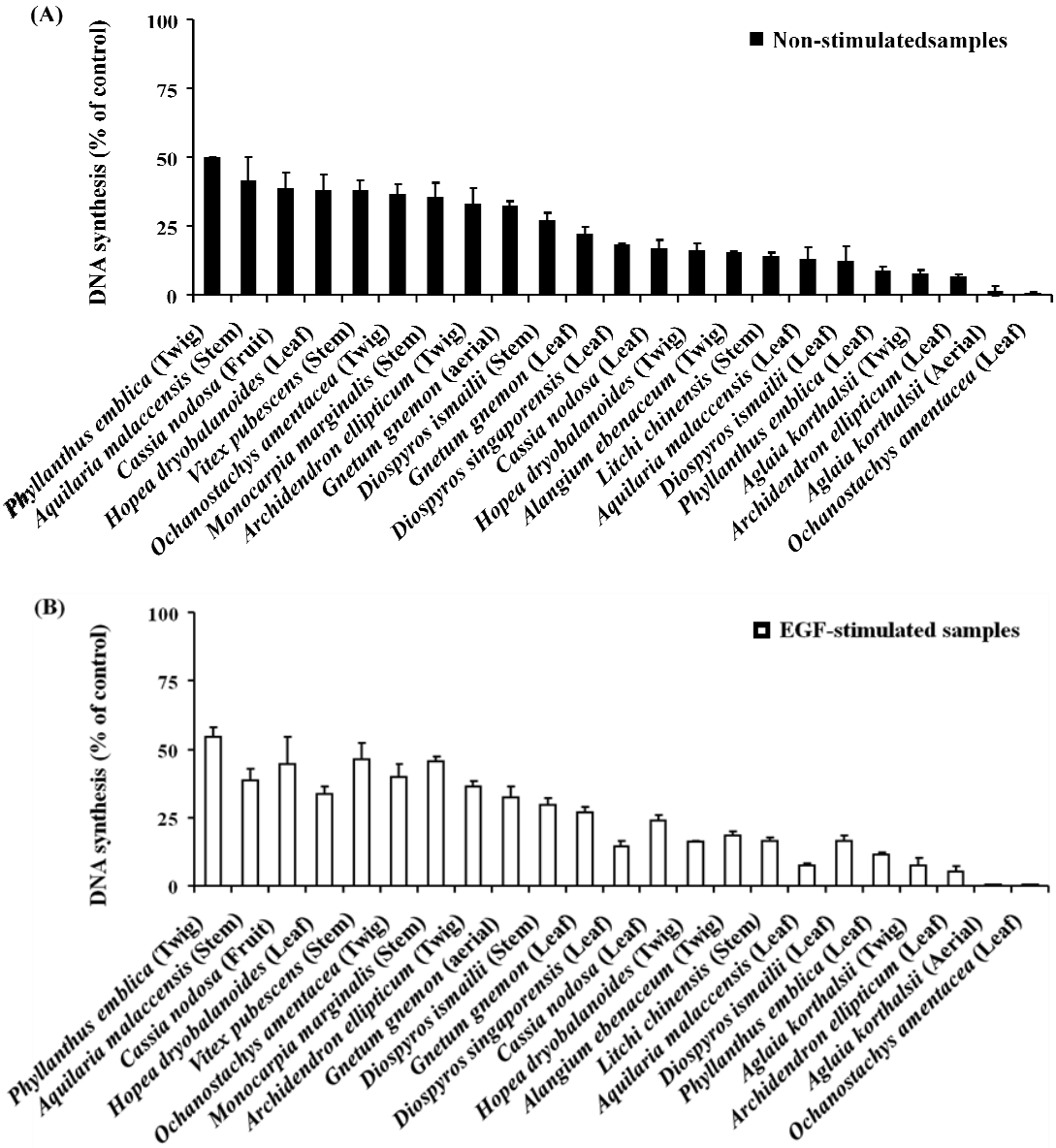


Figure 3.7 Inhibitory effects of 5 µg/ml of plant extracts on DNA synthesis. Quiescent cultures of MCF-7 Neo cells were treated with 5 µg/ml of plant extracts or vehicle control (0.05% DMSO) for 15 mins without stimulation **(A)** or stimulated with 25 ng/ml EGF **(B)**. Cells were then incubated for 15 hrs and then with [^{3}H]-thymidine (0.5 µCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, $p<0.05$ for all samples *versus* control. Similar results were observed in three independent experiments.

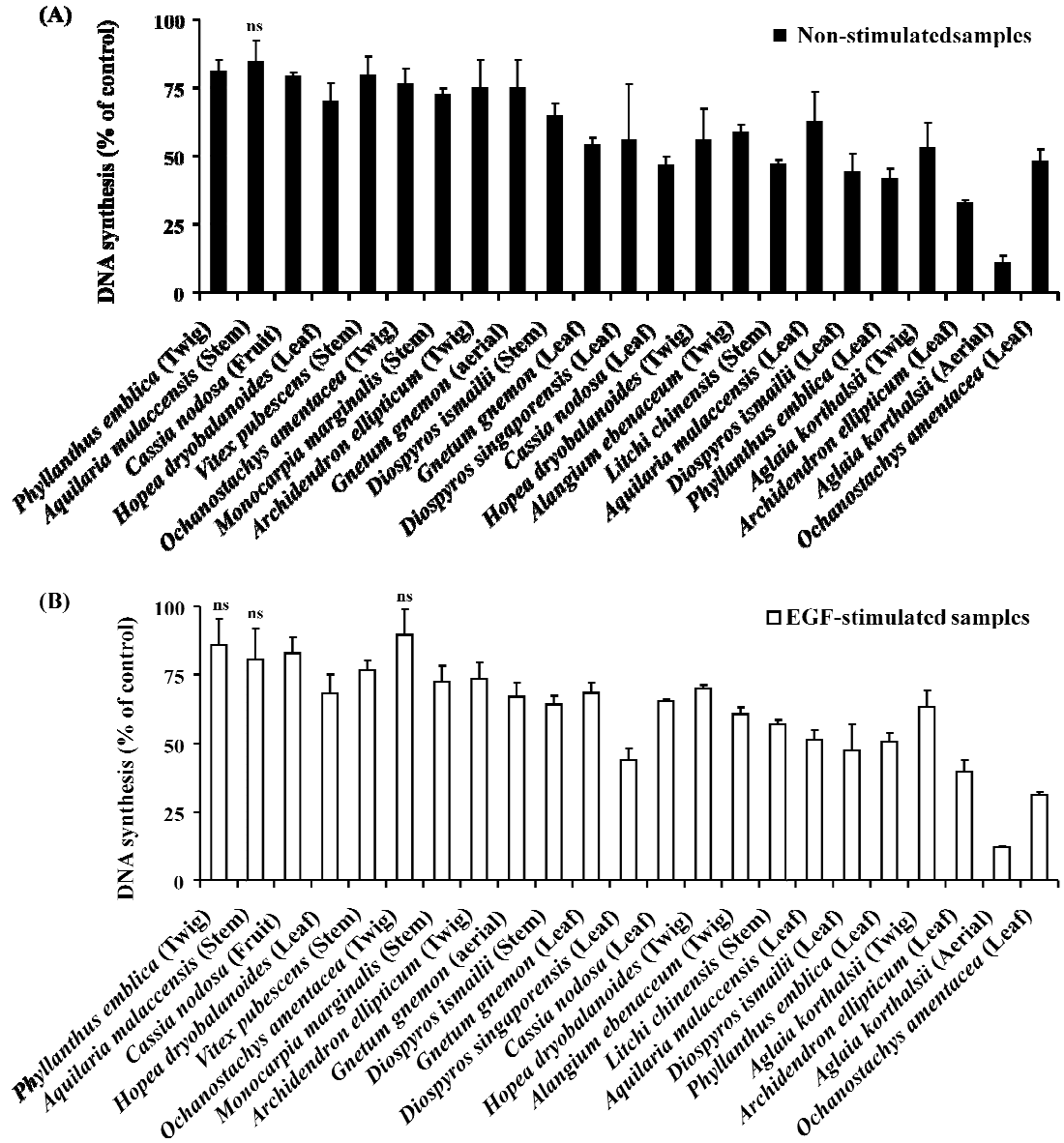
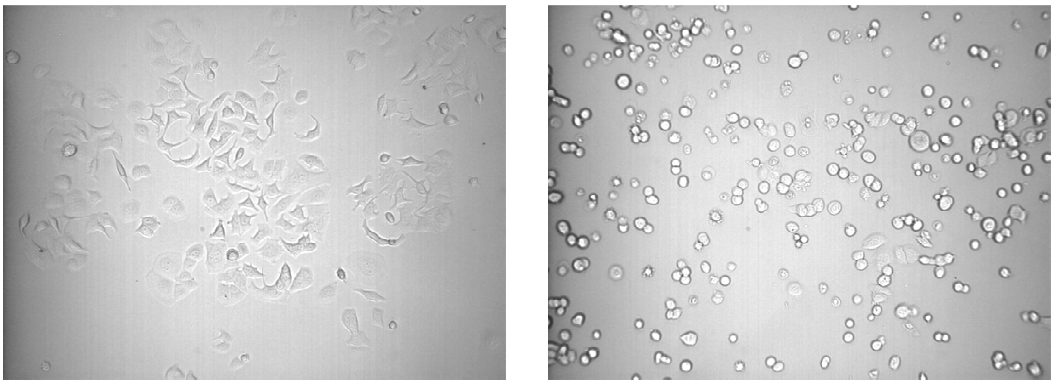


Figure 3.8 Inhibitory effects of 1 µg/ml of plant extracts on DNA synthesis. Quiescent cultures of MCF-7 Neo cells were treated with 1 µg/ml of plant extracts or vehicle control (0.05% DMSO) for 15 mins without stimulation (A) or stimulated with 25ng/ml EGF (B). Cells were then incubated for 15 hrs and then with [^{3}H]-thymidine (0.5 µCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, $p<0.05$ for all samples *versus* control, except those labelled with "ns", not significant, $p>0.05$. Similar results were observed in three independent experiments.

Visible changes in cellular morphology could be observed in cells treated with *Ochanostachys amentacea* leaf extract (Fig. 3.9B). Cells were observed to be rounded which could be due to detachment from monolayers. Pre-treatment with EGF did not reverse the changes in cell morphology (Fig. 3.9D). Investigation of biochemical changes such as caspase activation could establish whether cell death was also induced. Of note, cell proliferation was arrested since DNA synthesis was completely abolished by the extract at 5 µg/ml (Fig. 3.7A).

(A) **(B)**



(C) **(D)**

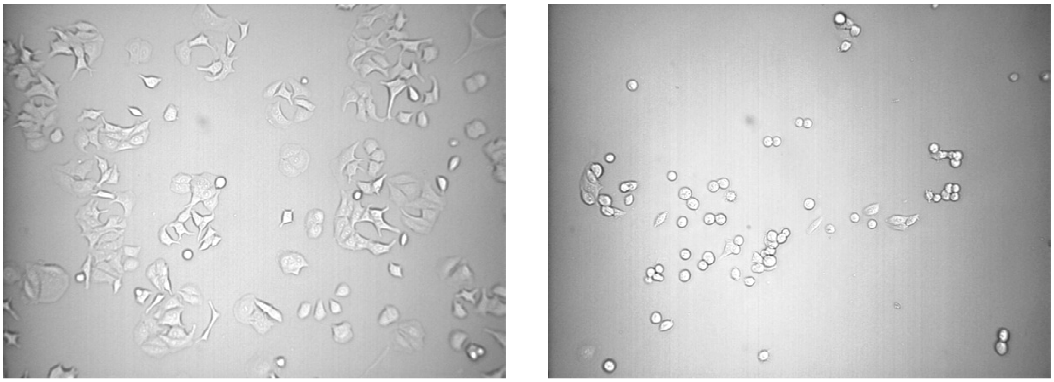


Figure 3.9 Effect of *Ochanostachys amentacea* leaf extract on cell morphology. Images were taken (at 100 times magnification under a light microscope) 15 hrs after quiescent MCF-7 Neo cells were treated with (A) 0.05% DMSO, (B) 5 µg/ml plant extract, (C) 25 ng/ml EGF and (D) 25 ng/ml EGF and 5 µg/ml plant extract. Results are representative of 3 independent experiments.

3.2.2 Effects of plant extracts on SK1 activity

To narrow down selection of plants for further investigations, an enzymatic assay that measures the activity of SK1 was performed using HEK 293 cells stably overexpressing SK1 (~20-fold increase in SK1 activity *versus* wild-type HEK 293 cells) (Fig. 3.10A). In each experiment, 10 μM of sphingosine and 250 μM of [^{32}P]-ATP were used as substrates including a positive control containing the competitive SK inhibitor, DMS and a vehicle control (5% DMSO). The assay measures radiolabelled [^{32}P]-S1P which partitions into the organic phase, with unreacted [^{32}P]-ATP remaining in the aqueous phase. In order to eliminate the possibility of DMSO interfering with activity measurement, increasing concentrations of DMSO were added in the assay buffer. As demonstrated in Fig. 3.10B, DMSO (up to a concentration of 15%) had no significant effect on SK1 activity in this assay, indicating that the assay can tolerate a high concentration of DMSO.

Initially, plant extracts were tested at 5 μg/ml but no appreciable effects were observed. Therefore, plant extracts of high concentration were tested. As shown in Fig. 3.11, several plant extracts such as *Phyllanthus emblica* (leaf), *Aquilaria malac*censis (stem) and *Hopea dryobalanoides* (leaf) extracts strongly inhibited SK1 activity with more than 80% inhibition. These plant extracts also inhibited SK1 activity in a concentration-dependent manner (Fig. 3.12). Therefore, these plants extracts were selected for further investigations. It has previously been demonstrated that SK1 is a pro-growth, pro-migratory and anti-apoptotic protein in MCF-7 cells (Sarkar *et al*., 2005; Long *et al*., 2010a). In the present study, MCF-7 Neo cells were treated with SK inhibitors (DMS and SKi) and *Hopea dryobalanoides* (Leaf) extract to investigate their effects on cell growth and ERK1/2 activation. As shown in Fig. 3.13, DMS, SKi and *Hopea dryobalanoides* (Leaf) extract inhibited basal and EGF-induced DNA synthesis, indicating they are effective cell proliferation inhibitors. Additionally, EGF and S1P stimulated proliferation of MCF-7 Neo cells (EGF typically induced ~2-fold increase in DNA synthesis, Fig. 3.6) and induced ERK1/2 phosphorylation (Fig. 3.14), which is reminiscent of their roles in promoting tumourigenesis of breast cancer cells (Nava *et al*.,

2002). Prolonged treatment with SK inhibitors or *Hopea dryobalanoides* (Leaf) extract induced cleavage of the nuclear enzyme poly(ADP-ribose)polymerase (PARP), a marker of apoptosis (Oliver *et al*., 1998). S1P and EGF-dependent ERK1/2 activation were also reduced (Fig.3.14). In the case of *Hopea dryobalanoides* (Leaf) extract, ERK1/2 activation induced by either S1P or EGF was also impaired. These data suggest that *Hopea dryobalanoides* (leaf) extract contained cytotoxic constituents.

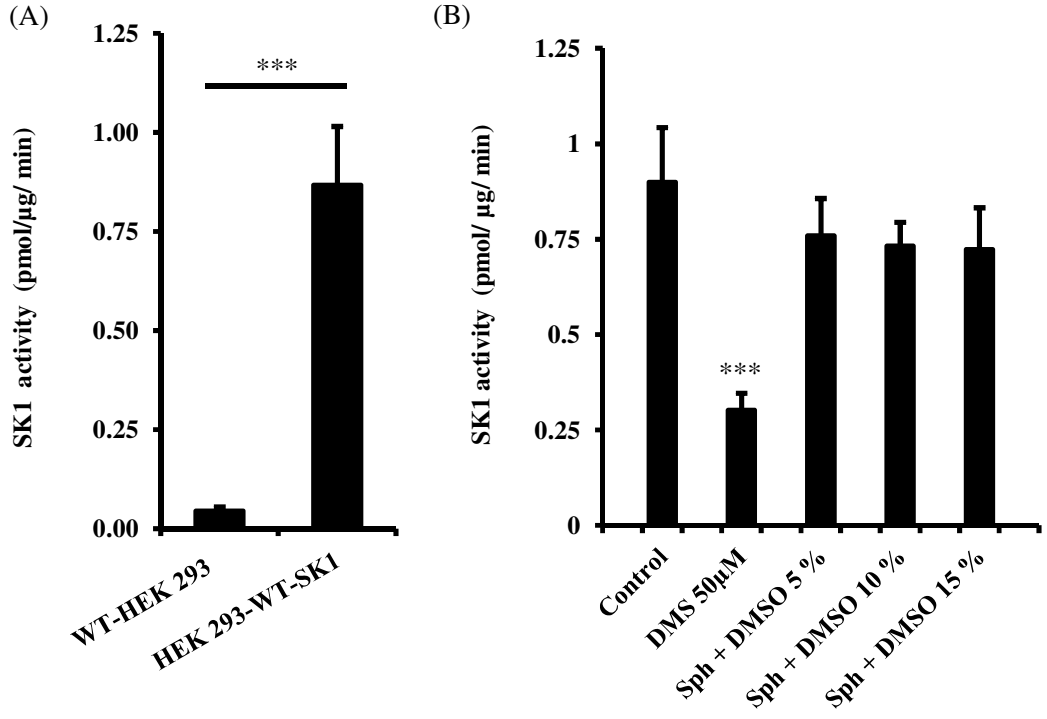


Figure 3.10 Activity of wild type-SK1 stably expressed in HEK 293 cells. (A) Wild type HEK 293 cells (WT-HEK 293) and HEK 293 cells stably expressing wild-type SK1 (HEK293-WT-SK1) were assayed for SK1 activity**,** ***p<0.001. **(B)** Effect of DMSO on SK1 activity**.** Activity was measured as described under Methods (section 2.2.15) without or with DMSO in varying concentrations using HEK-293-WT-SK1 in the presence of 10 µM sphingosine and 250 µM [^{32}P]-ATP as the substrates. DMS (50 µM) was included as a positive control. Data are expressed as means and standard deviations of triplicate determinations, ***p<0.001 *versus* control. No significant difference exists between control and samples containing different concentrations of DMSO, p>0.05.

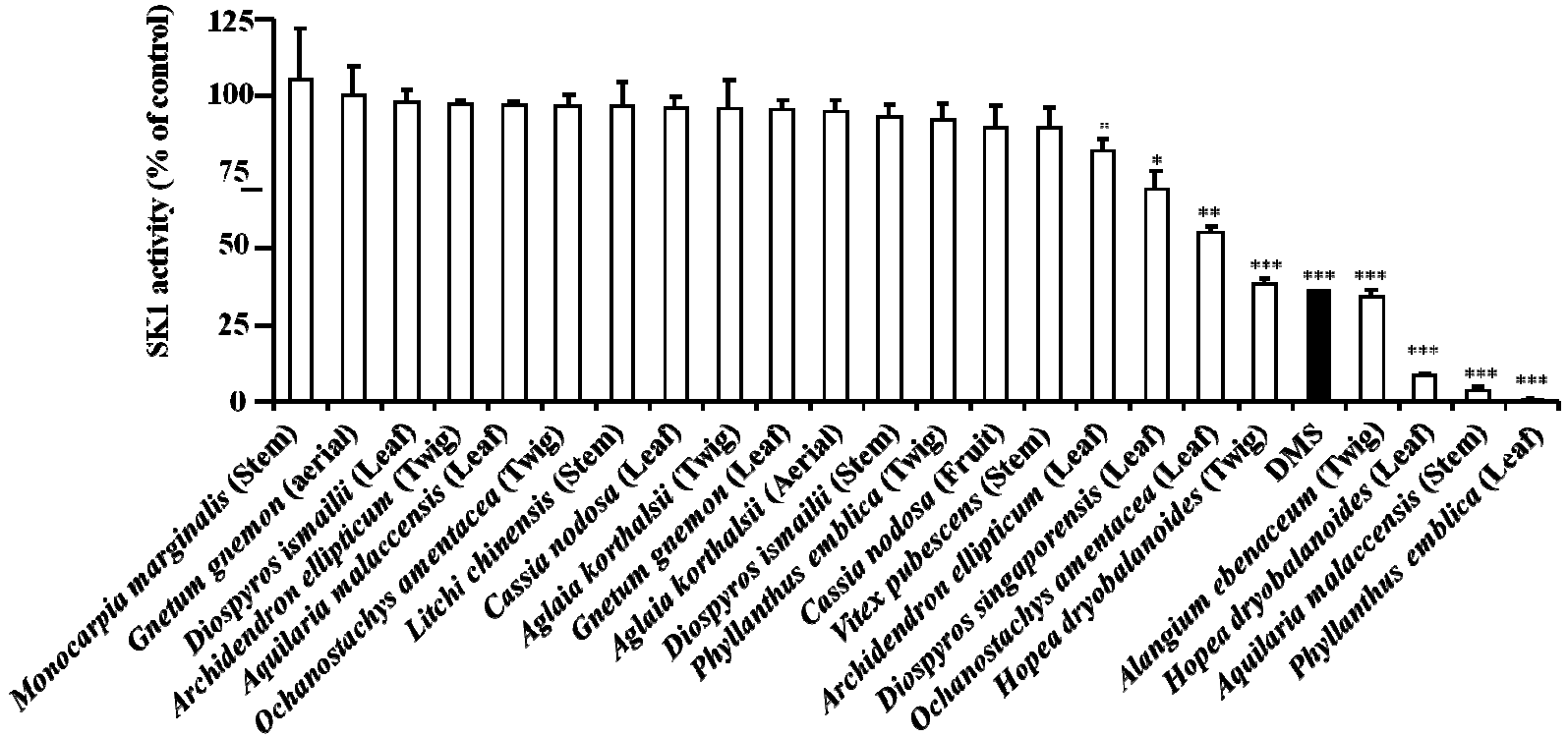


Figure 3.11 Effects of plant extracts on SK1 activity. SK1 activity was measured as described under Methods (section 2.2.15) using 10 µM sphingosine and 250 µM [^{32}P]-ATP as substrates in the presence of plant extracts (500µg/ml) or positive control (DMS, 50 µM; full bar) and HEK 293 cell lysates containing over-expressed recombinant SK1. Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, *p<0.05, **p<0.01, ***p<0.001 *versus* control. Similar results were obtained in three independent experiments.

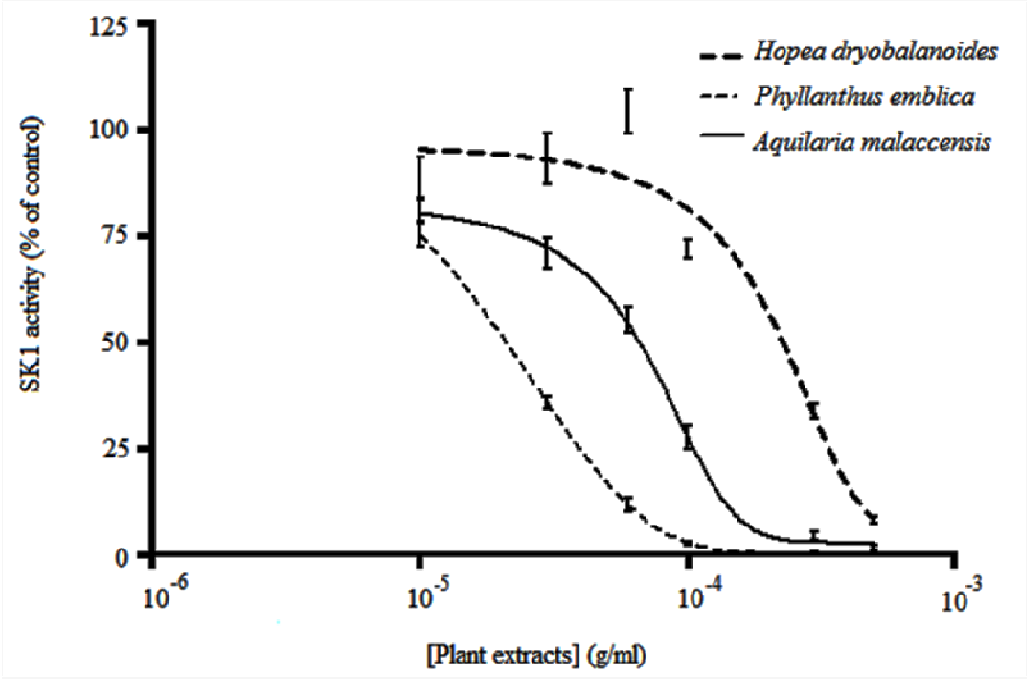


Figure 3.12 Concentration-dependent inhibitory effects of plant extracts on SK1 activity. Enzyme activity was measured as described under Methods (section 2.2.15) using 10 µM sphingosine and 250 µM [^{32}P]-ATP as the substrates and HEK 293 cell lysates containing over-expressed recombinant SK1 with increasing concentration of plant extracts. Data are expressed as means +/- standard deviations of triplicate determinations.

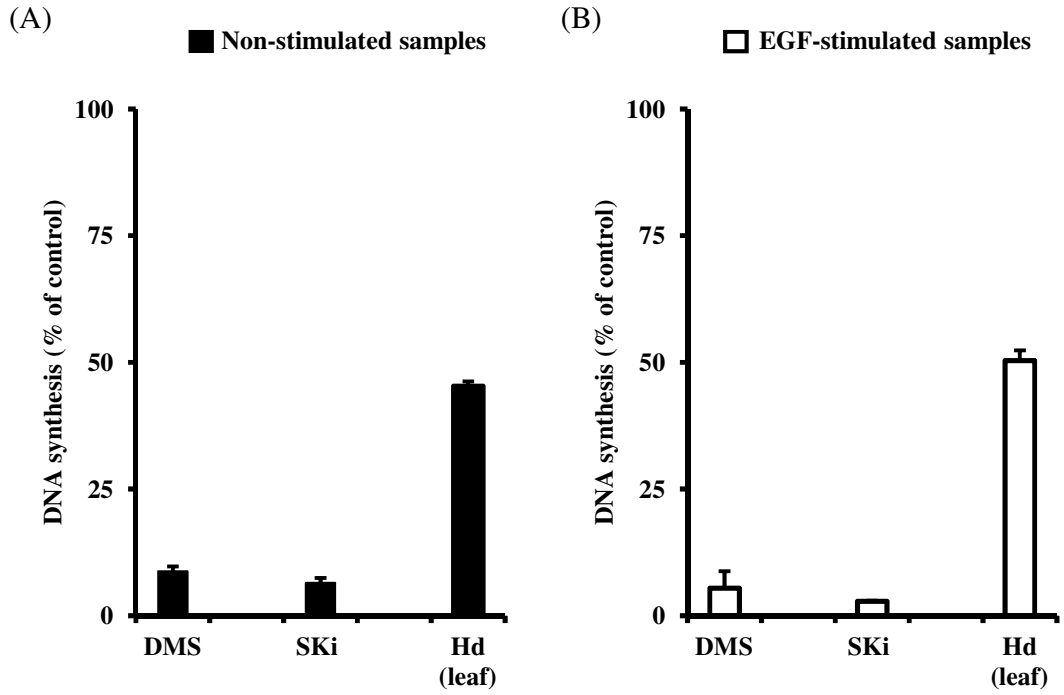


Figure 3.13 Inhibition of DNA synthesis by SK inhibitors. Quiescent MCF-7 Neo cells were treated with 10 µM DMS, 10 µM SKi, 5 µg/ml Hd (leaf), *Hopea dryobalanoides* leaf extract or vehicle control (0.05% DMSO) for 15 mins without stimulation **(A)** or stimulated with 25 ng/ml EGF **(B)**. Cells were then incubated for 15 hrs and then with [^{3}H]-thymidine (0.5 µCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, $p<0.001$ for all samples *versus* controls. Similar results were obtained in three independent experiments.

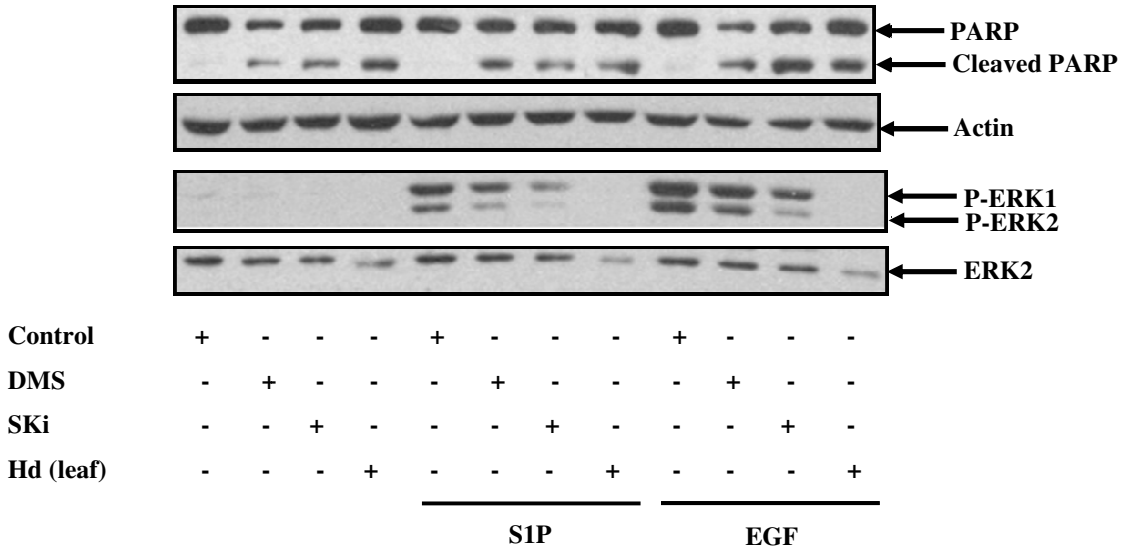


Figure 3.14 Inhibition of S1P or EGF-induced ERK1/2 phosphorylation and induction of PARP cleavage by SK inhibitors. Quiescent MCF-7 Neo cells were incubated with vehicle control (0.1% DMSO) or indicated inhibitors (10 µM DMS, 10 µM SKi and 500 µg/ml *Hd* leaf, *Hopea dryobalanoides* leaf extract) for 48 hrs before being stimulated by 25 ng/ml EGF or 1 µM S1P for 5 mins. Cell lysates were separated by SDS-PAGE and immunoblotted with anti-PARP or anti-phospho-ERK1/2 antibodies according to Methods (section 2.2.10 and 2.2.11). Blots were then stripped and reprobed with anti-actin or anti-ERK2 antibodies to ensure comparable protein loading. Results are representative of three independent experiments.

3.2.3 Novel synthetic compounds inhibit SK1 and/or SK2

Meanwhile, previous screening of QAB compound series (SKI analogues) has identified two novel compounds, QAB061 and QAB108 which inhibited ~30% of SK1 activity (Fig. 3.15A). However, QAB061 and QAB108 inhibited DNA synthesis of MCF-7 Neo cells to a different extent (Fig.3.15B); QAB061 was more effective than QAB108. Therefore, it is important to compare the effects of hits in a cell-based assay to verify their potential to be considered as leads for further drug development.

It has also been reported that the immunosuppressant, FTY720 inhibits partially purified SK1 activity isolated from rat heart (Vessey *et al*., 2007). In the present study, FTY720 was found to inhibit purified SK1 activity (Fig 3.16). FTY720 also competed with sphingosine as a substrate for SK2, thereby reducing SK2 activity (Billich *et al*., 2003). In addition, several synthetic analogues such as (*S*)-FTY720-vinylphosphonate (more effective than (*R*)-isomer) also inhibited purified SK1 whereas (*R*)- or (*S*)-FTY720 phosphonate were inactive (Fig.3.16). In contrast, (*R*)-FTY720-OMe inhibited SK2 but not SK1; the enantiomer, (*S*)-FTY720-OMe did not inhibit SK1 or SK2, demonstrating stereospecific inhibition with (*R*)-FTY720-OMe. SKi and (*S*)-FTY720 vinylphosphonate also inhibited purified SK2 effectively, while (*R*)-FTY720 vinylphosphonate and (*S*)-FTY720 phosphonate were moderately active (20-30% inhibition) and (*R*)-FTY720 phosphonate was inactive (Fig. 3.16). Collectively, these findings show that (*R*)-FTY720-OMe is a selective SK2 inhibitor while SKi and (*S*)-FTY720 vinylphosphonate are dual SK1 and SK2 inhibitors.

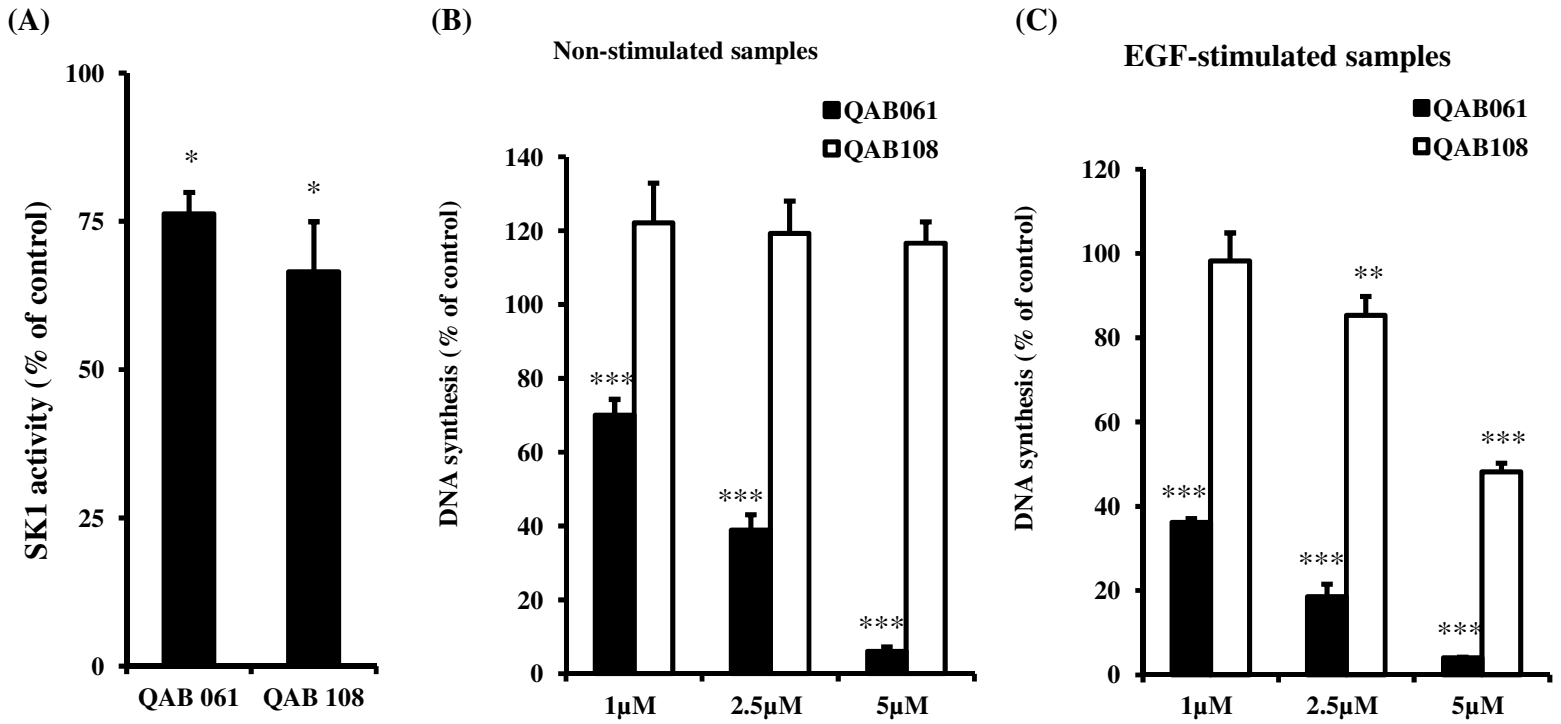


Figure 3.15 Effects of QAB compounds on SK1 activity and DNA synthesis. (A) SK1 activity was assayed as described under Methods (section 2.2.15) using 10 µM sphingosine and 250 µM [^{32}P]-ATP as the substrates and HEK 293 cell lysates containing over-expressed recombinant SK1 in the presence of QAB 061 and QAB 108 (both at 50 µM). Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, *p<0.05 *versus* control. Quiescent MCF-7 Neo cells were treated with QAB061 and QAB108 at the indicated concentrations for 15 mins and then stimulated without **(B)** or with EGF 25 ng/ml **(C)**. Cells were then incubated for 15 hrs and then with [^{3}H]-thymidine (0.5 µCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations, **p<0.01, ***p<0.001 *versus* control.

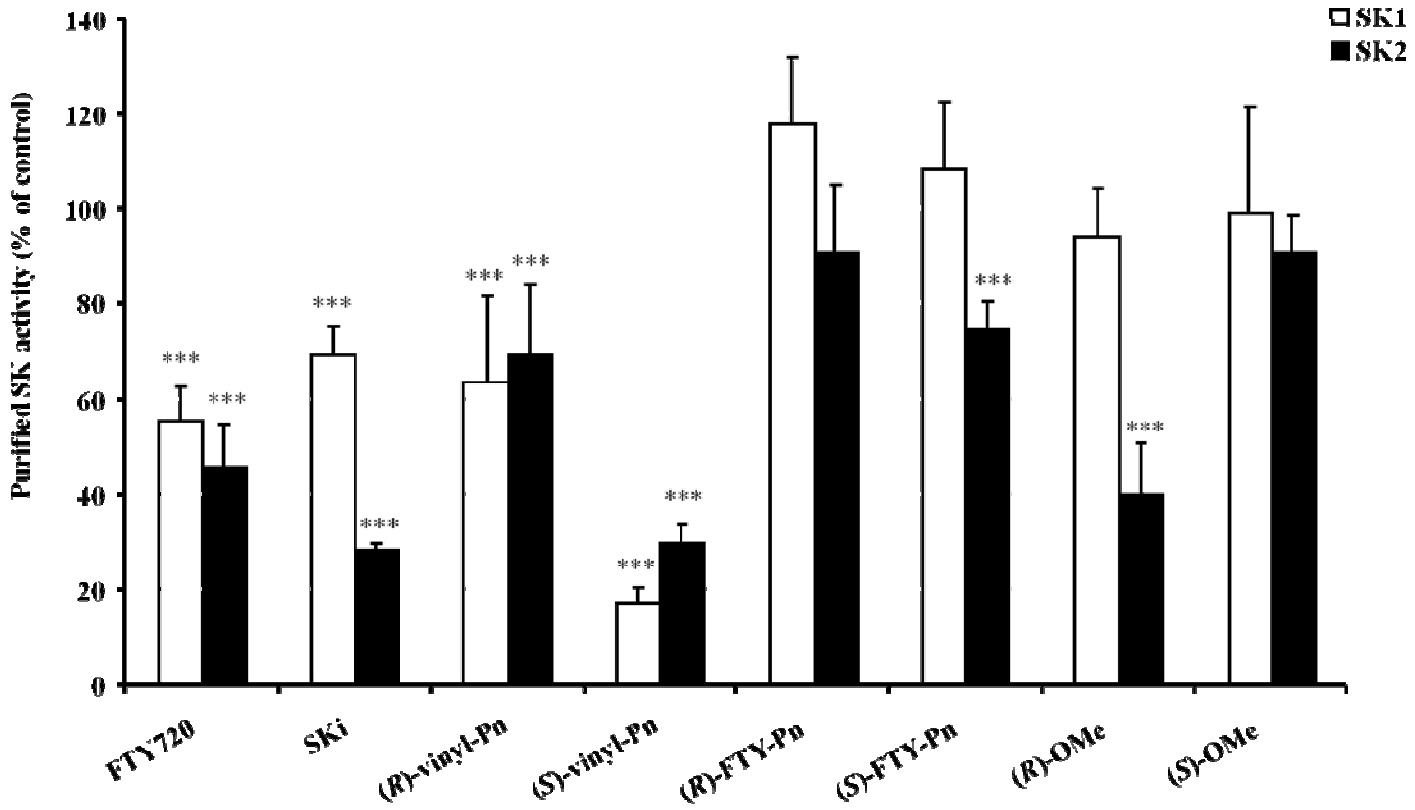


Figure 3.16 Inhibition of SK1 or SK2 activity by SKi, FTY720 and FTY720 analogues. Effect of SKi, FTY720 and six FTY720 analogues: (*R*)-FTY720 vinylphosphonate, (*R*)-vinyl Pn; (*S*)-FTY720 vinylphosphonate, (*S*)-vinyl Pn; (*R*)-FTY720 phosphonate, (*R*)-FTY Pn; (*S*)-FTY720 phosphonate, (*S*)-FTY Pn; (*R*)-FTY720-OMe, (*R*)-OMe; (*S*)-FTY720-OMe, (*S*)-OMe on purified SK1 or SK2 activity. SK activity was assayed as described under Methods (section 2.2.15) using 10 μM sphingosine and 250 μM [^{32}P]-ATP as substrates. All inhibitors were screened at 50 μM. Data are expressed as percentage of control and represent means and standard deviations of six determinations, ***p<0.001 *versus* control.

In the course of identifying novel SK inhibitors from the thiazole-like compounds, QAB 801A was identified as the most effective SK1 inhibitor (Fig. 3.17) achieving almost 50% inhibition of purified SK1 activity. Of note, a close structural analogue, QAB 801 which differs in only one substituent at the carboxylic functional group (a methyl side chain rather than a hydroxyl group; Fig. 3.2) renders the molecule inactive. This indicates that the hydroxyl group may be essential for efficient binding to SK1. Interestingly, QAB 143A appeared to stimulate SK1activity but the effect was not significant (Fig. 3.17).

Finally, another screening effort also yielded several SK1 inhibitors with moderate efficacy (Fig. 3.18). HB 67-87a and HB 67-88a inhibited ~30% of purified SK1 *versus* control whereas a related analogue HB 67-90a was inactive. Despite showing significant inhibition, GS246P, LR230, LR232, HB 55-21 and HB 55-22 are weak SK1 inhibitors. By contrast, bodipy-sphingosine (LZ566d) and (*S*)-FTY720 regioisomer did not inhibit but stimulated purified SK1 activity (Fig. 3.18). These compounds were also weak substrates for SK1 (see chapter 4, Fig. 4.5).

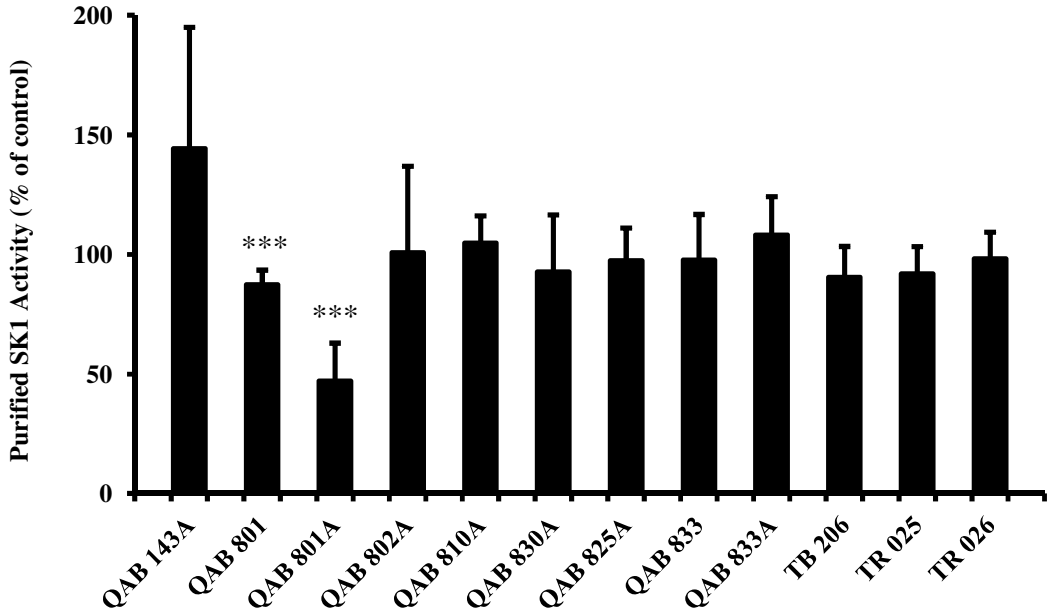


Figure 3.17 Inhibition of SK1 activity by QAB and TR compounds. Purified SK1 activity was assayed as described under Methods (section 2.2.15) using 10 μM sphingosine and 250 μM [^{32}P]-ATP as substrates. All inhibitors were tested at 50 μM. Data are expressed as percentage of control and represent means and standard deviations of six determinations, ***p<0.001 *versus* control.

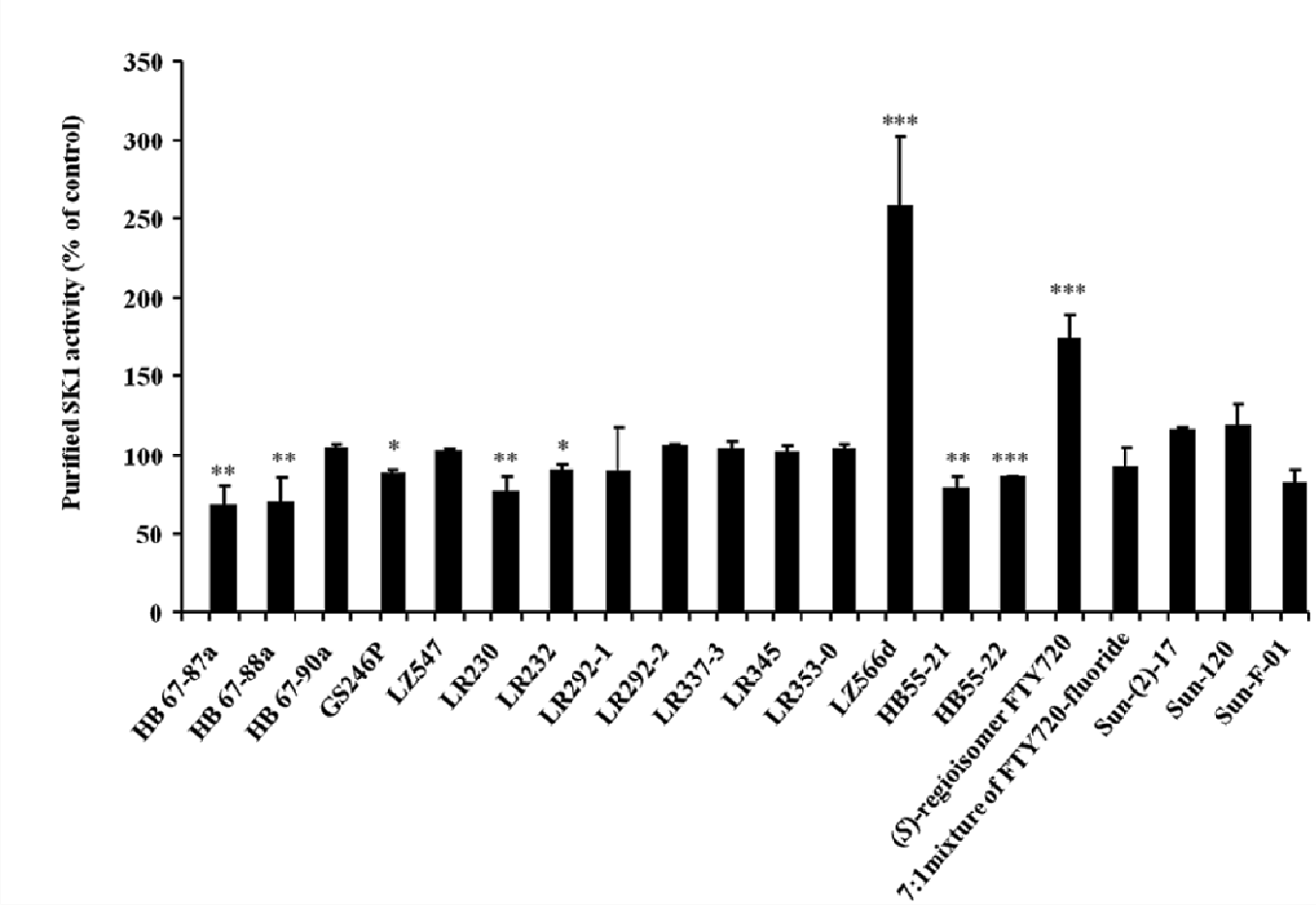


Figure 3.18 Effects of novel synthetic compounds on SK1 activity. Purified SK1 activity was assayed as described under Methods (section 2.2.15) using 10 μM sphingosine and 250 μM [^{32}P]-ATP as substrates. All inhibitors were tested at 50 μM. Data are expressed as percentage of control and represent means and standard deviations of two to four determinations, *p<0.05, **p<0.01, ***p<0.001 *versus* control.

3.3 Discussion

The presence of SK inhibitors from plants has been investigated in this study. The major finding of the preliminary screening is that plants produce compounds which can inhibit SK1 activity. In addition, FTY720 and its analogues inhibit SK1 and/or SK2. Several synthetic compounds such as QAB 801A, HB 67-87a and HB 67-88a are also effective SK1 inhibitors. Unexpectedly, two compounds (bodipy-sphingosine and (*S*)-FTY720 regioisomer are activators of SK1 activity. Therefore, several hits from both natural and synthetic sources have successfully been discovered. These findings will be discussed with the effects of these compounds on MCF-7 cell growth.

3.3.1 Inhibition of SK1 and MCF-7 cell growth by plant extracts

S1P signalling is evolutionarily conserved in many organisms such as yeasts, worms, plants and animals. In plants, S1P is involved in the regulation of seed germination and stomata opening (Worrall *et al*., 2008). However, S1P receptors have not been found in plants. Therefore, in contrast to human, S1P may have different cellular targets in other organisms. Additionally, isolation of SK inhibitors from bacteria and fungi indicates a role for these molecules in regulating immune function since S1P is required for lymphocyte recirculation, a blockage of which could be advantageous for the pathogens. Therefore, successful isolation and characterisation of the effects of these compounds from plants could provide valuable insights into the roles of SK/S1P signalling in cell biology.

Preliminary data indicate that potential useful anticancer compounds are produced by several species of plants, which may act on SK/S1P signalling pathway to inhibit cancer cell proliferation. MCF-7 cells have previously been shown to be dependent on SK1 for proliferation, survival and migration (Sarkar *et al*., 2005). Moreover, high expression of SK1 in ER^+ breast cancer patients has been correlated with decreased survival rate and reduced recurrence time (i.e. the development of resistance to tamoxifen) (Long *et al*., 2010a). Therefore, use of this cell line for preliminary

screening is appropriate to identify SK1 inhibitors. All selected plant extracts inhibit basal and EGF-induced DNA synthesis of MCF-7 cells, suggesting that these plants produce cytotoxic metabolites. This is in agreement to some of the anticancer effects reported on these plants (see section 3.1.1). Since only crude plant extracts were examined in this study, it is difficult to conclude whether the effects were due solely on a single cytotoxic constituent. Indeed, plants produce more than one cytotoxic compound. For example, at least 10 distinct cytotoxic compounds have been isolated from *Phyllanthus emblica*; these compounds were active (IC50: 2-50µg/ml) against the growth of gastric, uterine and skin cancer cells (Zhang *et al*., 2004). Similarly, *Hopea dryobalanoides* also produces polyphenolic compounds which are cytotoxic against several tumour cell lines (see chapter 5). Therefore, further studies are needed to isolate these cytotoxic compounds and examine their biological effects.

In the present study, EGF has been used to stimulate DNA synthesis. EGF induces a higher uptake of [^{3}H]-thymidine into the cells than that of S1P. It has previously been shown that S1P only moderately stimulates DNA synthesis in MCF-7 cells, depending partly on type II insulin-like growth factor (IGF-II) secretion (Nava *et al*., 2002). S1P also binds to $S1P_3$ and activates ERK1/2 in MCF-7 cells to stimulate cell migration (Long *et al*., 2010a). Acting directly on the EGFR/Ras/MAPK pathway, EGF also induces ERK1/2 phosphorylation which is stronger than that induced by S1P (Fig. 3.10; Long *et al*., 2010a). EGF can also activate SK1 (Sarkar *et al*., 2005) and may participate in sequential or criss-cross signalling between oestrogen, EGF and S1P (Sukocheva *et al*., 2006). Taken together, the present study confirms the role of EGF as an effective mitogen in MCF-7 cells.

Twenty three plant extracts have been screened in this study. In total, fourteen plants have been investigated because some extracts have been prepared from different parts of the same plant. This allows better characterisation of the constituents produced by different parts of the plants. Indeed, extracts obtained from different parts of a plant do not have the same activity against DNA synthesis, suggesting that different compounds are produced. For example, leaf extract of *Ochanostachys amentacea* is more effective than the twig extract in inhibiting DNA synthesis.

Among all these extracts, only *Ochanostachys amentacea* leaf extract induces morphological changes at the specified time-course and concentration (Fig. 3.9). Cell rounding and membrane disruption are typical of cells treated with the plant extract. Biochemical tests that examine activation of apoptotic proteins such as caspase activation and PARP cleavage can validate whether apoptosis is induced. It has previously been reported that potent cytotoxic polyacetylenes such as minquartynoic acids were isolated from the twig of *Ochanostachys amentacea* which inhibited the growth of a panel of human cancer cell lines including breast cancer (Ito *et al*., 2001a). Thus, it was of interest to investigate whether the plant extract could inhibit SK1 due to the presence of these lipidic inhibitors. Besides, all plant extracts were active in reducing [^{3}H]-thymidine uptake in MCF-7 cells. Therefore, other biochemical tests are needed to select candidate plants for further investigation.

Plant extracts at low concentration were generally inactive at inhibiting SK1 activity and subsequently higher concentrations had to be used. There are several reasons for modifying the concentrations of plant extracts used in cell-based *versus in vitro* enzymatic assays. First, crude plant extracts contain many compounds, some of which are inactive and the active components may present in a low quantity. Second, living cells may actively take up inhibitors from the extracellular environments through functional proteins and transporters which effectively concentrate inhibitors within specific cell compartments. Third, the amount of substrate present in a cell can be very different from that of an *in vitro* enzymatic experiment. Due to different substrate concentrations, the potency of an enzyme inhibitor will vary. Therefore, higher concentration may have to be used when screening plant extracts against an artificial substrate concentration in an enzymatic assay. In this regard, the amount of sphingosine in cells is not known but may usually be low as its production is rapidly balanced by metabolic conversion to ceramide or S1P. Nevertheless, unlike cell-based assays which can only tolerate low concentration of DMSO (<1%), there is less stringent control on the SK1 activity assay as evidenced by good tolerability to high concentrations of DMSO (Fig. 3.10).

Several hits have been identified by screening all plant extracts in the SK1 activity assay. Despite not being the most active extract, *Ochanostachys amentacea* leaf extract also inhibited SK1 moderately (~45% inhibition). However, the twig extract from the same plant was inactive even though it inhibited DNA synthesis in MCF-7 cells. This suggests different compounds are present in these extracts. The major outcome of this screening is that several extracts such as *Hopea dryobalanoides* (leaf), *Aquilaria malaccensis* (stem), *Phyllanthus emblica* (leaf) extracts, strongly inhibited SK1 activity. These extracts also inhibited SK1 in a concentration-dependent manner (Fig. 3.12). Since the active compounds responsible for the inhibition are not known, these extracts warrant further study which includes fractionation of the extracts and purification of active compounds to establish their effects on SK/S1P dependent cell proliferation and migration.

The effects of SK inhibitors and *Hopea dryobalanoides* extract on ERK1/2 activation were also investigated. The mitogenic MAPK, ERK1/2 plays a pivotal role in cell proliferation and migration which are implicated in cancer progression (Boutros *et al*., 2008). In MCF-7 cells, the growth-promoting effect of S1P is closely linked to EGFR signalling because S1P can activate ERK1/2 indirectly via EGF receptor transactivation and directly by binding to $S1P_{1-3}$ receptors (Sarkar *et al*., 2005; Sukocheva *et al*., 2006; Long *et al*., 2010a). EGF induces ERK1/2 activation primarily by binding to cognate cell surface receptors. For example, the Ras/MAPK pathway is activated when EGFR is ligated by EGF. EGF can also stimulate SK which in turn contributes to EGF-mediated calcium signalling (Meyer zu Heringdorf *et al.*, 1999). In addition, EGF-induced cell migration and ERK1/2 activation, is partly dependent on S1P signalling (Le Stunff *et al*., 2004). In the present study, treatment of MCF-7 Neo cells with SK inhibitors (SKi and DMS) reduced S1P and EGF-dependent activation of ERK1/2 (Fig. 3.11). Paugh and colleagues (2008) found that a selective SK1 inhibitor, SK1-I not only inhibits ERK1/2 activation but also deactivates Akt and induces profound apoptosis in leukaemia cells. In agreement, SKi also induced apoptosis in MCF-7 cells as evidenced by PARP cleavage (Fig. 3.11). Moreover, SKi also inhibited DNA synthesis (Fig. 3.10) and has been shown by others to inhibit MCF-7 cell proliferation and clonogenic survival (Antoon *et al*.,

2011). Therefore, impaired ERK1/2 activation and induction of apoptosis could be linked to impaired SK/S1P signalling.

Depending on cell type, agonists and the use of either genetic or chemical tools to perturb SK/S1P signalling, the subsequent effects on agonists-induced ERK1/2 activation may vary. For example, treatment with DMS, siRNA knockdown of SK1 and over-expression of a SK1 (G81D) mutant in T24 bladder cancer cells reduced VEGF-induced ERK1/2 activation, suggesting that VEGF signalling to ERK1/2 is dependent on SK1 (Shu *et al*., 2002). Similarly, siRNA knockdown of SK1 in MCF-7 Neo cells reduced S1P-induced but not EGF-induced ERK1/2 activation (Long *et al*., 2010a). Over-expression of S1P phosphatases 1 in HEK 293 cells reduced intracellular S1P levels and inhibited both S1P and EGF-induced ERK1/2 activation (Le Stunff *et al*., 2004). In this regard, prolonged treatment of MCF-7 Neo cells with SKi reduced both S1P and EGF-induced ERK1/2 activation, suggesting that SKi inhibits SK1 and reduces intracellular S1P to impair S1P and EGF signalling (Fig. 3.14). Alternatively, SKi may have additional effects/targets. Indeed, SKi has been suggested to bind to the antagonist-binding domain on oestrogen receptor, down-regulating transcriptional expressions of progesterone receptor and steroid-derived factor 1, thereby modulating oestrogen signalling in MCF-7 cells (Antoon *et al*., 2011). Oestrogen-induced ERK1/2 activation in MCF-7 cells may also be reduced by SK1 inhibitors since S1P export is induced by oestrogen-dependent SK1 activation which is required for S1P-mediated EGFR transactivation (Sukocheva *et al*., 2006). Indeed, siRNA knock down of SK1 or over-expression of a catalytically deficient G82D SK1 reduced oestrogen-dependent ERK1/2 activation (Sukocheva *et al*., 2006). Collectively, SK1 inhibitors reduce agonist-induced ERK1/2 activation, inhibit cell proliferation and induce apoptosis. Interestingly, *Hopea dryobalanoides* extract also showed a similar inhibitory profile as SK inhibitor, suggesting that the plant could produce compounds that inhibit SK/S1P signalling pathway.

More recently, Loveridge *et al*., (2010) demonstrated that SKi induces proteasomal degradation of SK1. There are at least two non-mutually exclusive models that explain these findings. First, catalytic inhibition of SK1 perturbs the dynamic balance

of sphingosine, ceramide and S1P in the cells such that formation of S1P is reduced whereas ceramide level is increased. Ceramide in turn activates proteasome and promotes degradation of polyubiquitinated SK1. Second, SKi not only activates proteasome but may also stabilise a conformational state of SK1 that is more readily recognised by the ubiquitin-proteasome system, hence accelerating SK1 down-regulation (see chapter 4; Loveridge *et al*., 2010). These important findings enable the development of a novel cell-based assay which can link the effects of SK1 inhibition to SK1 down-regulation. Therefore, formal investigation is needed to establish whether novel SK1 inhibitors (both synthetic and natural origins) identified from this study can affect SK1 expression.

3.3.2 Novel synthetic compounds as SK1/SK2 inhibitors

Meanwhile, several synthetic thiazole analogues have also been investigated in the present study. QAB 061 and QAB 108 are thiazoles, as is the commercially available SK inhibitor, SKi. Interestingly, even though both QAB compounds inhibit SK1 activity, they do not have the same efficacy in inhibiting DNA synthesis. This may be due to different solubility of the compounds. Solubility is an important consideration in the development of many drug leads. An effective lead must be sufficiently water soluble and possess the optimal partition coefficient for it to permeate cells. It may also be due to different three-dimensional configuration of these molecules because inhibitor binding to target is stereospecific. Another possibility is that different enzyme isoforms (SK1 *versus* SK2) may be inhibited by these compounds resulting in different actions on DNA synthesis. Therefore, further study on these compounds together with other synthetic or novel SK inhibitors isolated from plants will provide valuable insights into the SAR of these inhibitors.

Another major finding from this study is that some S1P agonists may also act as SK inhibitors. Apart from binding to S1P receptors after phosphorylation, FTY720 also inhibits partially purified SK1 activity from rat heart (Vessey *et al*., 2007). However, the findings from the present study are unequivocal as they have been obtained using purified SK1 (Fig. 3.16). These findings also suggest a close functional relationship

between SK inhibitors and S1P receptor ligands. Indeed, (*S*)-FTY720 vinylphosphonate not only inhibits SK1 but also acts as a pan-antagonist for S1P receptors (Valentine *et al*., 2010). (*S*)-FTY720 vinylphosphonate partially antagonises $S1P_2$ and $S1P_5$ but fully antagonises $S1P_1$, $S1P_3$ and $S1P_5$ (Valentine *et al*., 2010). Interestingly, (*S*)-FTY720 vinylphosphonate is a more effective SK1 inhibitor than FTY720. At the same concentration and under the same assay condition, (*S*)-FTY720 vinylphosphonate causes ~80% reduction of SK1 activity compared to ~40% inhibition by FTY720. Therefore, further investigations including kinetic studies are needed to address the difference in efficacy for these closely related structural analogues.

Unlike FTY720 which is a substrate for SK2, (*S*)-FTY720 vinylphosphonate cannot be phosphorylated and is therefore not a competing substrate. The reduction in SK2 activity by (*S*)-FTY720 vinylphosphonate and SKi indicate that these inhibitors are dual SK1 and SK2 inhibitors. (*R*)-FTY720 vinylphosphonate has moderate efficacy in inhibiting both SK1 and SK2, suggesting that the (*S*)-isomer has a better fit to the binding site on the enzymes. Other stereoselective inhibition has been observed with (*S*)-FTY720 phosphonate which is a weak SK2 inhibitor whereas the (*R*)-isomer is inactive. Another significant finding from this study is that (*R*)-FTY720-OMe is the only effective and selective SK2 inhibitor among all analogues tested in the present study. Taken together, it is possible to develop effective and selective SK1 and SK2 inhibitors from structural modification of FTY720. The fact that different isomers have different efficacy further confirms that SK behaves as a typical enzyme where catalysis and ligand binding are stereospecific.

In another screening effort, QAB 801A is the most effective SK1 inhibitor identified from the QAB compound series so far, achieving ~ 50% inhibition of SK1 activity. More work is needed to assess whether QAB 801A is selective against SK1. HB 67-87a, HB 67-88a, LR230, HB 55-21 and HB 55-22 have all been found to inhibit SK1 but they only inhibit <30% of SK1 activity. It should be noted that SKi also inhibits ~30-40% of SK1 activity, yet it is a very potent anticancer agent *in vivo* (French *et al*., 2006). Therefore, the identification of these novel compounds as hits against SK1

warrants further investigation on their selectivity and biological effects on cell-based assays. Eventually, the anticancer effects should be confirmed by *in vivo* experiments using xenograft tumour models.

In summary, preliminary screening of plant extracts, FTY720 analogues, thiazole analogues and other novel compounds has led to the discovery of several selective SK1 and SK2 inhibitors. Due to promising outcomes from this study, the next stage of the project included fractionation of active plant extracts in order to identify and isolate active compounds. Once the isolation and purification of the compounds are achieved, structural elucidation by NMR/MS can then be performed. Specific *in vitro* and cell-based bioassays can be used to characterise mechanisms of action of novel synthetic and natural compounds. For example, *in vitro* enzymatic assay can establish the inhibition kinetics of SK inhibitors. Further characterisation of these compounds is also needed to establish their specificity for SK. Kinetic characterisation and pharmacological evaluation of these inhibitors will reveal important biological functions such as SAR for different SK isoforms. Successful characterisation of SK1 and/or SK2 inhibitors may open up new opportunities for developing potent drug leads.

Chapter 4 Characterisation of novel sphingosine kinase 1 and 2 inhibitors

4.1 Introduction

Enzymes are important targets in drug discovery due to their pivotal roles in many signalling pathways, which if deregulated can contribute to the development of various pathological conditions such as cancers. To gain a better understanding of the biological functions of a target enzyme, such as SK which catalyses the formation of S1P, perturbation of the activity of the enzyme is needed. There are generally two approaches which are used to modulate the activity of an enzyme resulting in gain or loss of functions. First, a genetic approach is widely used to delete the gene encoding the enzyme (genetic knockout or elimination of mRNA); in cell-based assay, this is frequently achieved by using siRNA to knock down the gene. The enzyme can also be over-expressed leading not only to a restoration of function but may also enhance any biological effects mediated by the enzyme. Second, pharmacological modulation of enzyme activity is achieved by using inhibitors or activators which can suppress or enhance enzyme catalytic activity, providing valuable insights into protein structure-function relationship. Of note, both approaches complement each other to provide a better understanding of the biology of an enzyme. These approaches have also been used to study other protein targets including receptors and ion channels. For instance, using SB649146 as a chemical probe (which binds to different conformational states of $S1P_1$) has revealed the complex pharmacology of $S1P_1$ (Pyne and Pyne 2011). Similarly, highly potent and selective chemical probes have been used to complement genetic approach in the study of $S1P_1$ receptor (Rosen *et al*., 2007). Many intracellular actions of S1P have been investigated with the use of SK inhibitors. However, experimental data from early studies which employ DMS or other substrate mimetic as SK inhibitors should be interpreted cautiously because these inhibitors are not specific for SK. For instance, DMS can also inhibit PKC (Kim *et al*., 2005). Moreover, it is important to establish the selectivity of SK inhibitor on different SK isoforms given that SK1 and SK2 have distinct cellular functions in certain cases. Therefore, this chapter aimed to characterise the inhibition kinetics of different SK inhibitors (discovered from Chapter 3). The anticancer effects of these inhibitors will be elucidated in relation to their inhibitory actions on SK1/SK2. This introduction will provide an overview of the study of enzyme kinetics and the rationale for establishing inhibitor selectivity between SK1 and SK2.

4.1.1 Enzyme kinetics

Enzymes are a specific group of proteins with high catalytic ability essential for life to exist. Due to their important roles in metabolism, enzymes demonstrate high substrate selectivity and are tightly regulated. The kinetic behaviour of enzymes can be characterised by a number of mathematical models, some of which are complex depending on the catalytic properties of the enzyme, such as the number of substrates and subunits of the enzyme participating in each catalytic cycle. For instance, sphingosine kinase is a two-substrate enzyme catalysing the formation of S1P by utilising sphingosine and ATP as substrates. Therefore, the mode of inhibition of an inhibitor with respect to which substrate needs to be specified. In this regard, inhibition experiments are usually performed in the presence of a fixed concentration (usually several folds higher than the K_m) of a substrate while varying the concentration of another substrate. Careful analysis of this data can then reveal the mode of inhibition of a ligand with respect to the substrate of interest.

To understand how inhibitors affect enzyme catalysis, the experiment is performed at steady-state condition (where $E + S \leftrightarrow ES \rightarrow E + P$; E, enzyme; S, substrate; P, product). Kinetic constants such as K_m can be calculated to describe the dependence of reaction rate on concentrations of substrate. For enzymes that obey Michaelis-Menten kinetic, the following equation can be used to obtain the rate of a reaction: $v=V_{max}*S/ (K_m + S)$ where v is the reaction rate; S is the substrate concentration; V_{max} is the maximum reaction rate and K_m is the Michaelis constant. In the presence of an inhibitor, a change in steady state is shown by alterations in the kinetic constants including V_{max} and K_m. Inhibition constants reflect the binding affinity of an inhibitor to a subset of enzyme conformational states. These constants, also known as K_{ic} and K_{iu} (competitive and uncompetitive inhibition constants respectively) can be obtained graphically (Cortés *et al*., 2001).

The current understanding of ligand binding and protein folding has evolved significantly in the field. Based on the funnel shape energy landscape model, macromolecules such as enzymes are present in a continuum of different conformations, each with a distinct energy level (Tsai *et al*., 1999). Inhibitors or activators are thought to bind to a conformation which results in a shift in

equilibrium to the most favourable bound-enzyme state. This model predicts that the tertiary structures of enzymes are not static but consists of a dynamic range of conformations. Of note, ligand binds to a conformer with the best fit; the most favourable conformation for ligand binding does not have to be the least energetic conformer. It follows that a competitive inhibitor may bind to a substrate-binding site and stabilise a conformation similar to that of a substrate-bound enzyme. In contrast, an allosteric inhibitor stabilises an inactive conformation of the enzyme at a site other than the active site. Allosteric binding can result in catalytic inhibition or activation, presumably by altering conditions at the active sites (e.g. by distorting the positions of key residues) to enhance or prevent efficient substrate binding. Hence, kinetic characterisation of SK1 inhibitors can reveal important information on the structure-function relationship of the enzyme.

4.1.2 Sphingosine kinase 1 and 2

Two highly homologous SK isoforms (termed SK1 and SK2) are responsible for the synthesis of S1P in many cell types. Studies on gene deletion in mice have shown that expression of SK is important in mammalian development where S1P receptors mediate diverse functions in vascular and nervous system development in the embryo (Kono *et al*., 2004). Interestingly, mice deficient in either SK1 or SK2 develop normally with no obvious defects whereas double knock-out of both SK1 and SK2 is embryonically lethal, suggesting that these enzymes play essential developmental roles (Mizugishi *et al*., 2005). However, SK1 and SK2 have different cellular functions and are often cited to play opposing roles in regulation of growth and survival of mammalian cells. In contrast to SK1 which promotes cell proliferation, SK2 has been found to promote apoptosis. Depending on the cell type and cell confluence, SK2 inhibits DNA synthesis and is mainly a nuclear protein because the N-terminus of SK2 contains a nuclear localization signal (Igarashi *et al*., 2003). The identification of a putative BH3 motif (largely found in pro-apoptotic proteins) in SK2 strengthens the notion that SK2 promotes apoptosis, by inducing cytochrome c release and caspase-3 cleavage (Liu *et al*., 2003). Moreover, subcellular localisation of SK2 and its catalytic activity are important determinants of its pro-apoptotic

function. For example, serum withdrawal led to relocalisation of SK2 to the ER membranes (Maceyka *et al*., 2005) and to the nucleus (Okada *et al*., 2005) which are associated with inhibition of DNA synthesis in various cell types.

Despite previous studies demonstrating a pro-apoptotic action of SK2, recent investigations into functional roles of SK2 have revealed that the enzyme can also mediate an anti-apoptotic action. For example, siRNA knock down of SK2 in MCF-7 cells induces apoptosis by activating caspase 7 and stimulating PARP cleavage (Sankala *et al*., 2007). In addition, SK2 up-regulates doxorubicin-induced p21 expression which promotes cell senescence and prevents apoptosis (Sankala *et al*., 2007). Furthermore, SK2 knockdown in human glioblastoma cell lines also promotes apoptosis and cell cycle arrest more effectively than SK1 knockdown by RNA interference (Van Brocklyn *et al*., 2005). Therefore, down-regulation of SK2 sensitises cancer cells to apoptosis, providing a rationale for targeting SK2 as a new approach for cancer treatment. More recently, ABC29460 has been found to inhibit SK2 selectively with antitumour activity against mammary adenocarcinoma xenograft model (French *et al*., 2010), demonstrating effectiveness in reducing cancer progression by inhibiting SK2.

Late-stage cancer is characterised by tissue invasion and tumour metastasis which are often attributed to treatment failure (Hanahan and Weinberg, 2011). The role of SK1 in cell migration has been well established. Particularly, SK1 is required for the formation of a migratory phenotype in MCF-7 cells (Long *et al*., 2010a). Under resting conditions, actin is clustered into focal adhesions in MCF-7 Neo (encoding a neomycin resistance gene) cells. Upon stimulation with S1P, marked rearrangement of actin at the membrane ruffles occurs. Knockdown of SK1 expression with siRNA prevents actin rearrangement induced by S1P, indicating an essential role for SK1 in S1P-induced cell migration of MCF-7 cells (Long *et al*., 2010a). SK2 has also been demonstrated to play a role in EGF-induced cell migration in HEK 293 and MDA-MB-431 cells; down-regulation of SK2 by siRNA completely eliminated migration of MDA-MB-431 cells towards EGF (Hait *et al*., 2005). Interestingly, treatment of a highly metastatic kidney cancer cell line (A-498) with a SK2 inhibitor (ABC29460) also reduced formation of lamellipodia and cell migration (French *et al*., 2010).

Taken together, SK1 and/ or SK2 inhibitor may also be added to the treatment armamentarium of cancer metastasis.

To date, several natural and synthetic compounds have been identified as SK inhibitors but there are still needs to develop potent compounds with high selectivity. For example, SKi has been shown to be an effective inhibitor against SK1 *in vitro* and exhibits potent *in vivo* anti-tumour properties. However, the molecular mechanisms underlying this are not fully elucidated. Furthermore, while focus has been placed on developing SK1 inhibitors, selective SK2 inhibitors are lacking. Therefore, this chapter aimed to characterise the mechanisms of action of SKi and several novel SK inhibitors which have been rationally designed or discovered in this drug discovery project. Particularly, the kinetic mechanism of inhibition by these inhibitors will be elucidated. In addition, the biological effects of these compounds will be investigated in relation to SK1/SK2 expression, promotion of apoptosis and inhibition of cell motility.

4.2 Results

4.2.1 Optimisation of assay condition for kinetic analysis

Optimisation of assay condition for purified SK1 obtained commercially was not successful, possibly due to the inclusion of insufficient amount of purified enzymes. Therefore, kinetic studies on SK1 were performed using cell lysates containing recombinant SK1 stably expressed in HEK 293 cells. In agreement to previous studies on SK1 (Olivera *et al*., 1998; Pitson *et al*., 2000a), the substrate kinetics of cell lysates containing stably over-expressed SK1 was shown to exhibit Michaelis-Menten kinetics (V_{max} = 0.3 pmol/μg/min; K_m = 2.9 μM for sphingosine) (Fig. 4.1A). The Hill coefficient for SK1 was also determined from reaction rates spanning 10-90% of the maximum reaction rate. As shown in Fig. 4.1B, the slope of the Hill plot (also known as the Hill coefficient) was estimated to be 0.9 +/-0.1, indicating that SK1 is not a cooperative protein. Thus, binding of substrates to SK1 is independent on bound substrates, i.e. SK1 obeys Michaelis-Menten kinetics. To ensure that the inhibition kinetics of SK1 inhibitors was assessed at the linear phase of the kinase reaction, the time-dependence of the increase of S1P was assessed in the presence of fixed concentrations of proteins and substrates. The mid-point of the reaction progress was between 15-25 minutes (Fig. 4.1C). To improve handling and performance of the radioactive assay, three inhibitor concentrations and five substrate concentrations (lower and higher than the K_m of sphingosine) were included in any single experiment. The inclusion of very low concentration of sphingosine (e.g. 0.5-2 μM) facilitates characterisation of compounds which act in a competitive manner with the substrate. In contrast, concentration of sphingosine higher that the K_m (e.g. 20 μM) is necessary to characterise compounds which exhibit an uncompetitive mode of inhibition. Therefore, kinetic analyses of SK1 inhibitors were performed for 15-20 minutes in the presence of 0.5-20 μM of sphingosine at a fixed concentration of [^{32}P]-ATP (250 μM).

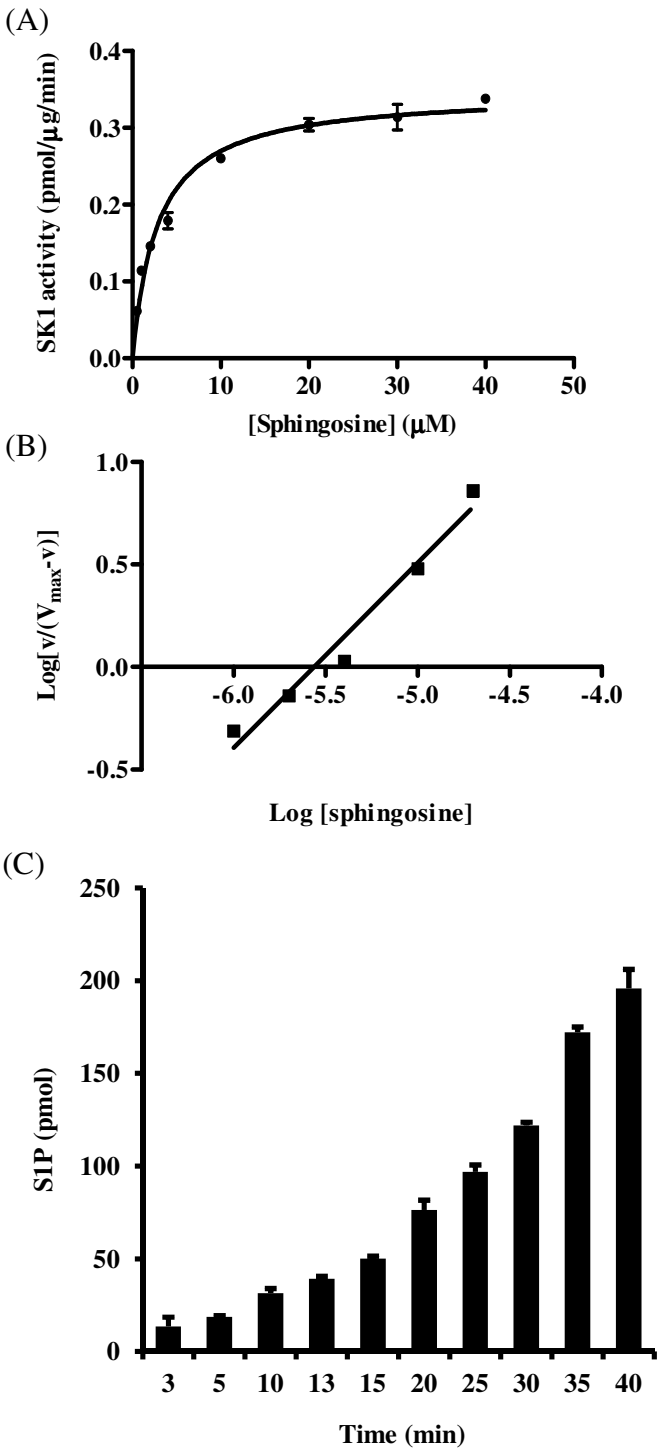


Figure 4.1 SK1 exhibits Michaelis-Menten kinetic behaviour. (A) Substrate titration (sphingosine) of steady state velocity for SK1 in the presence of 250 µM [^{32}P]-ATP for 20 mins. (B) Hill plot for SK1. Reaction rates were derived from (A) and plotted on a logarithmic scale. (C) Time-course for the formation of S1P in the presence of fixed concentration of substrates (10 µM sphingosine and 250 µM [^{32}P]-ATP). SK1 activity was assayed using cell lysate containing stably over-expressed SK1 as described under Methods (section 2.2.15). Data are means and standard deviations of duplicate determinations. Similar results were obtained in three independent experiments.

4.2.2 FTY720 analogues display different inhibition modalities

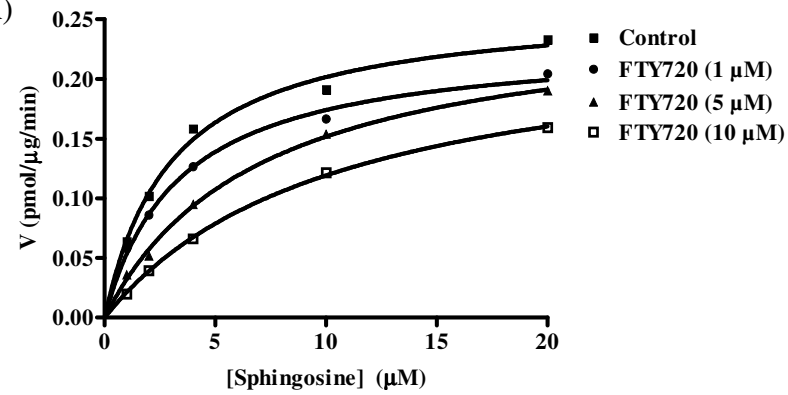
To elucidate the kinetic mechanisms of different SK1 inhibitors, varying concentrations of inhibitor were tested to obtain the inhibition constants (Table 4.1). As shown in Fig. 4.2A, FTY720 acts as a competitive inhibitor (with sphingosine) as V_{max} was unchanged while the K_m increased with increasing concentration of FTY720. The competitive inhibition constant (K_{ic}) for FTY720 was found to be 2.0 +/- 0.5 µM (Fig. 4.2B; Table 4.1). Due to its structural similarity to sphingosine, FTY720 has been shown to bind to SK2 which phosphorylates FTY720 (Billich *et al*., 2003). In agreement to previous studies, FTY720 also inhibited purified SK1 (Fig. 3.16). These data not only confirm the inhibitory effect of FTY720 on SK1—albeit a partially purified enzyme (Lee *et al*., 2004; Vessey *et al*., 2007) but also reveal that FTY720 competes with sphingosine and prevents sphingosine from binding to SK1.

Table 4.1 Inhibition constants for sphingosine kinase inhibitors

Inhibitor	Inhibition mechanism	Inhibition constant(s)
FTY 720	Competitive	K_{ic} = 2.0 +/- 0.5 µM
(*S*)-FTY720 vinylphosphonate	Uncompetitive	K_{iu} = 14.5 +/- 4.4 µM
SKi	Mixed	K_{ic} = 17.0 +/- 3.5 µM; K_{iu} = 48.3 +/- 11.5 µM

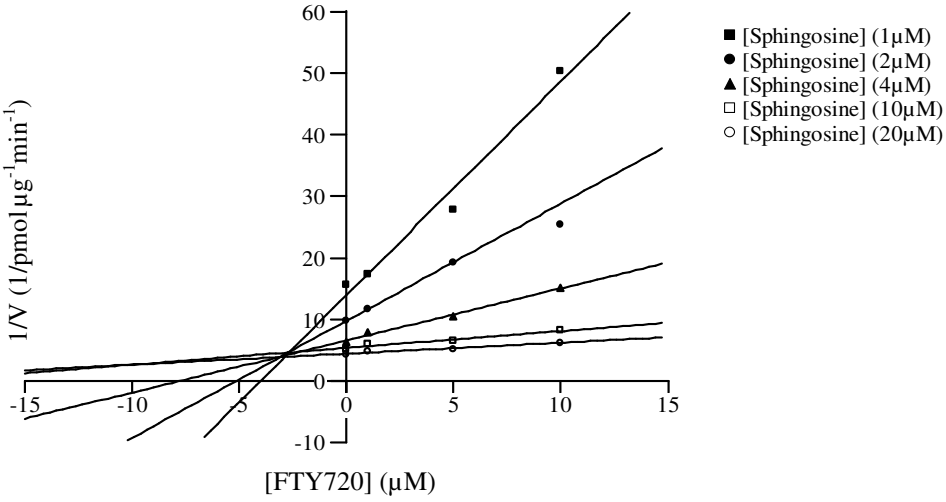
K_i values are means and standard deviations for n=3 experiments

(A)



FTY720 (µM)	0	1	5	10
V_{max} (pmol/µg/min)	0.26	0.23	0.26	0.24
K_m (µM)	3.04	3.37	7.03	10.53

(B)



(C)

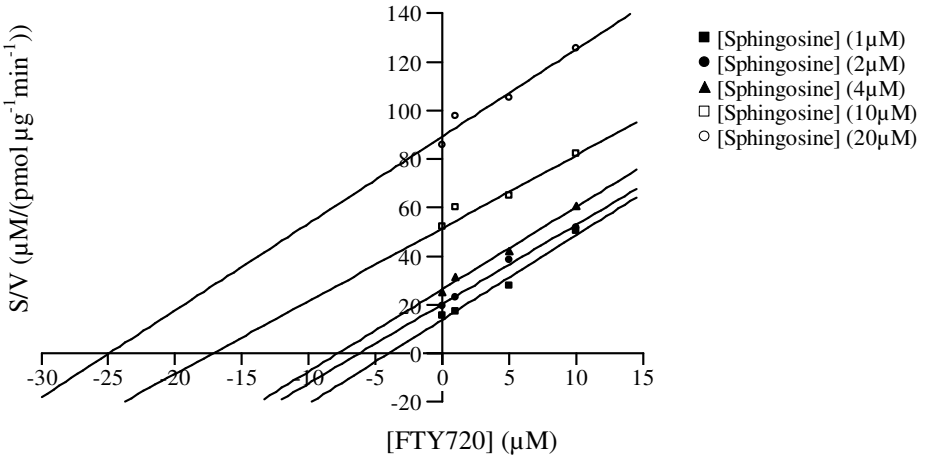
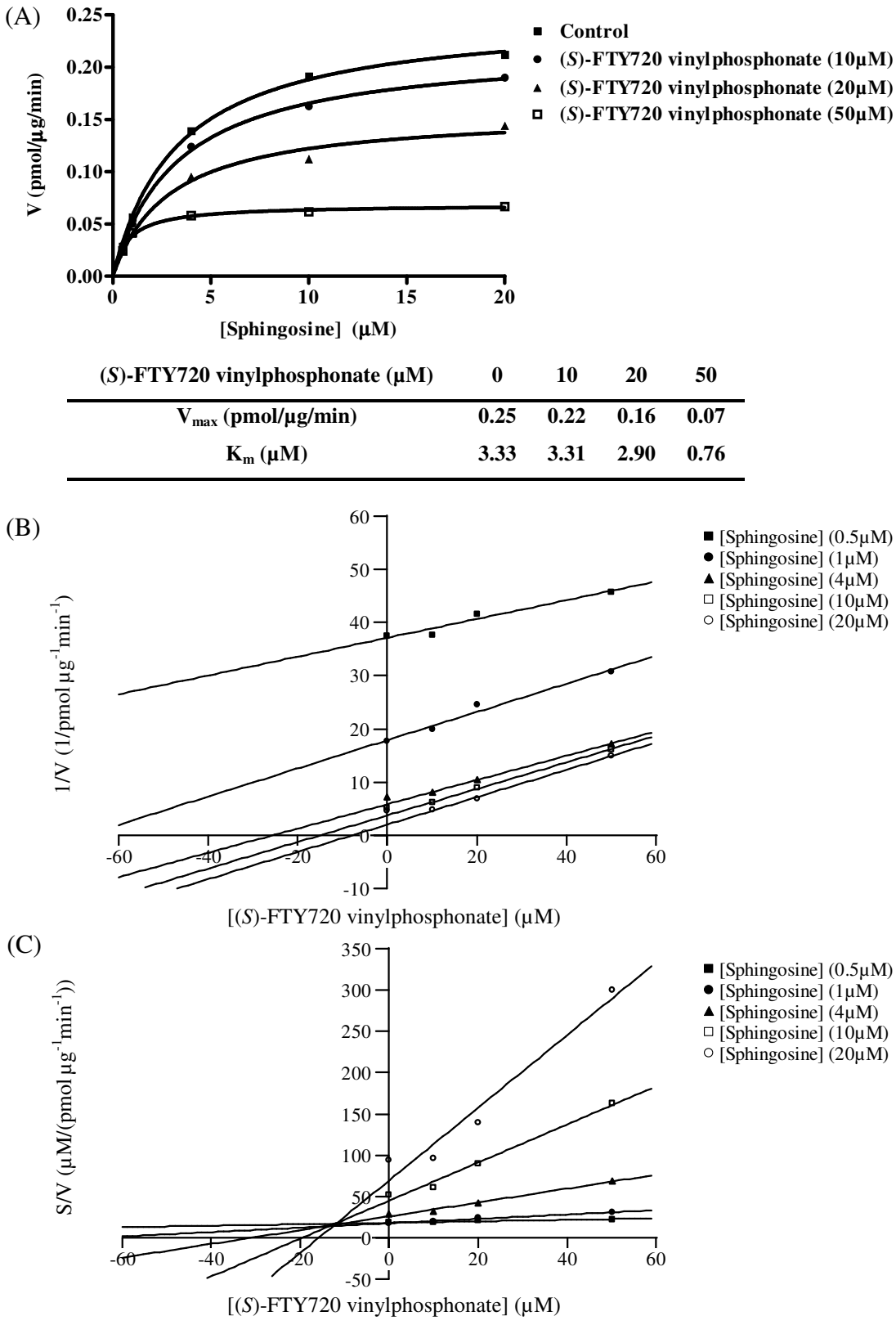


Figure 4.2 Inhibitor kinetic analysis of FTY720 for stably expressed SK1 in HEK 293 cells. (A) Non-linear regression analysis. **(B)** Dixon plot. **(C)** S/V *versus* [FTY720] plot. Similar results were obtained in three independent experiments.

In the initial drug discovery stage, compounds with different inhibition modalities are important for the success of developing effective compounds for preclinical studies. In this regard, *(S)*-FTY720 vinylphosphonate inhibited SK1 uncompetitively (with sphingosine), as evidenced by reduced K_m and V_{max} with increasing concentration of inhibitor (Fig. 4.3A). The uncompetitive inhibition constant (K_{iu}) was found to be 14.5 +/- 4.4 μM (Fig. 4.3C; Table 4.1). Therefore, in contrast to FTY720 which is predicted to bind to the active site, *(S)*-FTY720 vinylphosphonate may bind to a putative allosteric site contingent on the formation of an enzyme-substrate complex (i.e. putative allosteric binding site for *(S)*-FTY720 vinylphosphonate is only available when SK1 is bound to sphingosine). Furthermore, at a fixed concentration of sphingosine, the concentration-response curve for *(S)*-FTY720 vinylphosphonate was sigmoidal (Fig. 4.4A) with a Hill coefficient of 1.9 +/- 0.2 indicating positive cooperativity at the inhibitor binding site(s). Similarly, a Hill coefficient of 2.2 +/- 0.3 was obtained at another fixed concentration of sphingosine (Fig.4.4B). Therefore, it appears that more than one binding site exists for *(S)*-FTY720 vinylphosphonate and these allosteric sites interact in a positively cooperative manner.



(*S*)-FTY720 vinylphosphonate (µM)	0	10	20	50
V_{max} (pmol/µg/min)	0.25	0.22	0.16	0.07
K_m (µM)	3.33	3.31	2.90	0.76

Figure 4.3 Inhibitor kinetic analysis of (*S*)-FTY720 vinylphosphonate for stably expressed SK1 in HEK 293 cells. (A) Non-linear regression analysis. **(B)** Dixon plot. **(C)** S/V *versus* [(*S*)-FTY720 vinylphosphonate] plot. Similar results were obtained in three independent experiments.

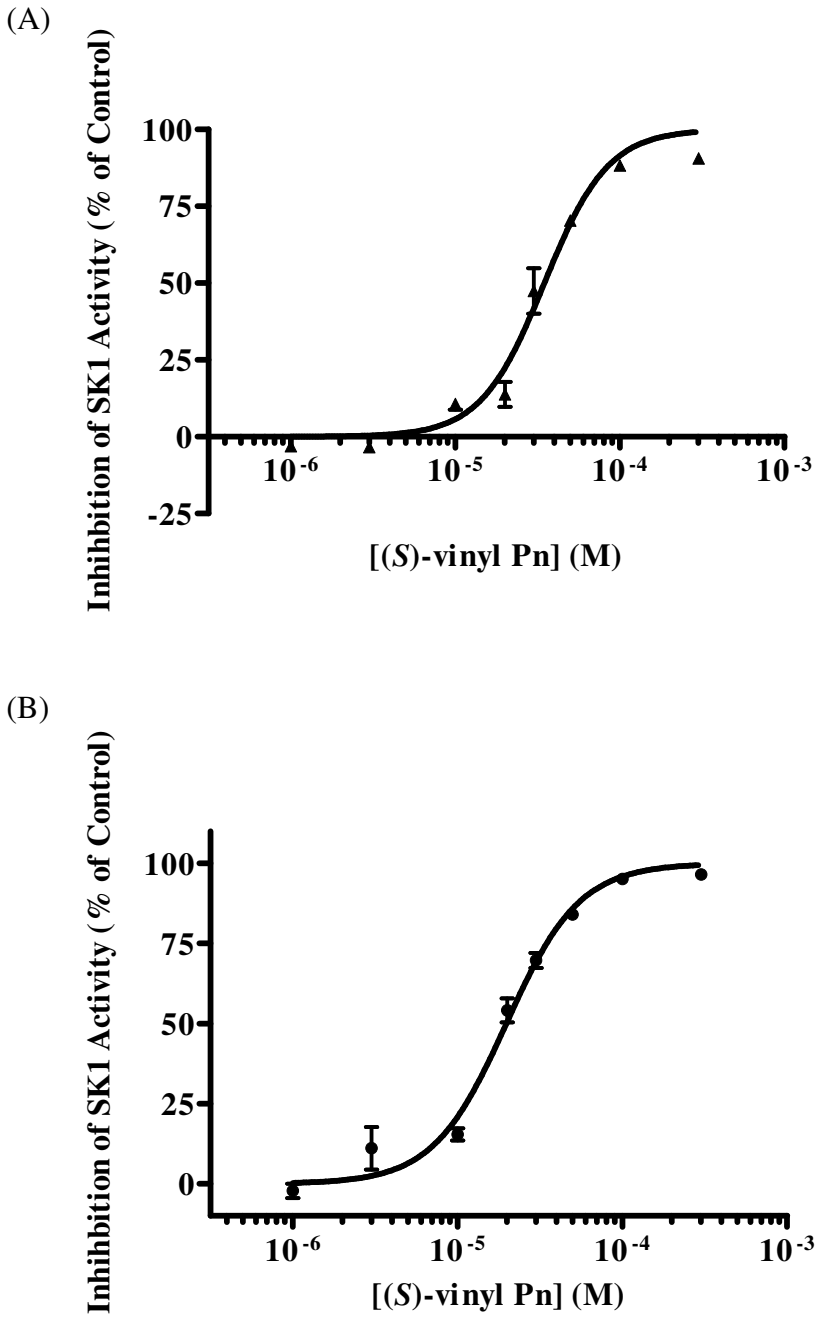


Figure 4.4 (*S*)-FTY720 vinylphosphonate exhibits positive cooperativity while inhibiting SK1. Concentration-dependent inhibition of stably expressed SK1 in HEK 293 cells by (*S*)-FTY720 vinylphosphonate in the presence of 250 µM [^{32}P]-ATP and **(A)** 10 µM or **(B)** 20 µM sphingosine as substrates. Data are expressed as percentage of control and represent means +/- standard deviations of triplicate determinations. Similar results were obtained in two to five independent experiments.

4.2.3 Novel compounds potentiate SK1 activity

Further evidence for the presence of an allosteric site(s) is supported by the discovery of two activators of SK1: (*S*)-FTY720 regioisomer and bodipy-sphingosine (Bdp-So, sphingosine conjugated to a fluorophore). Both compounds were also substrates for SK1 but were relatively weak substrates compared to sphingosine (Fig. 4.5A). Interestingly, Bdp-So activated purified SK1 in a concentration-dependent manner with a Hill coefficient of 4.5 +/-2.7 (Fig. 4.5B).

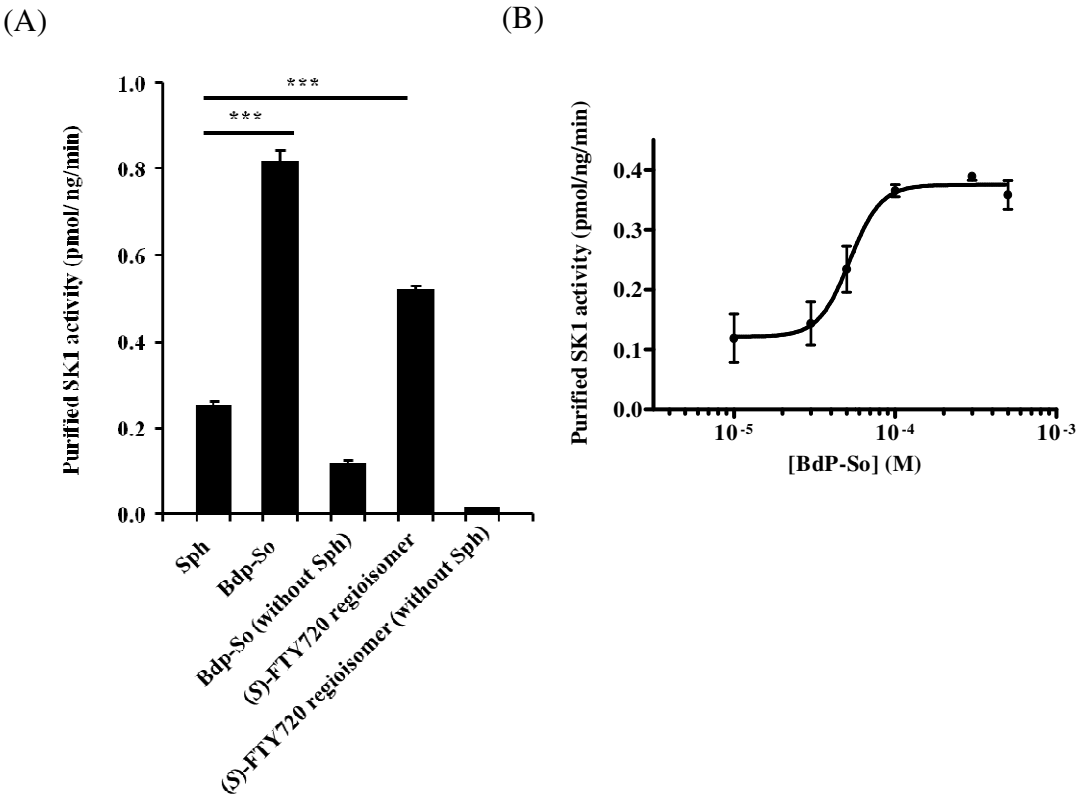


Figure 4.5 Activators of SK1. (A) The effect of bodipy-sphingosine (Bdp-So) and (*S*)-FTY720 regioisomer (both 50 µM) on the activity of purified SK1 assayed with and without 10 µM sphingosine (Sph). *** $p<0.001$ compared to control (sphingosine alone). **(B)** Concentration-dependent activation of purified SK1 by bodipy-sphingosine assayed in the presence of 10 µM sphingosine and 250 µM [^{32}P]-ATP. Data are expressed as percentage of control and represent means and standard deviations of duplicate determinations, ***$p<0.001$ *versus* control. Similar results were obtained in three independent experiments.

4.2.4 Oligomerisation of SK1

It has previously been demonstrated that SK1 is capable of forming oligomers (Kihara *et al*., 2006). The present study established that mutant SK1 can form oligomers. Myc-tagged G81D SK1 (mutated in the ATP binding site) and FLAG-tagged D178N SK1 (mutated in the sphingosine binding site) were transiently co-expressed and immunoprecipitated with either anti-myc or anti-FLAG antibodies. Co-immunoprecipitation analyses revealed that myc-tagged G81D SK1 was present in anti-FLAG immunocomplexes whereas FLAG-tagged D178N SK1 was present in anti-myc immunocomplexes (Fig. 4.6).

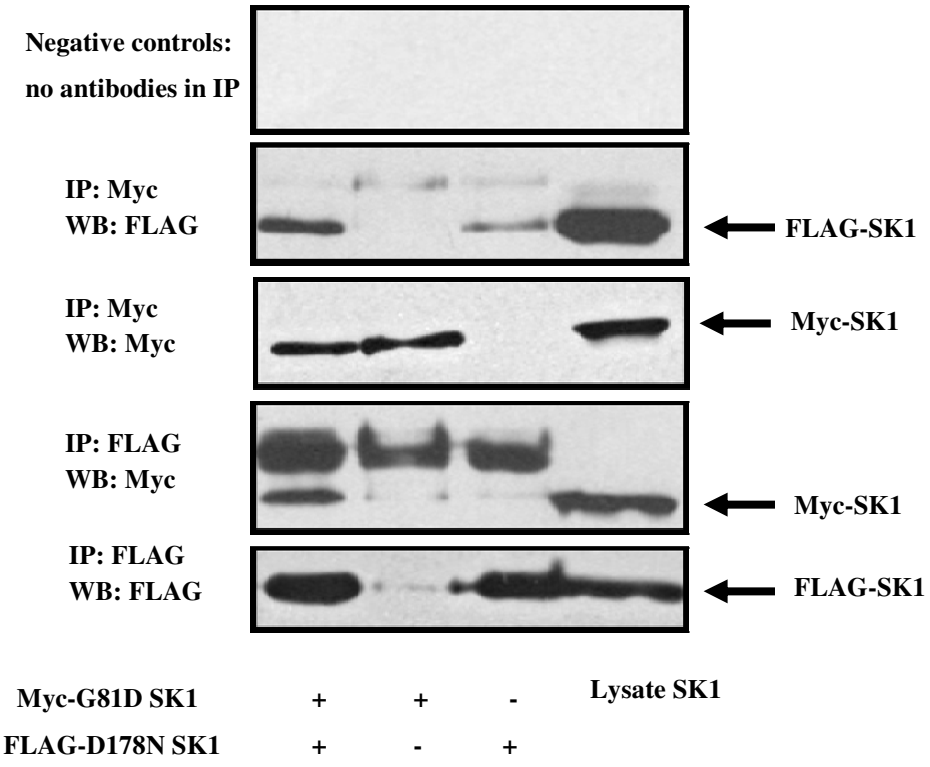


Figure 4.6 Mutant-SK1 forms oligomers. Lysates from HEK 293 cells co-transfected with myc-tagged G81D SK1 and/or FLAG-tagged D178N SK1 were immunoprecipitated with anti-myc or anti-FLAG antibodies. SK1 present in the immunoprecipitates was detected by Western blotting using anti-myc or anti-FLAG antibodies. "Lysate SK1" represents cell lysates co-expressing both myc-tagged and FLAG-tagged SK1. Results are representative of three independent experiments.

If hybrids of mutant SK1 and wild-type (WT) SK1 are created, then it is possible that oligomers will possess more allosteric sites than catalytic sites. Moreover, if the allosteric site exerts an auto-inhibitory effect on the enzyme, such hybrids will exhibit less catalytic activity. To test this hypothesis, oligomerisation of SK1 was also confirmed with the use of WT myc-tagged and FLAG-tagged SK1 plasmid constructs (Fig. 4.7A, B). Hybrid SK1 oligomers were created by co-transfecting HEK 293 cells with WT SK1 and myc-tagged G81D SK1 or FLAG-tagged D178N SK1. Both myc-tagged G81D and FLAG-tagged D178N SK1 showed less than 10% activity compared with WT SK1 whereas the myc-tagged G81D/FLAG-tagged D178N SK1 oligomers were catalytically deficient (Table 4.2). This nullifies the notion where each mutant may contribute one substrate binding site (either the ATP or sphingosine) to form a functional catalytic site in an oligomeric arrangement. In agreement with inhibition kinetics established using stably expressed WT SK1, FTY720 and *(S)*-FTY720 vinylphosphonate also displayed similar inhibition kinetics against transiently over-expressed WT-FLAG/Myc-tagged SK1. In this regard, FTY720 was competitive with sphingosine, K_{ic} ~7 µM (Fig. 4.7C), whereas *(S)*-FTY720 vinylphosphonate was uncompetitive with sphingosine, K_{iu} ~7 µM (Fig. 4.7 D).

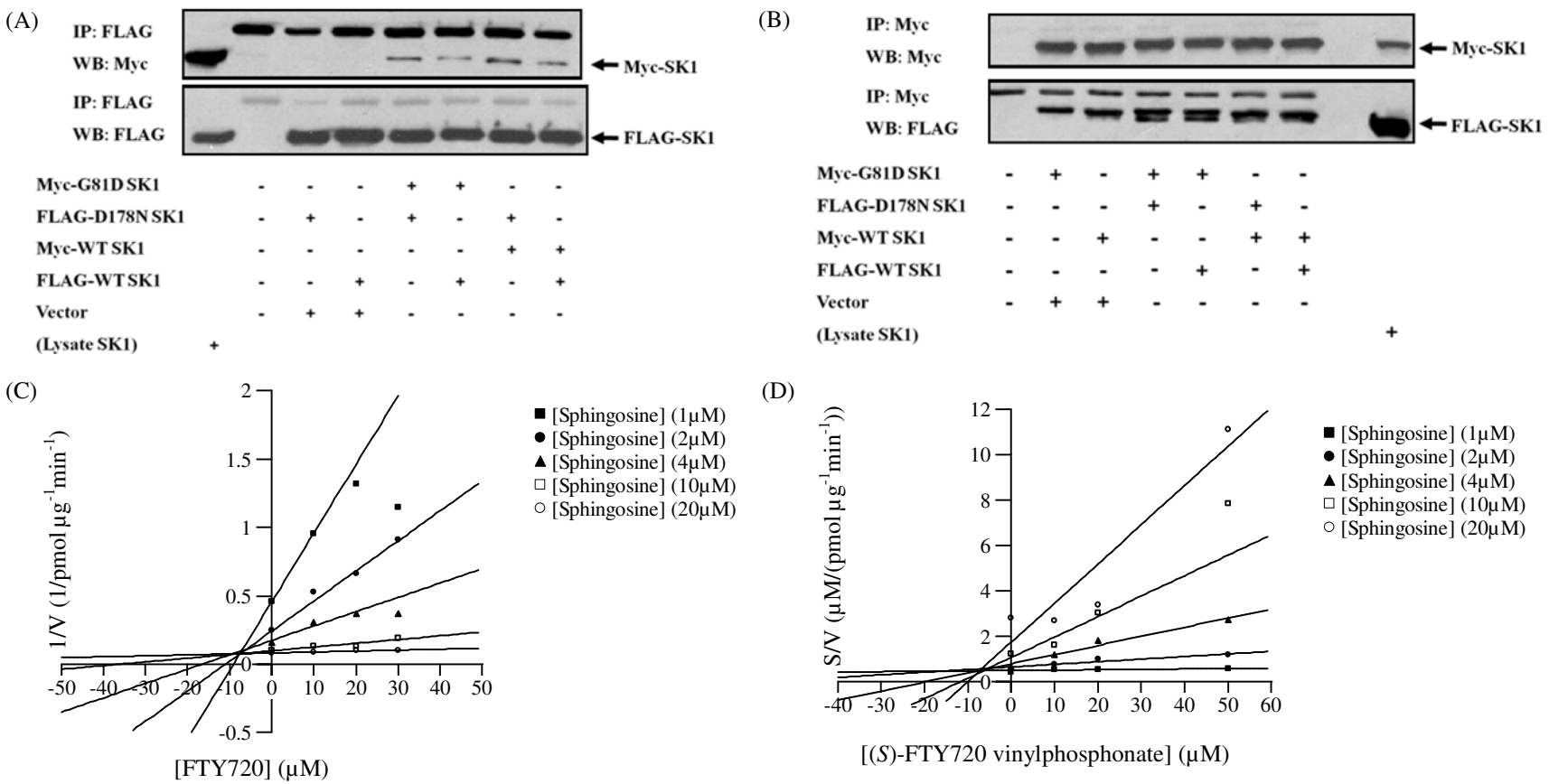


Figure 4.7 WT-SK1 and mutant-SK1 form oligomers. Lysates from HEK 293 cells transiently transfected with myc-tagged G81D SK1, FLAG-tagged D178N SK1, myc-tagged WT SK1, and/or FLAG-tagged WT SK1 were immunoprecipitated with **(A)** anti-FLAG, **(B)** anti-myc antibodies. 'Lysate SK1' represents cell lysates co-expressing both myc-tagged and FLAG-tagged SK1. Results are representative of three independent experiments. **(C)** Dixon plot for K_{ic} determination of FTY720 of WT enzyme **(D)** S/V *versus* [inhibitor] plot for K_{iu} determination of (*S*)-FTY720 vinylphosphonate of WT enzyme. SK1 activity was assayed in HEK 293 cells transiently expressing WT FLAG/Myc tagged SK1 as described under Methods (section 2.2.15) in the presence of 10 µM sphingosine, 250 µM [^{32}P]-ATP.

Table 4.2 SK1 activity measured in SK1 immunoprecipitates.

	Anti-myc immunoprecipitates (pmol/min)	Anti-FLAG immunoprecipitates (pmol/min)
myc-tagged G81D SK1-transfected cells	0.23	0.14
myc-tagged G81D SK1/ FLAG-tagged D178N SK1-transfected cells	1.65	2.00
Myc-tagged WT SK1-transfected cells	33.0	0.19
FLAG-tagged D178N SK1-transfected cells	0.04	2.80
FLAG-tagged WT SK1-transfected cells	0.09	20.6

SK1 activity was measured by incubating immunoprecipitates directly in SK1 reaction buffer following the procedures as described under Methods (section 2.2.15). All values are representative data from 2 to 3 independent experiments.

To substantiate the notion that dominant-negative SK1 oligomerises with WT SK1 and inhibits the catalytic activity of the oligomers, hybrid SK1 was created in HEK 293 cells by transiently over-expressing hybrid SK1 with a fixed amount of myc-tagged WT SK1 plasmid constructs (1 µg) and varying amount of FLAG-tagged D178N SK1 plasmid constructs (1-8 µg). Under this condition, expressions of WT SK1 were maintained constant while expressions of mutant SK1 were increased (Fig. 4.8A, B). The activity of WT SK1 was reduced in the presence of high amount of mutant SK1 (Fig. 4.9), suggesting that allosteric sites which are in excess exerted an inhibitory effect on the catalytic activity of the oligomers.

The present study also found that SKi is a mixed SK1 inhibitor (with sphingosine) (Fig. 4.10A-C). The mixed inhibition was characterized by two inhibition constants: 17.0 +/- 3.5 µM and 48.3 +/- 11.5 µM for K_{ic} and K_{iu} respectively. This further supports the presence of allosteric site(s) in SK1 to which SKi may bind.

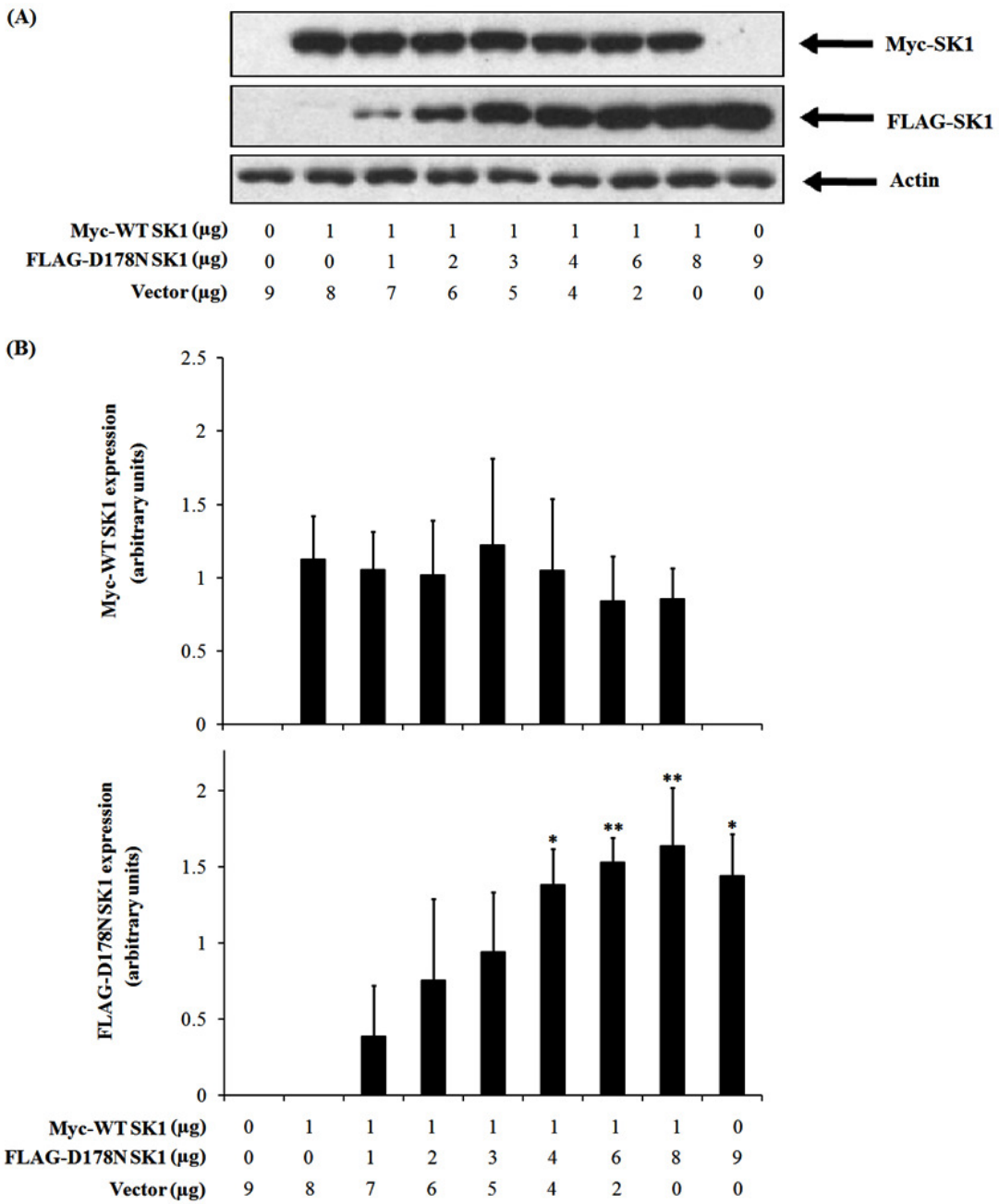


Figure 4.8 Co-expression of WT SK1 with increasing amount of mutant SK1. (A) HEK 293 cells were transiently transfected with increasing amount of FLAG D178N SK1 in the presence of a fixed amount of myc-WT SK1. Cell lysates were separated by SDS-PAGE and immunoblotted with anti-myc or anti-FLAG antibodies according to Methods (section 2.2.10 and 2.2.11). Blots were then stripped and reprobed with anti-actin to ensure equal protein loading. **(B)** Bar graphs showing quantification of myc-WT SK1 and FLAG-D178N SK1 expressions. Data were normalized with respect to the actin band and are expressed in arbitrary units. No significant difference observed in myc-WT SK1 expressions ($p=0.874>0.05$). Expressions of FLAG-D178N SK1 were increased, $*p<0.05$, $**p<0.01$ *versus* cells transfected with 1µg of FLAG-D178N plasmid, lane 3. Results are representative of three experiments.

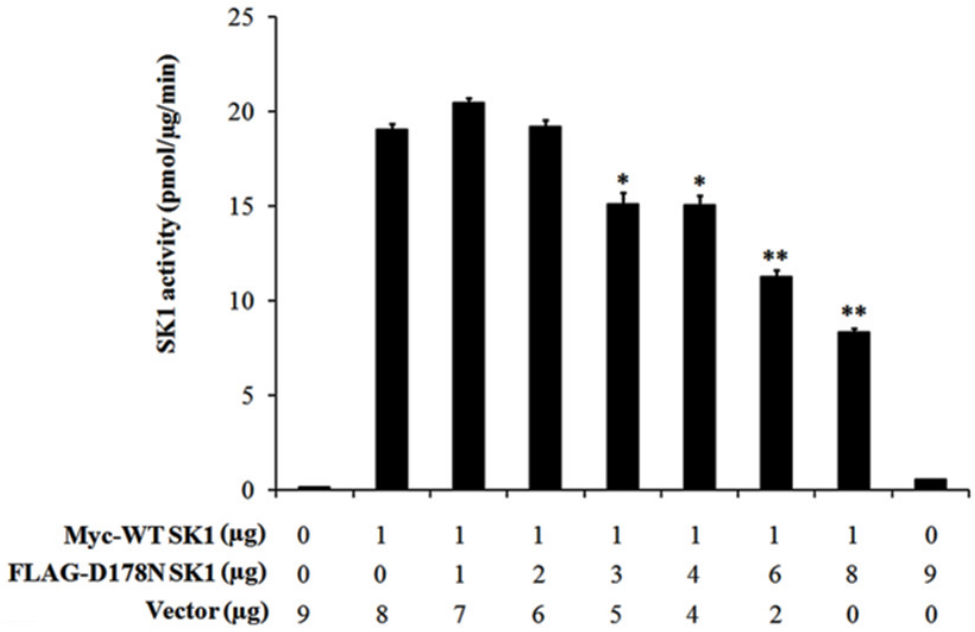
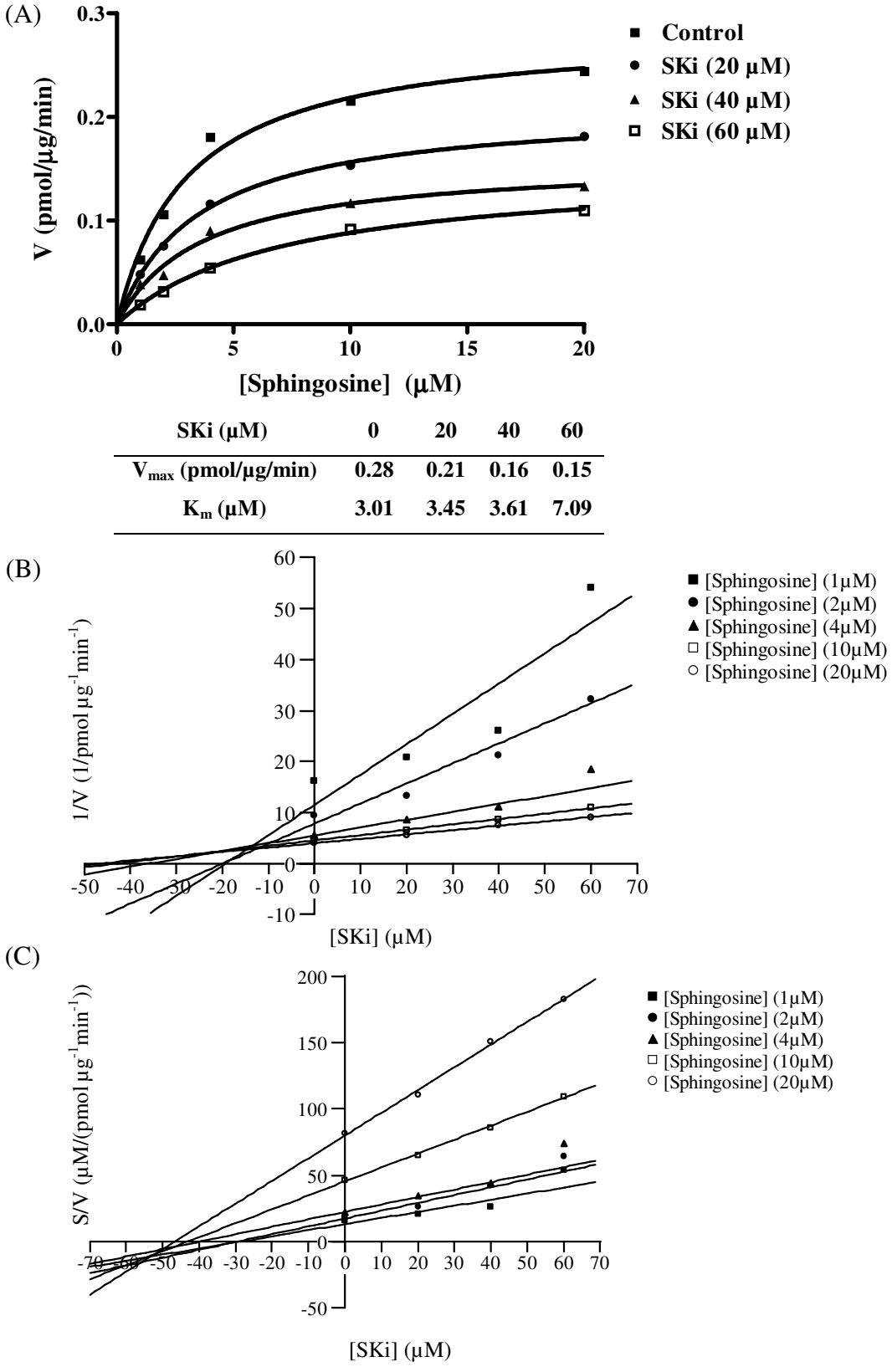


Figure 4.9 Mutant SK1 inhibits WT SK1 activity. SK1 activity was measured as described under Methods (section 2.2.15) using 10 µM sphingosine and 250 µM [^{32}P]-ATP as substrates. Data are expressed as percentage of control and represent means and standard deviations of duplicate determinations, *p<0.05, **p<0.01 versus lane 2. Results are representative of three independent experiments.



SKi (µM)	0	20	40	60
V_{max} (pmol/µg/min)	0.28	0.21	0.16	0.15
K_m (µM)	3.01	3.45	3.61	7.09

Figure 4.10 Inhibitor kinetic analysis of SKi for stably expressed SK1 in HEK 293 cells. (A) Non-linear regression analysis. **(B)** Dixon plot. **(C)** S/V *versus* [SKi] plot. Similar results were obtained in three independent experiments.

Finally, several experiments were performed to identify the allosteric domain of SK1 which exerts a regulatory control over SK1 activity. It has been demonstrated that G113A mutation of SK1 leads to an increase of activity for up to 1.7–fold greater than WT SK1 activity (Pitson *et al*., 2001). G113A mutant may be trapped in an "activated" conformation, rendering further activation more difficult or reducing sensitivity to catalytic inhibition. Thus, it was of interest to determine if G113A SK1 has similar sensitivity as WT SK1 to allosteric regulators. However, both wild-type SK1 and G113A SK1 were inhibited by *(S)*-FTY720 vinylphosphonate to a similar extent (Fig. 4.11). Bdp-So also activated both enzymes, indicating that G113A is not involved in allosteric regulation of SK1.

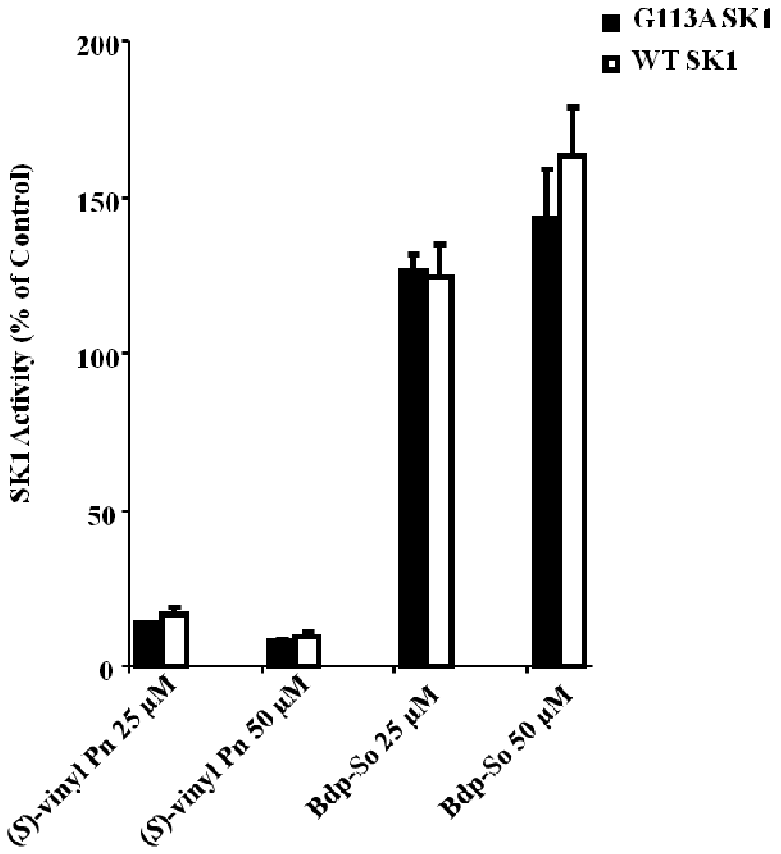


Figure 4.11 Effects of allosteric regulators on G113A SK1 activity. Wild-type SK1 and G113A SK1 were transiently expressed in HEK 293 cells. Equal amount of cell lysates were then measured for SK1 activity as described under Methods (section 2.2.15) in the presence of 10 μM sphingosine, 250 μM [^{32}P]-ATP and Bodipy-sphingosine (Bdp-So) or (*S*)-FTY720 vinylphosphonate ((*S*)-vinyl Pn)) at indicated concentrations. Data are expressed as percentage of control and represent means and standard deviations of triplicate determinations. Significant differences exist between all samples and control, $p<0.001$.

A prokaryotic homologue of eukaryotic SK and DGK is the phosphatidylglycerol kinase, YegS. Analysis of the amino acid sequence of YegS reveals that several conserved motifs are present in the catalytic domain between YegS and eukaryotic DGK, indicating that they may adopt a similar fold (Nichols *et al*., 2007). Therefore, studies on this enzyme may provide structural insights into how SK1 is allosterically regulated since the crystal structure of YegS is available (Bakali *et al*., 2007; Nichols *et al*., 2007). However, SKi, FTY720, *(S)*-FTY720 vinylphosphonate and Bdp-So did not alter YegS catalytic activity significantly (Fig. 4. 12)

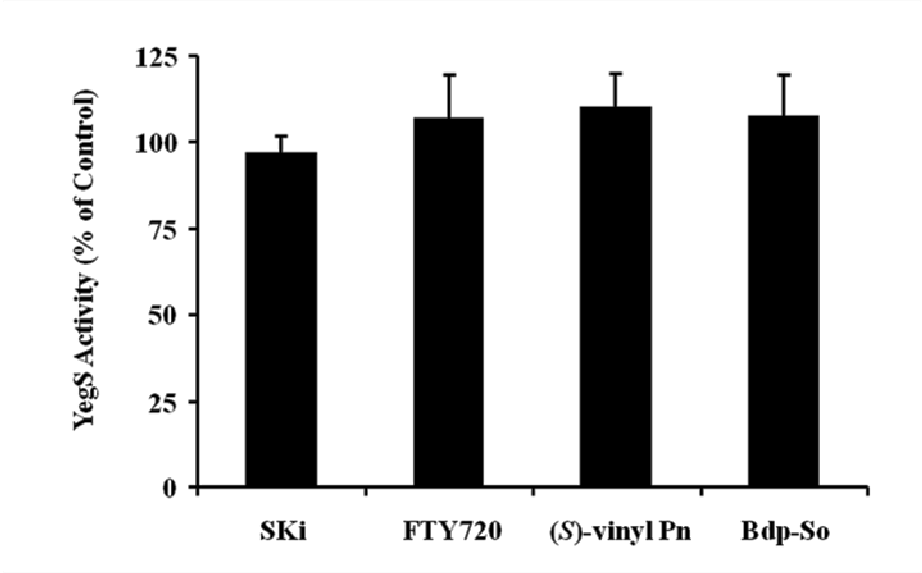


Figure 4.12 Effects of SK inhibitor/activator on YegS activity. YegS activity was measured as described under Methods (section 2.2.15) in the presence of 10 µM phophatidylglycerol, 250 µM [^{32}P]-ATP and SKi, FTY720, (*S*)-FTY720 vinylphosphonate ((*S*)-vinyl Pn) or bodipy-sphingosine (Bdp-So) (all at 50 µM). Data are expressed as percentage of control and represent means and standard deviations of three to six determinations. No significant difference exists between each inhibitor on YegS activity, $p>0.05$.

4.2.5 SK1 inhibitors induce down-regulation of SK1 and apoptosis

SKi has been shown to down-regulate SK1 expression in mammalian cells including breast and prostate cancer cells (Loveridge *et al*., 2010). It has also been established that the HER2 oncogene up-regulates SK1 expression in MCF-7 cells stably expressing HER2 (MCF-7 HER218 cells). SK1 in turn reduces the expression of HER2 and this phenomenon is termed "oncogenic tolerance" (Long *et al*., 2010a). Therefore, it was of interest to examine whether SK1 expression can be down-regulated in both MCF-7 Neo and MCF-7 HER218 cells, and whether the newly identified SK inhibitor, *(S)*-FTY720 vinylphosphonate behaved similarly. Here, the effects of FTY720, *(S)*-FTY720 vinylphosphonate and SKi on SK1 expression were assessed in MCF-7 cells expressing either the Neo vector (MCF-7 Neo) or the HER2 oncogene. As shown in Fig. 4.13, all SK1 inhibitors reduced SK1 expression. Down-regulation of SK1 was independent of apoptosis because the caspase 3/7 inhibitor Ac-DEVD-CHO (Garcia-Calvo *et al*., 1998) did not affect inhibitor-induced SK1 proteasomal degradation (Loveridge *et al*., 2010; see Chapter 5, Fig. 5.3). This effect is however dependent on the formation of ceramide which accelerates the rate of proteasomal degradation of polyubiquitinated SK1 (Loveridge *et al*., 2010). Despite an up-regulation of SK1 expression on MCF-7 HER218 cells, the effects of SK1 inhibitors on SK1 down-regulation are similar in both MCF-7 Neo and MCF-7 HER218 cells (Fig. 4.13B). Furthermore, SK inhibitors-induce degradation of SK1 in MCF-7 HER218 cells was also MG-132 sensitive, indicating involvement of the ubiquitin-proteasome system (Fig. 4.13C).

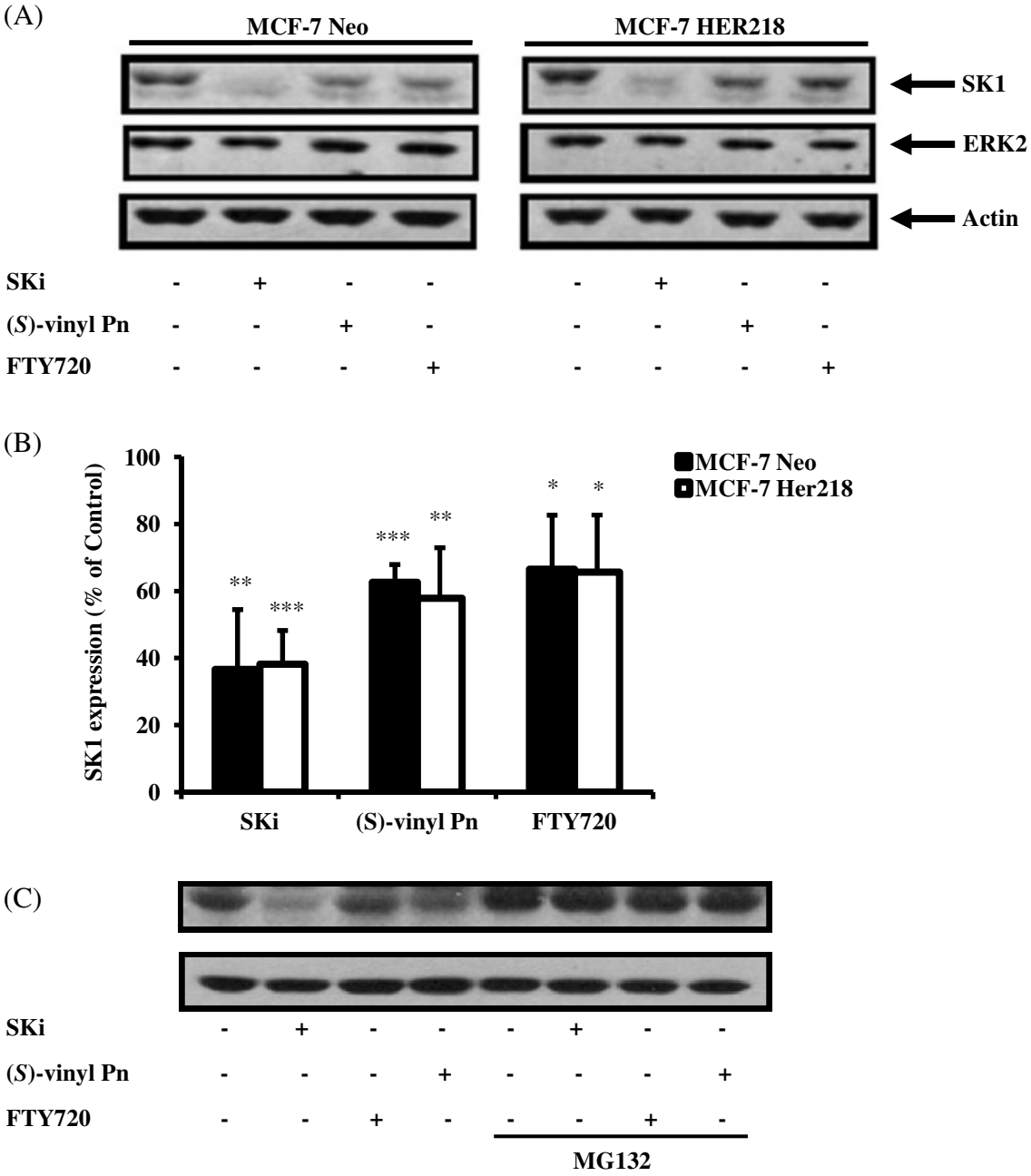


Figure 4.13 SK inhibitors induce down-regulation of SK1. (A) MCF-7 Neo and MCF-7 HER218 cells were treated with SKi, (*S*)-FTY720 vinylphosphonate ((*S*)-vinyl Pn) or FTY720 (all at 10 μM) for 24 hrs. Equal amount of cell lysates were resolved by SDS-PAGE. Immunoreactive bands were detected using anti-SK1 as described under Methods (section 2.2.11). Blots were then stripped and reprobed with anti-ERK2 and anti-actin antibodies to ensure comparable protein loading. **(B)** Bar graphs showing quantification of the effects of SK inhibitors on SK1 expression by quantifying the SK1: actin ratio for cells treated with SK inhibitors and are expressed as a percentage of control, *p<0.05, **p<0.01, ***p<0.001 for SK inhibitor-treated cells *versus* control. **(C)** MCF-7 HER218 cells were pre-treated with MG132 (10 μM, 30 mins) prior to addition of SK1 inhibitors. Results are representative of 3 independent experiments.

SK inhibitors also induced apoptosis in MCF-7 Neo cells. SKi was the most effective inhibitor in inducing PARP cleavage, followed by FTY720 and *(S)*-FTY720 vinylphosphonate (Fig. 4.14A). This relationship correlates well with the ability of the inhibitors in down-regulating SK1. However, despite down-regulation of SK1 expression by SK inhibitors, no significant PARP cleavage was observed in MCF-7 HER218 cells, even with the use of a potent cytotoxic agent, etoposide (Fig. 4.14A). This suggests that other signalling pathway might operate to compensate for the loss of SK1/S1P signalling in MCF-7 HER218 cells to prevent apoptosis. Indeed, MCF-7 HER218 had high basal phospho-ERK1/2 activity (Fig. 4.14B). This is in contrast to MCF-7 Neo cells, where ERK1/2 activation can only be induced by exogenous agonists such as S1P and EGF (see Chapter 3; Fig. 3.14).

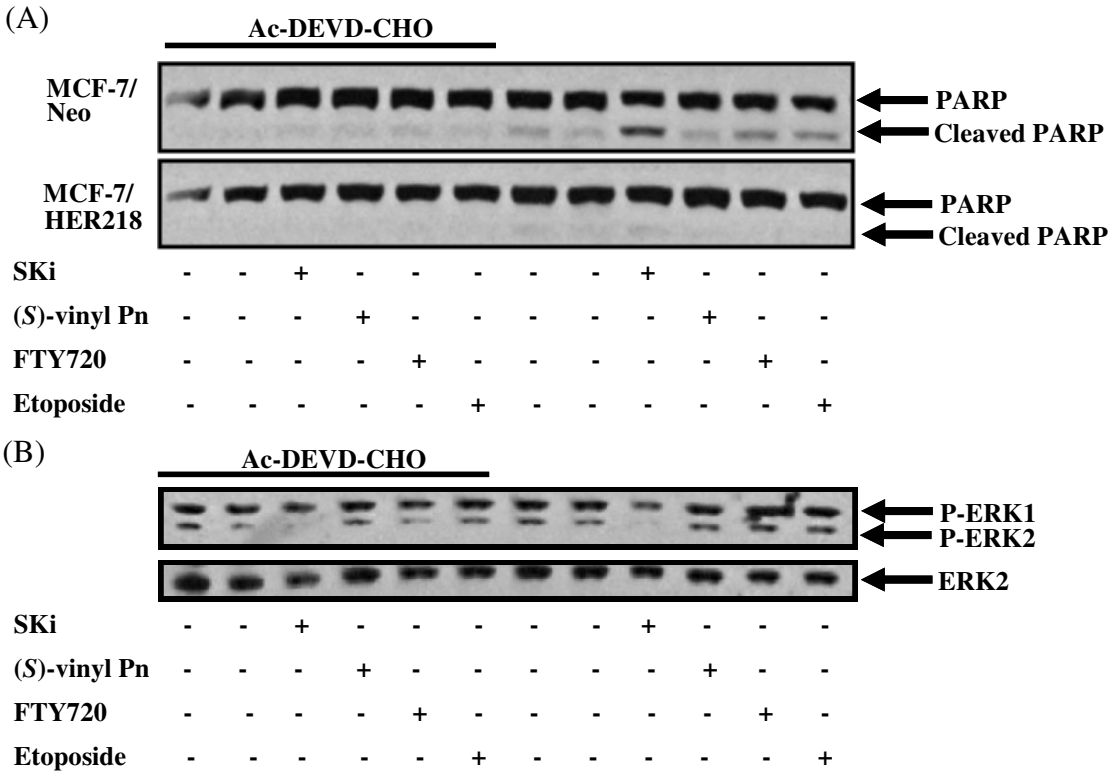


Figure 4.14 SK inhibitors induce PARP cleavage. (A) MCF-7 Neo and MCF-7 HER218 cells were pre-treated with 100 µM Ac-DEVE-CHO or vehicle control (0.2% DMSO) for 30 mins. Then, cells were treated with SKi, (*S*)-FTY720 vinylphosphonate ((*S*)-vinyl Pn), FTY720 (all at 10 µM) or vehicle control (0.1% DMSO) for 24 hrs. Etoposide (100 µM) was included as a positive control to induce PARP cleavage. Equal amount of cell lysates were resolved by SDS-PAGE. Immunoreactive bands were detected using anti-PARP as described in Methods (section 2.2.11). **(B)** Blots from MCF-7 HER218 cells were then stripped and reprobed with anti-phospho ERK1/2 and anti-ERK2 antibodies. Results are representative of three independent experiments.

Previously, EGF- or S1P-induced translocation of SK1 to lamellipodia has been shown to be involved in regulating the migration of MCF-7 cells (Sarkar *et al*., 2005; Long *et al*., 2010a). Subsequent localised production of S1P is essential for cell migration since S1P receptors mediate cell motility through differential coupling to various G proteins (Sugimoto *et al*., 2003). MCF-7 Neo cells express abundant pro-migratory $S1P_3$ but low levels of anti-migratory $S1P_2$ mRNA (Long *et al*., 2010a). S1P stimulates membrane ruffling (actin-rich lamellipodia) through $S1P_3$ ligation. Moreover, siRNA directed against SK1 inhibits S1P-induced actin polymerisation and leads to its reorganisation into adhesion foci in MCF-7 Neo cells (Long *et al*., 2010a). Therefore, it was of interest to investigate whether biologically active SK1 inhibitors could inhibit S1P-induced membrane ruffling since these inhibitors reduce formation of S1P through catalytic inhibition of SK1. Indeed, pre-treatment of MCF-7 Neo cells with SKi, FTY720 and *(S)*-FTY720 vinylphosphonate prevented S1P-induced actin polymerisation and membrane ruffling but induced formation of actin adhesion foci at the cell periphery (Fig. 4.15). These results support the role for SK1 in promoting cell migration and survival.

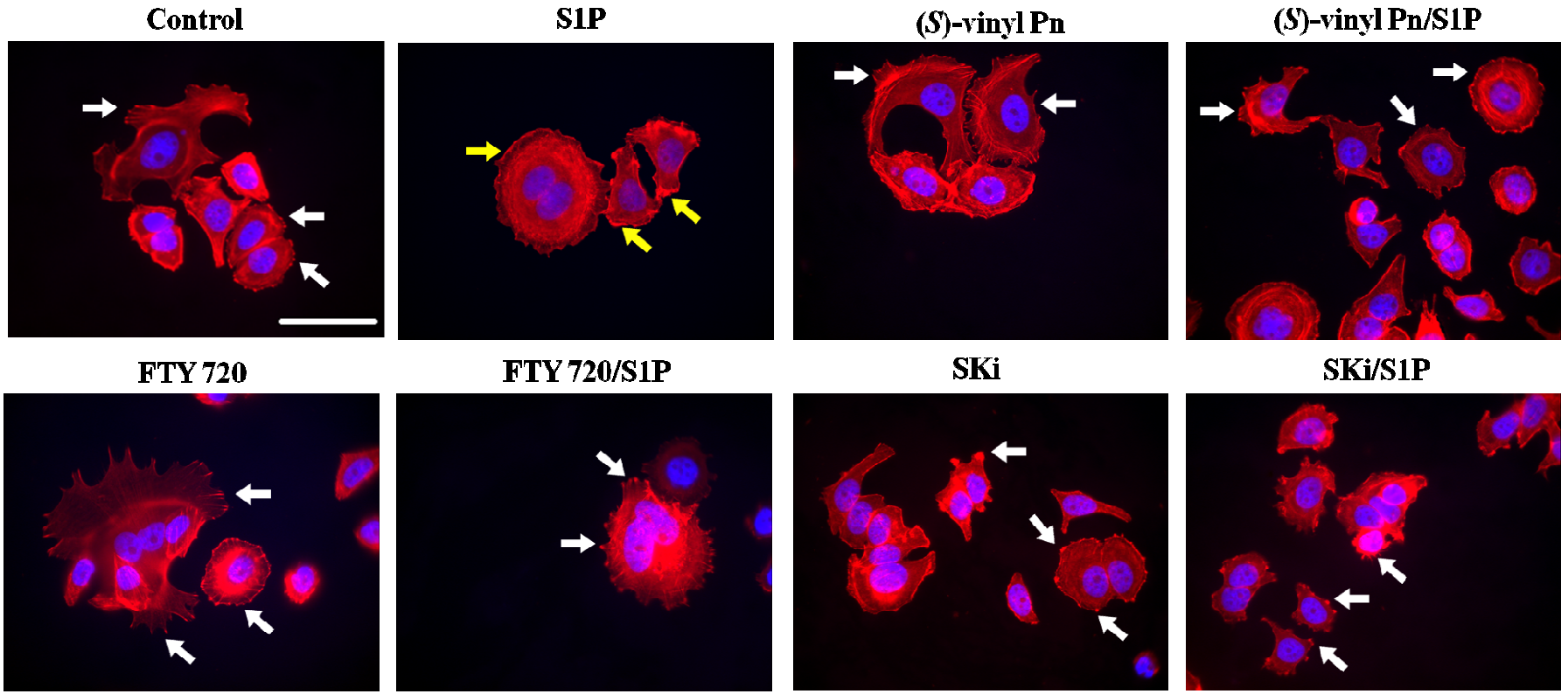
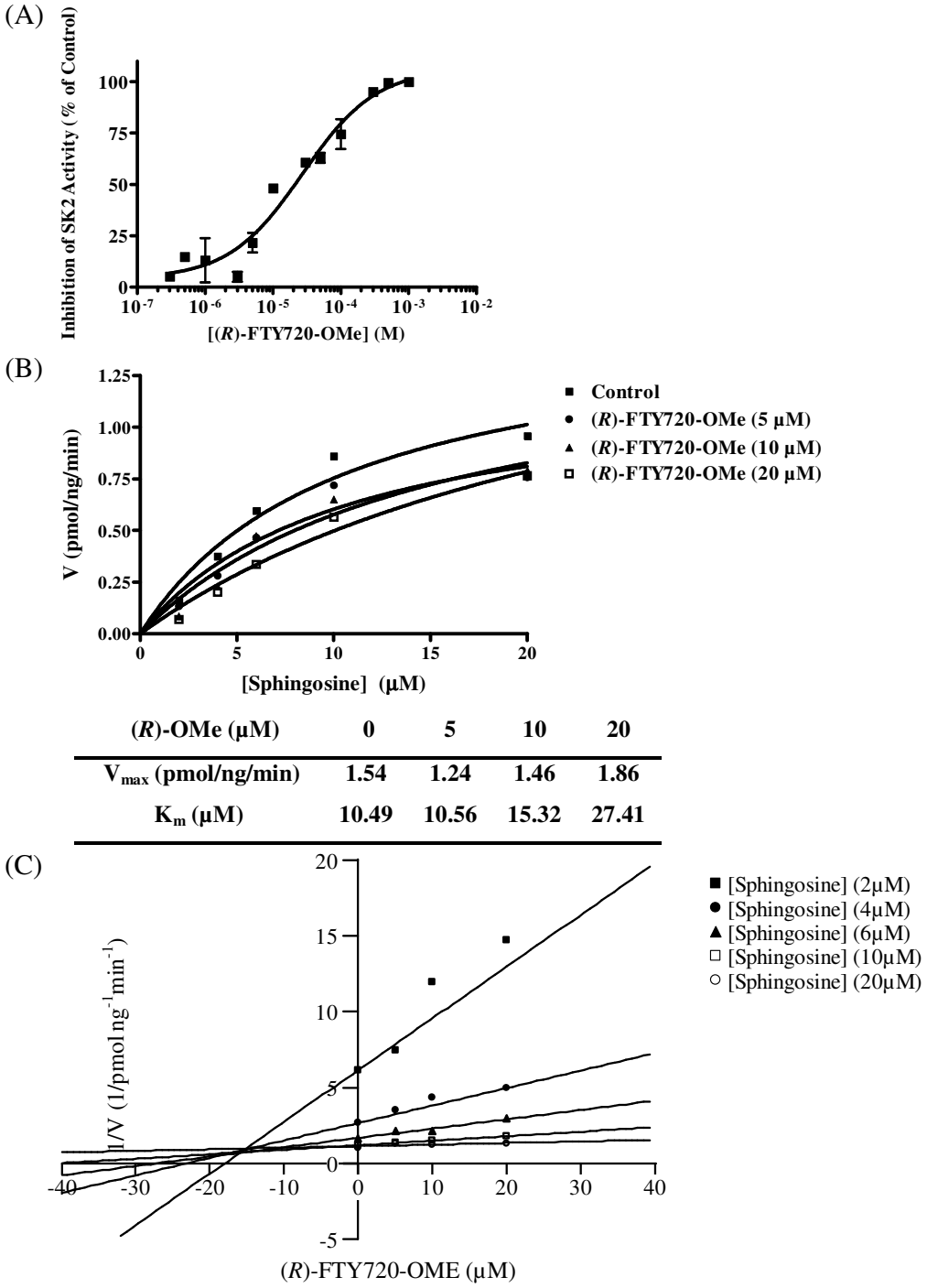


Figure 4.15 SK inhibitors inhibit membrane ruffling in MCF-7 Neo cells. Quiescent cells were pre-treated with FTY720 or SKi or (*S*)-FTY720 vinylphosphonate ((*S*)-vinyl Pn) (all at 10 µM, 30 mins) prior to addition of S1P (1 µM, 5 mins). Cells were fixed and actin was detected by phalloidin red staining as described under Methods (section 2.2.13). Nuclei were co-stained with DAPI. Yellow arrows identify actin localised to lamellipodia/membrane ruffles; white arrows identify actin clustered into focal adhesions. Bar, 50 µm. Results are representative three independent experiments.

4.2.6 (*R*)-FTY720-OMe is a selective SK2 inhibitor

During the preliminary screening of SK inhibitors, (*R*)-FTY720-OMe was found to inhibit purified SK2 but not SK1 activity. Since FTY720 is a substrate for SK2, it was of interest to investigate the activity of an analogue bearing a methyl ether group in place of one of the prochiral hydroxyl group on FTY720. (*R*)-FTY720-OMe inhibited SK2 in a concentration-dependent manner (IC_{50}: 27 +/- 1.3 μM; Hill coefficient: 0.8 +/- 0.2; Fig. 4.16A). Further investigation on the inhibition of SK2 reveals that (*R*)-FTY720-OMe exhibits competitive inhibition against sphingosine with a K_{ic}= 16.5 +/- 1.0 μM (Fig. 4.16B, C). For example, in the presence of 20 μM (*R*)-FTY720-OMe, the K_m for SK2 increased from 10.49 μM to 27.41 μM whereas the V_{max} was not significantly altered.

The present study also investigated the effect of (*R*)-FTY720-OMe on SK2 expression. As shown in Fig. 4.17A, (*R*)-FTY720-OMe reduced SK2 expression in HEK 293 cells over-expressing myc-tagged SK2. Interestingly, down-regulation of SK2 by (*R*)-FTY720-OMe was also associated with PARP cleavage suggesting that apoptosis may be induced. This indicates that SK2 plays a pro-survival role in HEK 293 cells. (*R*)-FTY720-OMe-induced reduction of SK2 expression was not overcome by pre-treating the cells with cathepsin B inhibitor (CA074Me) or proteasome inhibitor (MG132) (Fig. 4.17B). Cells pre-treated with MG132 showed increased SK2 expression *versus* control, indicating that SK2 is regulated by the ubiquitin-proteasomal pathway under basal conditions. The effect of (*R*)-FTY720-OMe on SK2 expression is specific since SK1 expression was not altered by (*R*)-FTY720-OMe (Fig. 4.17C).



(*R*)-OMe (µM)	0	5	10	20
V_{max} (pmol/ng/min)	1.54	1.24	1.46	1.86
K_m (µM)	10.49	10.56	15.32	27.41

Figure 4.16 Inhibitor kinetic analysis of (*R*)-FTY720-OMe for purified SK2. **(A)** Concentration-dependent inhibition of SK2 by (*R*)-FTY720-OMe ((*R*)-OMe). Experiments were performed in the presence of 10 µM sphingosine and 250 µM [^{32}P]-ATP. **(B)** V *versus* S non-linear regression analysis **(C)** Dixon plot. Results are representative of 3 independent experiments.

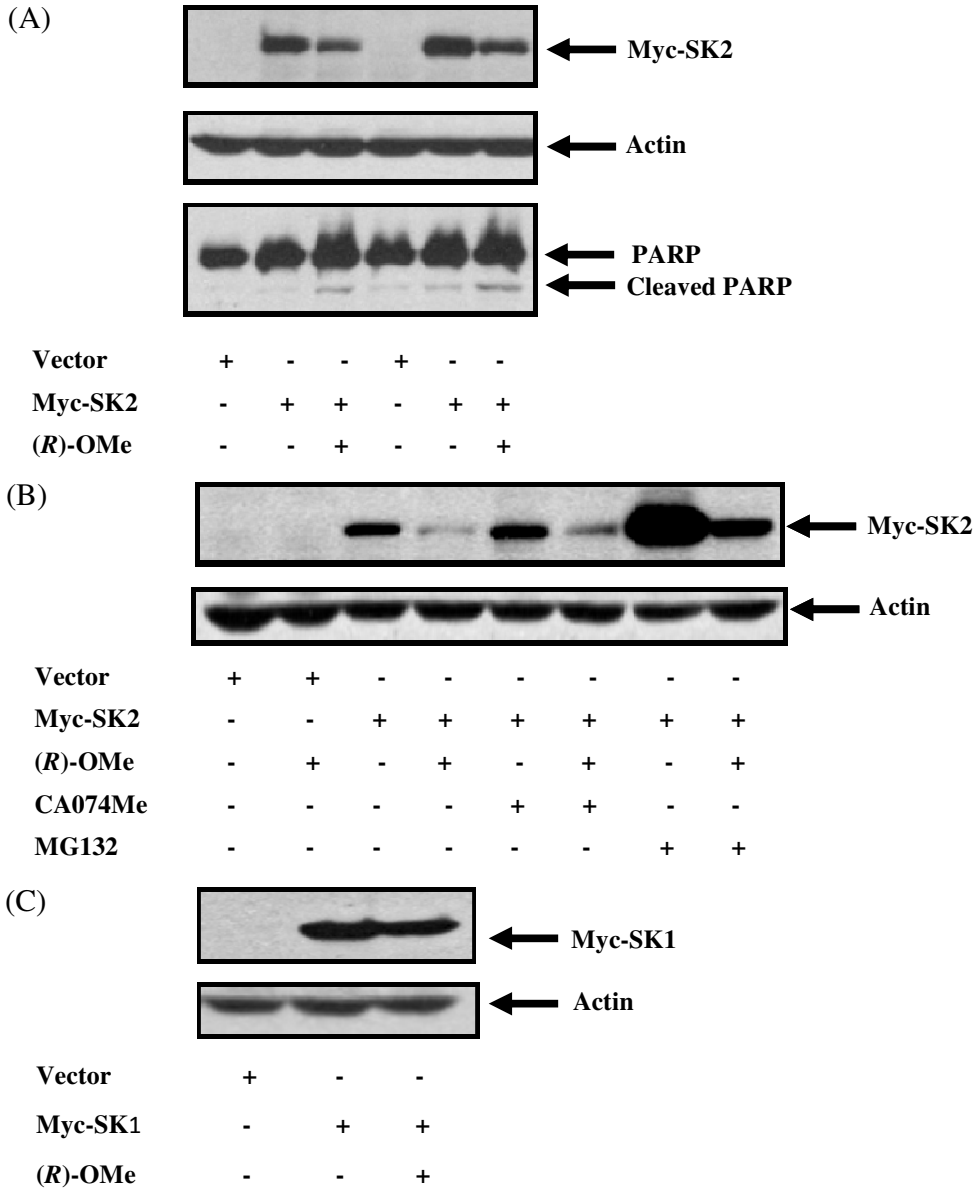
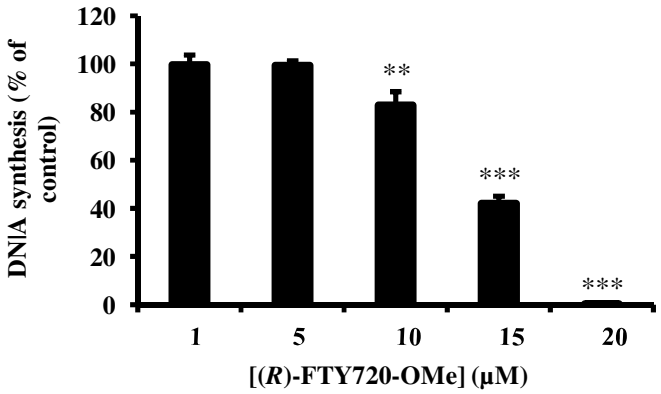


Figure 4.17 Effects of (*R*)-FTY720-OMe on SK2 and SK1 expression. Western blots showing the effects of **(A)** HEK 293 cells over-expressing Myc-SK2 in the presence of 10 µM (*R*)-FTY720-OMe, ((*R*)-OME) for 24 hrs, **(B)** HEK 293 cells over-expressing Myc-SK2 with pre-treatment with MG132 (10 µM, 30 mins) and CA074Me (10 µM, 30 mins) prior to addition of (*R*)-FTY720-OMe (10 µM, 24 hrs) and **(C)** HEK 293 cells over-expressing Myc-SK1 in the presence of 10 µM (*R*)-FTY720-OMe, ((*R*)-OME) for 24 hrs. Equal amount of cell lysates were resolved by SDS-PAGE. Immunoreactive bands for SK2 and SK1 were detected using anti-Myc antibody; PARP was detected using anti-PARP antibody. Blots were then stripped and reprobed with anti-actin antibody to ensure comparable protein loading. Results are representative of 3 to 5 independent experiments.

The pro-apoptotic effect of (*R*)-FTY720-OMe on HEK 293 cells prompted the investigation of its effect on MCF-7 cell proliferation since SK2 is endogenously expressed in this cell type (Sankala *et al*., 2007). As expected, (*R*)-FTY720-OMe inhibited DNA synthesis of MCF-7 cells in a concentration-dependent manner (Fig. 4.18A). As previously demonstrated in this study (Fig. 4.15), SK1 inhibitors mimic the action of siRNA targeted at SK1, i.e. they produce a reduction in S1P-induced actin rearrangement in MCF-7 Neo cells. Therefore, it was of interest to examine the effect of (*R*)-FTY720-OMe on cell migration of this cell line. As shown in Fig. 4.18B, S1P stimulated membrane ruffling and this was inhibited by pre-treatment of MCF-7 Neo cells with (*R*)-FTY720-OMe. This suggests that SK2 also affects actin rearrangement involved in cell migration.

(A)



(B)

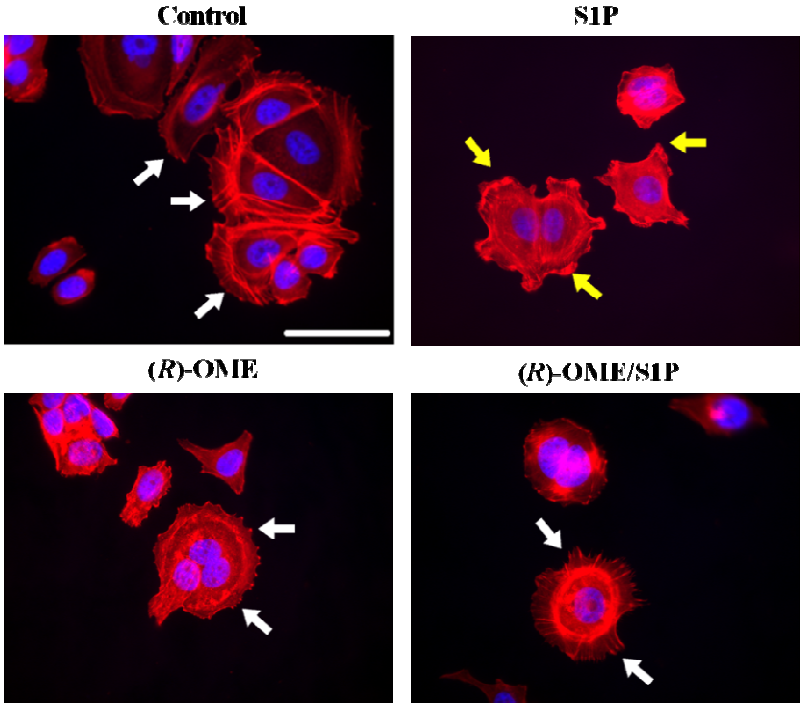


Figure 4.18 Effects of (*R*)-FTY720-OMe on DNA synthesis and actin-rearrangement (A) MCF-7 cells were treated with increasing concentration of (*R*)-FTY720-OMe or control (DMSO 0.01%) for 24 hrs and then with [^{3}H]-thymidine (0.5µCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control, which represent means and standard deviations of triplicate determinations, **p<0.01, ***p<0.001 *versus* control. **(B)** MCF-7 Neo cells were treated with (*R*)-FTY720-OMe ((*R*)-OME), (10 µM, 15 mins) prior to stimulation with and without S1P (1 µM, 5 mins). Actin was visualised using phalloidin red staining as described under Methods (section 2.2.13). Nuclei were co-stained with DAPI. Yellow arrows identify actin localised to lamellipodia/membrane ruffles, while white arrows identify actin clustered into focal adhesions. Bar, 50 µm. Results are representative of three independent experiments.

4.3 Discussion

The studies described in this chapter characterised the inhibition kinetics of FTY720, *(S)*-FTY720 vinylphosphonate and SKi on SK1. In addition, the presence of putative allosteric site(s) on SK1 was investigated. The latter part of this chapter presents the characterisation of the kinetics of inhibition of SK2 by (*R*)-FTY720-OMe. The effects of these inhibitors on SK1 and SK2 will be discussed in relation to their anticancer effects on MCF-7 cells.

4.3.1 Kinetic characterisation of SK inhibitors

One of the major findings is that FTY720 inhibits SK1 in a competitive manner (with sphingosine, K_{ic} = 2 μM). This suggests that FTY720 may compete with sphingosine at the catalytic site of SK1. Indeed, FTY720 can be phosphorylated by SK2 which is 30-fold more efficient than SK1 in using FTY720 as a substrate (Billich *et al*., 2003). Kinetic characterisation of SK1 inhibition by *(S)*-FTY720 vinylphosphonate reveals that in contrast to FTY720, *(S)*-FTY720 vinylphosphonate is an uncompetitive inhibitor (K_{iu} = 14.4 μM). The uncompetitive inhibition modality suggests that *(S)*-FTY720 vinylphosphonate only binds to the enzyme-substrate complex. In addition, similar modes of inhibition (competitive for FTY720 and uncompetitive for *(S)*-FTY720 vinylphosphonate with sphingosine against SK1) have also been established using WT-FLAG/Myc tagged SK1 transiently over-expressed in HEK 293 cells. The inhibition constant, K_i is a measure of the affinity of an inhibitor to specific conformational state of an enzyme during a catalytic cycle. Due to different inhibition modalities, it can be concluded that FTY720 and *(S)*-FTY720 vinylphosphonate do not bind to the same enzyme conformational state. SKi displays mixed competitive inhibition (with sphingosine) against SK1, suggesting that it has affinity for both free enzyme and the enzyme-sphingosine complex. Indeed, two kinetic constants were obtained to define the mode of inhibition of SK1. For the free enzyme species, the inhibition constant is 17 μM whereas for the enzyme-sphingosine complex, the inhibition constant is 48.3 μM. Therefore, SKi has a higher affinity for the free enzyme species.

4.3.2 Oligomerisation and allosteric regulation of SK1

Despite the identification of lipids and proteins which interact with SK1 and regulate its activity, little is known about the domain(s) involved in these regulations. Interaction of SK1 with PA has been mapped to the C-terminal half of SK1 since the N-terminal fragment of SK1 fails to bind PA (Delon, *et al*., 2004). Stahelin and colleagues (2005) have also shown that phosphorylation of Ser^{225} improves membrane targeting of SK1 to PS which interacts with Thr^{54} and Asn^{89} on SK1. Anionic lipids possibly stimulate the activity of SK1 through improved substrate presentation. Some intracellular proteins are able to interact with SK1 (see Chapter 1, section 1.2.1). One important observation is that phosphorylation of Ser^{225} by ERK1/2 enhances SK1 catalytic activity which is essential for its translocation to the plasma membrane (Pitson *et al*., 2003). It is well known that targeting SK1 to different subcellular compartments dictates its signalling function (Wattenberg *et al*., 2006). Indeed, some SK1-interacting protein such as SKIP has been proposed to act like scaffolding or anchoring protein for SK1 (Lacaná *et al*., 2002). However, conformational change in SK1 might be induced, which results in activation or inhibition of its catalytic activity (see below).

In the present study, SK1 has been demonstrated as a kinase capable of forming oligomers in agreement to a previous study conducted by Kihara and colleagues (2006). Using immunoprecipitation, myc-tagged SK1 was successfully pulled down by anti-FLAG antibody whereas FLAG-tagged SK1 was pulled down by anti-myc antibody when myc and FLAG-tagged SK1 were co-expressed in HEK 293 cells (Fig. 4.6, 4.7). Interestingly, when a mutant SK1 (FLAG-tagged D178N SK1; mutated at the sphingosine binding site) was co-expressed with WT SK1, the activity of the WT enzyme was inhibited by the mutant SK1 in a concentration-dependent manner (Fig. 4.8, 4.9). This suggests that SK1 contains an auto-inhibitory domain at an allosteric site that regulates its catalytic activity. There are three alternative interpretations of these findings which can be excluded. First, dimerisation may be required for the formation of two competent catalytic sites. Co-expression of a mutant SK1 with WT SK1 would reduce the activity of the oligomers. If this were the case, co-expression of two different

mutants, one mutated at the sphingosine-binding site (D178N SK1) and another one mutated at the ATP-binding site (G81D SK1) should reconstitute ~50% of activity by domain swapping of functional catalytic sites from each monomer in a dimeric arrangement. For example, the ATP-binding site from D178N SK1 may form competent catalytic sites with the sphingosine-binding site from G81D SK1. However, the activity of such oligomers (~10% of WT SK1) was equivalent to that of D178N SK1 (Table. 4.2). Therefore, domain swapping of oligomeric SK1 is not involved in the formation of competent catalytic sites. Second, it is possible that oligomerisation can facilitate positive cooperativity between catalytic sites from each monomer. Co-expression of catalytically inactive mutant SK1 with WT SK1 may reduce activity of the oligomers by disrupting cooperativity between catalytic sites. However, SK1 displays simple Michaelis-Menten kinetics with a Hill coefficient close to unity (Fig. 4.1), indicating that there is no cooperativity between catalytic sites on SK1. Third, formation of competent catalytic sites might be dependent on oligomerisation of SK1 monomers and mutagenesis of Asp^{178} might impair oligomerisation. However, D178N SK1 still forms oligomers with the WT SK1 (Fig. 4.7). Since the concentration of WT SK1 is constant in the experiment, the inhibition of WT SK1 catalytic activity by D178N SK1 can be attributed to putative allosteric site(s) which exerts an auto-inhibition on WT SK1 activity.

The presence of putative allosteric site(s) or auto-inhibitory domain on SK1 is further supported by kinetic data showing that *(S)*-FTY720 vinylphosphonate is an uncompetitive inhibitor of SK1, i.e. it binds only to SK1-sphingosine complex. In addition, (*S*)-FTY720 regioisomer and bodipy-sphingosine stimulated SK1 activity. Therefore, a model is proposed in which SK1 contains allosteric site(s) which auto-inhibits catalytic activity. Binding of (*S*)-FTY720 vinylphosphonate to an "on" conformational state shifts the equilibrium in favour of the "auto-inhibitory" state of SK1. Binding of bodipy-sphingosine and (*S*)-FTY720 regioisomer to an "off" conformational state relieves auto-inhibition (Fig. 4.19). The auto-inhibitory domain may block SK1 activation and/or reduce the efficiency of catalysis by occluding its

catalytic site. SK1 has been reported to have low basal activity independent of post-translational modification (Pitson *et al*., 2000a). Hence, it appears that this putative allosteric site may also regulate activation of SK1 by phosphorylation catalysed by ERK1/2. The Hill coefficient is 2 for inhibition of SK1 by *(S)*-FTY720 vinylphosphonate, indicating that the allosteric sites may function in a cooperative manner. Similarly, stimulation of SK1 by bodipy-sphingosine shows a Hill coefficient of 4.5. Therefore, binding of bodipy-sphingosine to the allosteric site(s) also displays positive cooperativity. *(S)*-FTY720 vinylphosphonate and bodipy-sphingosine may not bind to the same allosteric site since the extent of cooperativity for ligand binding is different for these two compounds. Alternatively, inhibitor or activator might act on the same site but they exhibit different efficiency of cooperative interaction with different enzyme conformations (the single site model is depicted in Fig. 4.19).

The identification of a putative allosteric site(s) which exerts an auto-inhibitory control over SK1 warrants further investigation as this offers new opportunities to develop highly selective and effective inhibitor targeting SK1. An interesting observation was made by Pitson and colleagues (2001) where a point mutant (G113A) of SK1 has higher catalytic efficiency than WT SK1. This constitutively active lipid kinase has been proposed to adopt a conformation that is more conducive for catalysis. Therefore, the present study investigated if Gly^{113} may be part of the allosteric site. Mutation of Gly^{113} to Ala may trap the allosteric site in an "off" mode (Fig. 4.19) where auto-inhibition is relieved, prolonging the lifetime of this activated conformational state. However, G113A SK1 activity is still sensitive to inhibition by *(S)*-FTY720 vinylphosphonate and activation by Bdp-So, thereby suggesting that Gly^{113} is not involved in allosteric regulation of SK1.

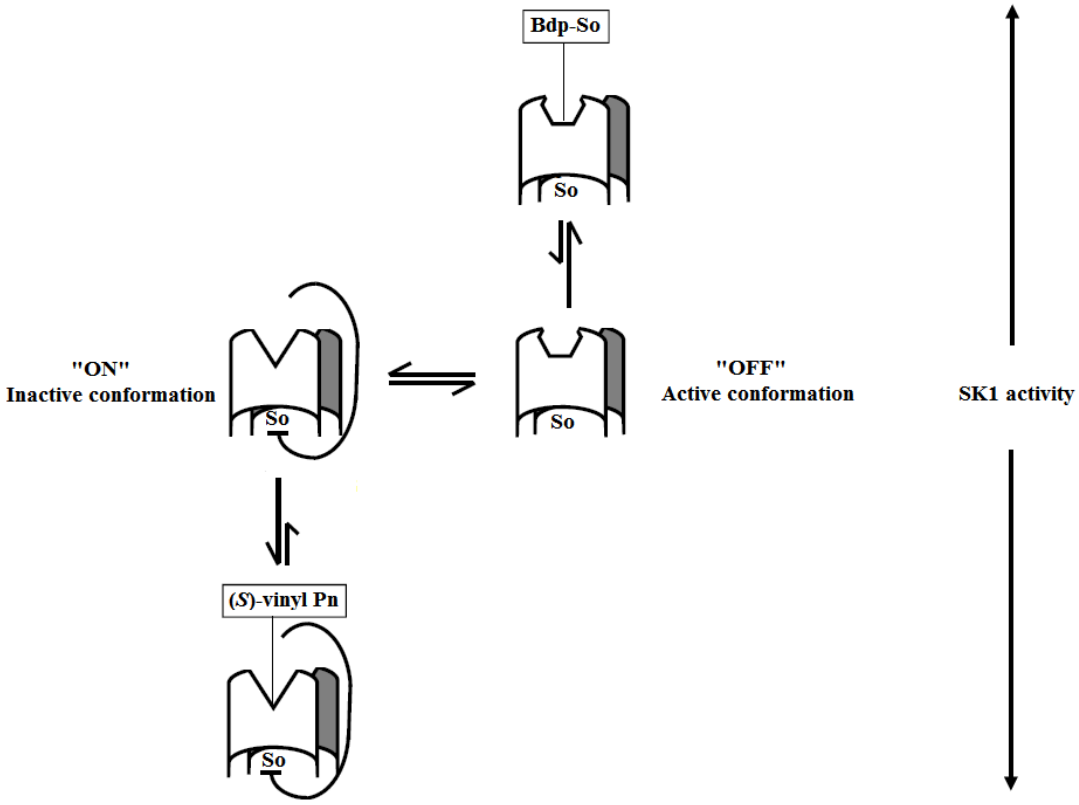


Figure 4.19 The "on" or "off" auto-inhibitory conformational states of SK1. Interaction of different SK1 conformational states with sphingosine (So), (*S*)-FTY720 vinylphosphonate ((*S*)-vinyl Pn), or Bdp-sphingosine (Bdp-So) are depicted. Binding of (*S*)-vinyl Pn to an inactive conformational state of SK1 shifts the equilibrium towards an enzyme conformation which has low catalytic activity. Conversely, Bdp-So binds an active enzyme conformation and increases SK1 activity. Adapted from Lim *et al*., (2011b).

High sequence homology between SK and other lipid kinases such as YegS and DGK predict that these enzymes may undergo a similar catalytic cycle while phosphorylating their respective substrates. Indeed, the ATP-binding site of SK shows high sequence similarity to that of DGK (Pitson *et al*., 2002). YegS is a close homologue to mammalian DGK. Therefore, the study of YegS may provide important structural information for SK. To this end, the present study investigated whether SK inhibitors such as *(S)*-FTY720 vinylphosphonate can inhibit YegS, thereby facilitates the identification of the location of the putative allosteric site. However, neither *(S)*-FTY720

vinylphosphonate nor the SK1 activator, Bdp-So altered YegS catalytic activity, indicating that YegS may not contain an allosteric site or that the site is conformationally constrained not to allow access to these compounds. More recently, a truncated SK1 mutant which has deletion of 21 amino acids at the C-terminus has been shown to have higher catalytic activity compared to the WT SK1 (Hengst *et al*., 2010a). It is tempting to speculate that this polypeptide at the C-terminus may be the auto-inhibitory domain. Whether this domain is involved in allosteric regulation of the enzyme awaits future studies.

Many proteins are regulated by phosphorylation which acts as a switch to turn on or turn off the activity of a protein. The switch is well characterised by an auto-inhibitor model in which phosphorylation of specific residues remove occlusion of an activation loop on the catalytic site (Adams, 2003). The development of auto-inhibitor model is based on structure-function studies on protein kinases using X-ray crystallography. Despite intense interests, no crystal structures have been reported for SK or eukaryotic DGK. However, structural information is available for YegS (Bakali *et al*., 2007; Nichols *et al*., 2007) and DgkB, a prokaryotic DGK (Miller *et al*., 2008). YegS shows significant amino acid sequence homology with eukaryotic SK, DGK and the N-terminus (phosphate-binding domain) of prokaryotic polyphosphate/ATP NAD kinases (Nichols *et al*., 2007). Interestingly, the crystal structures of YegS and DgkB reveal that these enzymes are also in a dimeric arrangement (Nichols *et al*., 2007; Miller *et al*., 2008). In addition, other related lipid kinases such as NAD kinases and 6-phosphofructokinase (PFK) are either dimeric or tetrameric (Labesse *et al*., 2002; Miller *et al*., 2008). Crystal structures of YegS and DgkB reveal that they share similar topology where the active site is located at an inter-domain cleft (hinge region) connected by two separate domains (Domain 1 and Domain 2). Since the active site is located away from the dimer interface, each monomer can independently bind substrates and facilitate catalysis (Miller *et al*., 2008). This might be true for SK1 since the present study found that SK1 is capable of forming oligomers (minimally a dimer) which contain non-cooperative catalytic sites.

Combining structural analysis and site-directed mutagenesis experiments, functional domains on YegS and DgkB have been mapped. For example, the protein chain of YegS traces from the N-terminus (Domain 1, nucleotide-binding region) into the C-terminal domain (Domain 2, lipid substrate-binding region) before returning to Domain 1. Therefore, both the N- and C-termini of YegS reside in Domain 1. Binding regions for activating lipids have been proposed to locate at a second conserved hydrophobic cleft in Domain 1 (Nichols *et al*., 2007). Some mammalian DGKs (e.g. DGKα, DGKβ and DGKγ) contain an EF-hand motif which changes conformation of the enzyme upon calcium binding (Yamada *et al*., 1997). Calcium is known to activate many enzymes including DGKα; the presence of EF-hand motif in DGKα suggests that this domain may be auto-inhibitory, acting like an allosteric regulator (Raben and Wattenberg, 2009). This type of motif has not been identified in YegS but distinct regulatory site has been linked to a novel metal-binding site which binds $Ca^{2+/}Mg^{2+}$ (Bakali *et al*., 2007). Interestingly, though distally related to DGK/YegS, PFK is an allosteric enzyme which contains a regulatory allosteric site in Domain 2 (Labesse *et al*., 2002). Substantial evidence has shown that SK1 activity can be stimulated by Ca^{2+}, anionic lipids (PA and PS) and post-translational modifications such as phosphorylation (see chapter 1, section 1.2.1). Taken together, it is possible that SK1 can be regulated by an auto-inhibitory domain in an allosteric manner. Structural studies on YegS and DgkB have revealed that such allosteric control may exert its effect through the hinge region which is linked to Domain 1 (putative ATP-binding site) and Domain 2 (putative sphingosine-binding site). Conformational change may induce movement of the two domains around the hinge region to regulate orientation of key residues which participate in substrate binding during catalysis (Raben and Wattenberg, 2009). The present study provides evidence for allosteric regulation of SK1 by analysing the kinetic behaviours of SK1 inhibitors/activators and performing functional studies on SK1 oligomers. Future work using site-directed mutagenesis and protein crystallography will further characterise this regulatory domain on SK1.

4.3.3 Anticancer effects of SK1 inhibitors

As discussed in Chapter 1, SK1 contributes to tumour progression and poor prognosis in breast cancer patients. In particular, Long *et al*., (2010a) recapitulated the biochemical pathways of SK1/$S1P_3$/ERK in ER^+ breast cancer patients using two cell lines, MCF-7 Neo (stably expressing the Neo vector) and MCF-7 HER218 (stably expressing the HER2/neu oncogene) and found that MCF-7 HER218 cells had higher SK1 expression. Previously, the pro-apoptotic effect of SKi has been linked to an increase of ceramide, activation of proteasome and subsequent degradation of SK1 through the ubiquitin-proteasome system (Loveridge *et al*., 2010). In the present study, FTY720 and (*S*)-FTY720 vinylphosphonate also induced proteasomal degradation of SK1 but to a lesser extent than that of SKi, demonstrating novel biological efficacy for these inhibitors. The difference in effectiveness observed with inhibitor-induced SK1 proteasomal degradation may be due to different extent of SK1 catalytic inhibition, activation of proteasome by SK1 inhibitors and conformation of inhibitor-SK1 complex (Loveridge *et al*., 2010). In this regard, it is difficult to correlate inhibitor potency alone with its cellular potency in down-regulating SK1 in cancer cells. Different physicochemical properties of the inhibitors can also affect cell permeability leading to varied bioavailability in cells. Nevertheless, SKi is the most effective compound in down-regulating SK1 in MCF-7 cells despite showing moderate inhibition of the catalytic activity of purified SK1. Up-regulation of SK1 in MCF-7 HER218 cells has no effect on inhibitor-induced SK1 down-regulation since all inhibitors down-regulate SK1 to a similar extent in both MCF-7 Neo and MCF-7 HER218 cells. Notably, SK inhibitors did not induce PARP cleavage in MCF-7 HER218 cells (Fig. 4.14). It has been suggested that EGF signalling might be involved, since pre-treatment of the cells with an EGF receptor tyrosine kinase inhibitor AG 1478, reduced basal ERK1/2 activation in MCF-7 HER218 cells (Long *et al*., 2010b). Interestingly, MCF-7 cells only express SK1a whereas LNCaP cells express two splice variants of SK1 (termed SK1a and SK1b). SKi induces proteasomal degradation of both SK1a and SK1b in LNCaP parental cells but only SK1a and not SK1b is down-regulated in LNCaP-AI (androgen-independent)

cells—a post translational modification of SK1b may be involved that protects SK1b from the proteasome in LNCaP-AI cells (Loveridge *et al*., 2010).

Previous studies in both *in vitro* and *in vivo* cancer models have established that SK1 inhibitors are effective anticancer compounds which inhibit cell growth and induce apoptosis. The present study reveals a novel mode of action for FTY720 (i.e. acting as a competitive SK1 inhibitor). Nagaoka and colleagues, (2008) found that FTY720 inhibited proliferation of various cancer cell lines including MCF-7 cells with IC_{50} between 5-7μM, and reduced activation of pro-survival MAPK (e.g. ERK1/2) but increased pro-apoptotic MAPK (e.g. JNK 1/2). SKi has been shown to inhibit cell proliferation and induce apoptosis (see Chapter 3 and Fig. 4.14). In common, FTY720 also induces PARP cleavage and down-regulates SK1 expression (Fig.4.13, 4.14). SK1 inhibitors act as potent apoptotic inducers possibly due to two non-mutually exclusive mechanisms.

First, catalytic inhibition of SK1 perturbs the dynamic balance between pro-apoptotic ceramide and pro-survival S1P. Indeed, treatment of LNCaP cells with SKi increases intracellular C22:0 ceramide and reduces S1P levels (Loveridge *et al*., 2010). This is reminiscent of the effects of SKi in reducing intracellular S1P levels in MDA-MB-231 breast cancer and JC mouse mammary adenocarcinoma cells (French *et al*., 2003; 2006). Other SK1 inhibitors have also been shown to perturb ceramide/S1P ratio: DMS, F12509A, B5354a, S-15183a and S-15183b reduce S1P formation in platelets (Yatomi *et al*., 1996; Kono *et al*., 2000a; Kono *et al*., 2000b; Kono *et al*., 2001); the new amidine-based SK1 inhibitors reduce S1P formation in human leukemia U937 cells at nanomolar range (Kennedy *et al*., 2011).

Second, a knock-on effect as a consequence of perturbation of ceramide/S1P ratio and SK1 down-regulation may operate. Exogenously added cell-permeable C-2 ceramide activates the proteasome and increases the rate of degradation of polyubiquitinated SK1. The effect of SKi can also be partially reversed by fumonisin B (a ceramide synthase

inhibitor) which reduces ceramide accumulation in cells (Loveridge *et al*., 2010). Furthermore, knockdown of SK1 by siRNA also increases ceramide formation and induces apoptosis (Taha *et al*., 2006b).

Besides, these inhibitors can act on additional targets. For example, SKi may also bind to the oestrogen receptor, acting as an antagonist to inhibit oestrogen-dependent signalling (Antoon *et al*., 2011). In addition, *(S)*-FTY720 vinylphosphonate is also a full $S1P_{1/3/4}$ antagonist (Valentine *et al*., 2010). Since $S1P_{1/3}$ is implicated in tumour growth and vascularisation, the dual actions of *(S)*-FTY720 vinylphosphonate ($S1P_{1/3}$ antagonism and SK1 inhibition) may contribute to its anti-cancer effects. Similarly, FTY720 is phosphorylated *in vivo* to FTY720 phosphate which is a $S1P_1$ functional antagonist. FTY720 has been shown to inhibit tumour growth and vascularisation in murine xenograft models possibly due to $S1P_1$ antagonism (LaMontagne *et al*., 2006).

The current study further relates the anticancer effects of SK1 inhibitors to inhibition of cancer cell migration. S1P (binding to $S1P_3$) stimulates re-localisation phospho-ERK1/2 into membrane ruffles and the nucleus to promote cell migration. The siRNA knockdown of SK1 not only reduces $S1P_3$ expression but also promotes focal adhesion and inhibits S1P-induced lamellipodia formation in MCF-7 Neo cells (Long *et al*., 2010a). This effect on actin reorganisation is recapitulated by the present study using SK1 inhibitors including SKi, FTY720 and (*S*)-FTY720 vinylphosphonate. Pre-treatment of MCF-7 Neo cells with these SK1 inhibitors prevents the formation of a "migratory phenotype", as evidenced by reduction in membrane ruffles/lamellipodia formations. This suggests that SK1 inhibitors may have anti-metastatic activity. Formal investigation is needed to validate the *in vivo* significance of these observations. However, in MCF7 HER218 cells, high expression of SK1 functions to desensitise S1P-induced cell migration. Furthermore, high SK1 expression in patients whose tumours are $ER^+/HER2^+$ was shown to correlate with improved patient survival. Therefore, this cautions against the use of SK inhibitors in patients whose breast cancer tumours are

$ER^+/HER2^+$ (Long *et al*., 2010a). Collectively, this part of the work successfully demonstrates SK1 inhibitors as effective anticancer compounds *in vitro*.

4.3.4 (*R*)-FTY720-OMe as a novel SK2 inhibitor

FTY720 is a prodrug which has to be phosphorylated *in vivo* by SK2 to produce FTY720 phosphate, the active compound responsible for the induction of lymphopenia (Billich *et al*., 2003). Despite being a substrate for SK2, FTY720 is not phosphorylated efficiently by SK1 (Billich *et al*., 2003). Indeed, FTY720 inhibits SK1 activity and acts in a competitive manner with sphingosine (Lim *et al*., 2011b). Therefore, it appears that FTY720 binds and interacts with catalytic residues in SK2; SK2 then catalyses phosphoryl transfer from ATP to one of the prochiral hydroxyl groups of FTY720. Modifying one of these hydroxyl groups on FTY720 should ablate phosphorylation by SK2. To test this hypothesis, a synthetic analogue has been synthesised by replacing one of the prochiral hydroxyl group on FTY720 with methyl ether (FTY720-OMe). Indeed, (*R*)-FTY720-OMe but not (*S*)-FTY720-OMe (see chapter 3, Fig. 3.16) inhibits SK2 activity competitively with sphingosine (Lim *et al*., 2011a).

The present study is the first to demonstrate that SK2 expression can also be reduced by a selective SK2 inhibitor. Prolonged treatment of HEK 293 cells transiently over-expressing myc-tagged SK2 with (*R*)-FTY720-OMe not only reduces SK2 expression but also induces PARP cleavage. Endogenous SK2 is expressed in HEK 293 cells and mediates cell migration towards EGF (Hait *et al*., 2005). (*R*)-FTY720-OMe induces PARP cleavage, suggesting that SK2 acts as an anti-apoptotic enzyme in this cell line. MCF-7 cells also express SK2 (Sankala *et al*., 2007) and indeed, the present study shows that (*R*)-FTY720-OMe inhibited DNA synthesis of MCF-7 cells in a concentration-dependent manner. The IC_{50} (concentration of compound which causes 50% inhibition) of (*R*)-FTY720-OMe on DNA synthesis is ~15 μM which is very similar to the K_i of (*R*)-FTY720-OMe (16.5 μM) for SK2, indicating good *in vitro* correlation of biological effect with catalytic inhibition. Autophagic death has been suggested to be the main

mechanism of cell death induced by a newly developed SK2 inhibitor, ABC294640 which does not induce apoptosis in some cancer cell lines (French *et al*., 2010). Of note, the present study provides evidence that (*R*)-FTY720-OMe may induce apoptosis in HEK 293 cells. Formal investigation is needed to assess whether (*R*)-FTY720-OMe can induce apoptosis and/or autophagic cell death in cancer cells.

SK2 is required for EGF-induced cell migration in mammalian cells (Hait *et al*., 2005) and treatment of cancer cells with a SK2 inhibitor inhibits cell migration (French *et al*., 2010). Since SK2 is also endogenously expressed in MCF-7 cells, the present study investigated the effect of the newly discovered SK2 inhibitor on redistribution of actin to that of a cell migratory phenotype in this cell line. Indeed, pre-treatment with (*R*)-FTY720-OMe reduced S1P-induced formation of a "migratory phenotype" (as evidenced by actin accumulating at lamellipodia) and induced a "stationary phenotype" (as evidenced by formation of actin focal adhesions) (Fig. 4.18B). These data are reminiscent of the effect of ABC29460 in reducing lamellipodia formation in cancer cells (French *et al*., 2010). Therefore, further studies are warranted to investigate the potential anti-metastatic activity of SK2 inhibitor *in vivo*.

This chapter presents evidence for allosteric control of SK1 activity by an auto-inhibitory domain. Due to high sequence homology between SK1 and SK2 and their overlapping functional roles in development, it is tempting to speculate that SK2 may also be allosterically regulated. Interestingly, a recent study has identified a lipid-binding domain for SK2 which may control subcellular localisation of the enzyme. Using sulfatide as a probe, the domain was mapped at the N-terminus (1-175 residues) of SK2. Endogenous glycolipid sulfatide (3-*O*-sulfogalactosylceramide) can bind to this domain and inhibits SK2 activity (Don and Rosen, 2009). Non-competitive inhibition of the catalytic activity of SK2 by sulfatide suggests that this domain may be involved in the allosteric regulation of SK2 activity. It will also be of interest to examine the effects of (*S*)-FTY720 vinylphosphonate and Bdp-So on SK2 activity. Similarly, SK2 may form oligomers which are conducive for allosterism.

In summary, (*R*)-FTY720-OMe has been discovered and characterised as a novel selective SK2 inhibitor. Using (*R*)-FTY720-OMe, the present study established SK2 as a pro-survival protein in MCF-7 cells. This reveals new opportunities for developing SK2 inhibitors, such as (*R*)-FTY720-OMe, for the treatment of breast cancer. Moreover, the development of these isoform-selective SK inhibitors will allow their use as chemical tools to dissect signalling pathways regulated by SK1 and SK2 in health and disease.

Chapter 5 Bioassay-guided isolation of anticancer compounds from plants

5.1 Introduction

Nature has nurtured the growth and survival of all life on earth. Driven by evolutionary forces, different organisms have developed various strategies to thrive when resources are scarce. For instance, plants invest heavily in the production of secondary metabolites which may have no apparent contribution to their survival. However, research has shown that biochemical pathways that produce these compounds are evolutionarily conserved and offer protection to the plants from predators (Agrawal, 2007). Chapter 3 provides evidence that plants can produce SK inhibitors. This is the first example to show that SK inhibitors can be produced by plants from unrelated families. It has previously been demonstrated that S1P is formed in plants and can regulate abscisic acid (ABA)-mediated stomatal opening, thereby regulating transpiration (Ng *et al*., 2001; Coursol *et al*., 2003). In addition, S1P can also regulate ABA-mediated seed germination, indicating that S1P plays an important role in plant cell signalling (Worrall *et al*., 2008). Therefore, plants may produce metabolites that can regulate the activities of SK in other organisms. This chapter will describe the isolation of cytotoxic compounds (potentially acting as SK inhibitors) from a plant hit identified in preliminary screening.

5.1.1 *Hopea dryobalanoides*

One of the first hits identified from the preliminary screening was *Hopea dryobalanoides* which belongs to the family Dipterocarpaceae. Dipterocarpaceae is a well known family of rainforest trees that comprises more than 500 species including over 100 species in the genus *Hopea* distributed mainly in tropical countries (Dayanandan *et al*., 1999). These trees also distribute widely in Southeast Asia, South America and Africa. The widespread distribution of dipterocarps has been proposed to originate from the Eurasian plate and migrated to Far East including South Asia and Africa where they eventually diversified (Dayanandan *et al*., 1999). Nevertheless, the number of species belonging to this family may be lower than expected due to discrepancies in species identifications resulting in replication. Therefore, some of these

species may apparently be the same even though they have been assigned with different names. Various attempts have also been used to study the phylogenetic relationships of different species in *Hopea* (Choong *et al*., 2008), in order to gain a better understanding of the diversification of these enormous but closely related trees.

5.1.2 Previous studies on resveratrol oligomers isolated from the *Hopea* genus

Oligostilbenoids such as resveratrol oligomers have been typically produced by plants in the *Hopea* genus which belong to the Dipterocarpaceae family. Compounds isolated from *Hopea dryobalanoides* are shown in Fig. 5.1. A common biosynthetic route of these resveratrol oligomers from plants has also been proposed (Sotheeswaran *et al*., 1993). One of the first compounds isolated was indeed a resveratrol tetramer, hopeaphenol from *Hopea odorata*, the structure of which was eventually elucidated using X-ray crystallography (Coggon *et al*., 1965; Coggon *et al*., 1970). Subsequently, this compound has been isolated from other species in the same genus, including *Hopea dryobalanoides*, *Hopea malibato* and *Hopea parviflora* (Sahidin *et al*., 2005; Dai *et al*., 1998; Tanaka *et al*., 2000). Interestingly, hopeaphenol has also been isolated from genera other than *Hopea* including *Neobalanocarpus heimii*, *Dipterocarpus hasseltii* and *Vitis vinifera* (Weber *et al*., 2001; Muhtadi *et al*., 2006; Yan *et al*., 2001) suggesting its common existence as an important metabolite for some plants. Hopeaphenol was found to be highly active against several cancer cell lines including human epidermoid nasopharynx carcinoma (KB), lung cancer carcinoma (A549) and breast cancer (MCF-7) (Ohyama *et al*., 1999). Hopeaphenol also strongly inhibited the proliferation of P-388 murine leukaemia cells (Muhtadi *et al*., 2006). Taken together, hopeaphenol with its interesting tetrameric structure is one of the most active anti-cancer compounds produced by these trees. However, its mechanism(s) of action remains to be elucidated. Another tetrameric resveratrol, known as Vaticanol B (Fig. 5.1) was also isolated from *Hopea dryobalanoides* and showed moderate cytotoxic activity against P-388 cells. (Muhtadi *et al*., 2006; Sahidin *et al*., 2005).

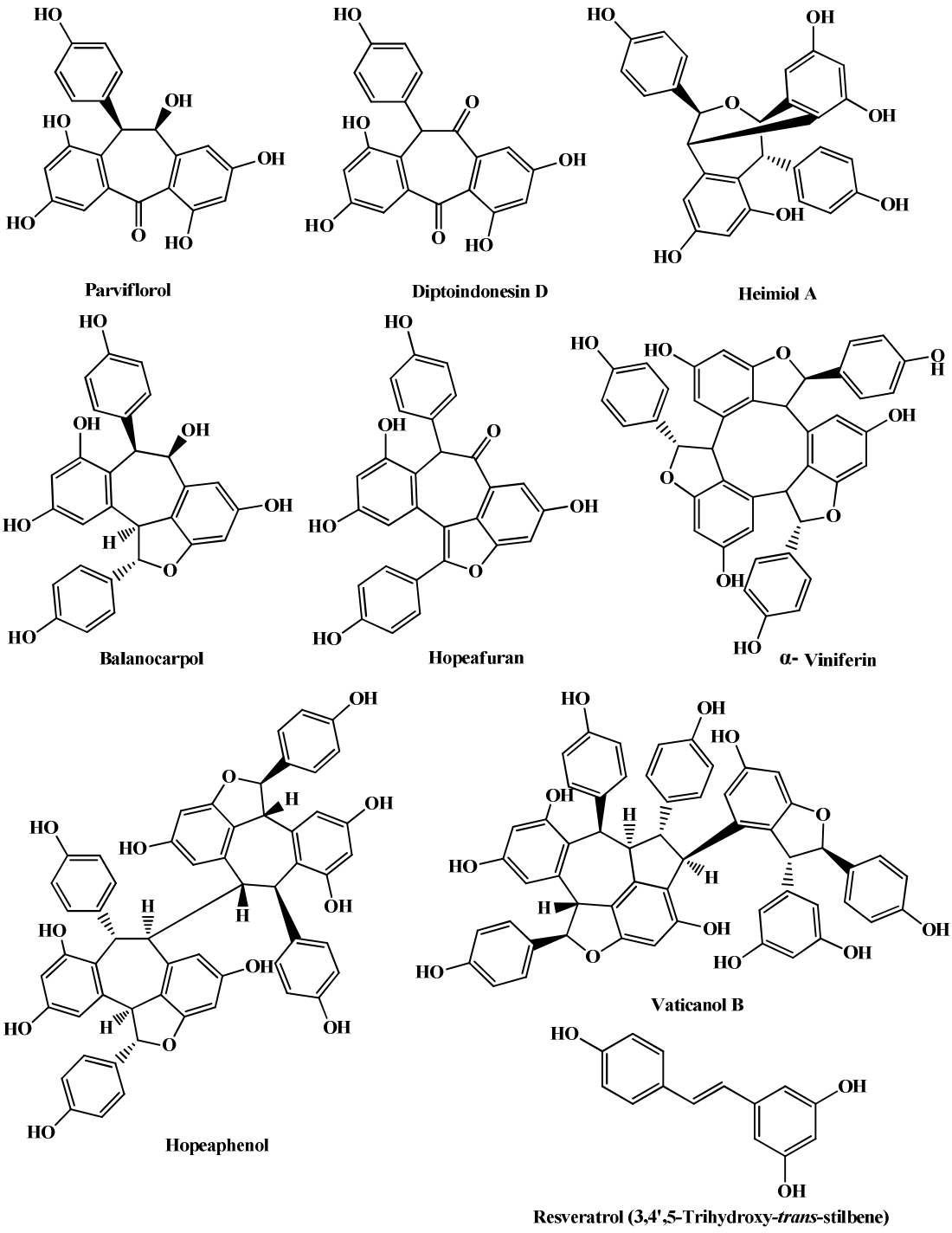


Figure 5.1 Structures of resveratrol and compounds isolated from *Hopea dryobalanoides*. Relative stereochemistry of each molecule was drawn if reported

Copalliferol A, also known as Vaticanol G is a resveratrol trimer isolated from several *Hopea* and *Shorea* spp. (Sotheeswaran *et al*., 1983; Ito *et al*., 2001b). α-viniferin is also a resveratrol trimer which was first isolated by Langcake and Pryce (1977a) from wine grapes (*Vitis vinifera*) as an antifungal agent. In addition, resveratrol, ε-viniferin (dimer) and α-viniferin (trimer) could be produced successively in a time-dependent manner under UV irradiation, indicating that the biosynthetic precursors were indeed resveratrol monomers (Langcake and Pryce, 1977b). Subsequently, α-viniferin has been isolated from *Hopea dryobalanoides* (Sahidin *et al*., 2005).

Several resveratrol dimers have also been isolated from *Hopea dryobalanoides* including parviflorol, diptoindonesin D, balanocarpol, heimiol A and hopeafuran (Fig. 5.1) (Sahidin *et al*., 2005). Parviflorol was first isolated from *Hopea parviflora* as a yellow solid (Tanaka *et al*., 2000). Diptoindonesin D was subsequently isolated by Sahidin and colleagues (2005) as a derivative (8-ketone) of parviflorol. Meanwhile, heimiol A appeared as a light brown solid isolated from *Neobalanocarpus heimii* (Weber *et al*., 2001). In fact, parviflorol and diptoindonesin D are modified dimers of resveratrol whereas balanocarpol and hopeafuran are derivatives of ampelopsin A. The Dictionary of Natural Products 2011 (version 20:1) documented ten derivatives of ampelopsin A (Table 5.1, Fig. 5.2).

Table 5.1 Variants and derivatives of ampelopsin A

Variants	Derivatives	Synonym
(+) form	7-epimer	(+)-balanocarpol
	8-deoxy	ampelopsin B
(-) form	13-O-β-D-glucopyranoside	laevifoside
	8-ketone	pauciflorol E
	7',8'-didehydro-8-ketone	hopeafuran
	8-deoxy	melanoxylin A
	7-epimer	(-)-balanocarpol
	8-epimer, 7',8'-Didehydro	malibatol A
	8-epimer, 3',14'-dihydroxy, 7',8'-didehydro	malibatol B
	7,8-Diepimer	hemsleyanol A

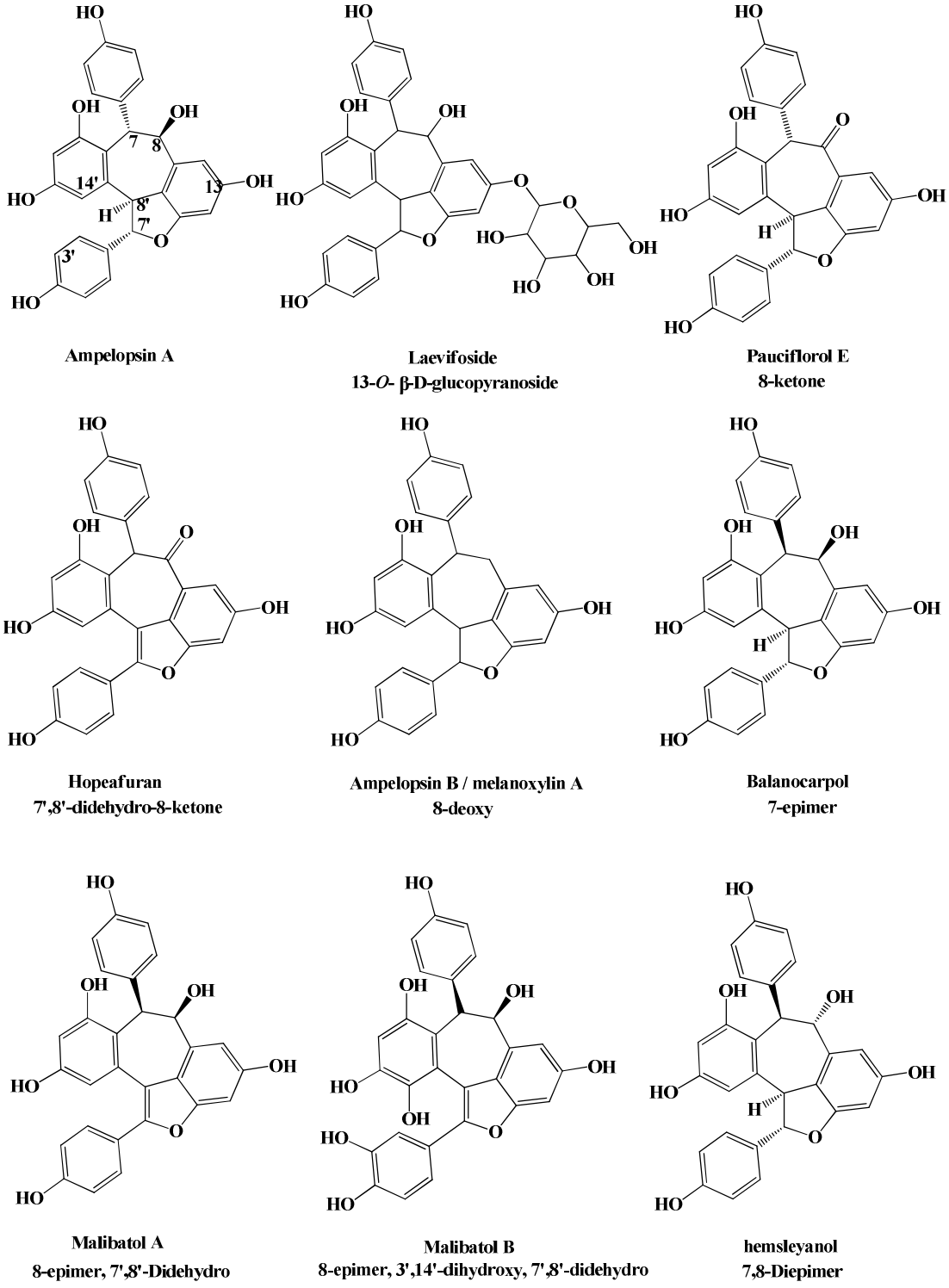


Figure 5.2 Structures of derivatives of ampelopsin A. Relative stereochemistry of each molecule is drawn if reported.

One of the most interesting observations was the action of malibatol against HIV infected cells (Dai *et al*., 1998). Isolated from *Hopea malibato*, malibatol A, malibatol B and balanocarpol share similar structures but only balanocarpol had modest activity against HIV. In fact, a search in the LASSO (Ligand Activity in Surface Similarity Order) database (www.chemspider.com) for malibatol A, malibatol B, ampelopsin A and balanocarpol gave a score of 0.83 when compared with other HIV protease inhibitors indicating 83% similarity of surface properties between these molecules and HIV protease ligands. In contrast, malibatol A and malibatol B retrieved a score of 0.98 when compared with known Src tyrosine kinase inhibitors whereas the score for ampelopsin A and balanocarpol was 0.01. Since LASSO is a virtual similarity tool that compares surface properties of different ligands without including two or three dimensional information, it only provides a clue on how similar these molecules are when compared with known ligands and there is no guarantee for activities. Even so, it is a very useful tool to obtain an idea of which targets may be bound by these molecules. Despite successful isolation of resveratrol oligomers, further study has been hampered by low yield of these compounds and intractable chemical synthesis. Conversely, resveratrol monomer has been extensively investigated.

5.1.3 Resveratrol and its biological actions

Resveratrol is found abundantly in grapes, peanuts and has been isolated from more than 70 plant species spanning 31 genera and 12 families (Jang *et al*., 1997). There is substantial evidence demonstrating that resveratrol (Fig. 5.1) is active against many types of cancer (Athar *et al*., 2007). Resveratrol has high antioxidant property owing to the possession of phenol rings which are strong scavengers of reactive oxygen species such as radicals induced by superoxide (Leonard *et al*., 2003). Resveratrol also inhibits cyclooxygenase 1 (COX-1) and COX-1 associated hydroxyperoxidase activity (Jang *et al*., 1997). This has been suggested to be linked with its chemopreventive property by inhibiting tumour initiation and progression. Indeed, resveratrol has been demonstrated to prevent tumourigenesis in mouse mammary glands and a two-stage skin cancer model

(Jang *et al*., 1997). Resveratrol has also been shown to inhibit COX-2 expression and activity in human mammary cells which could be responsible for its anti-inflammatory activity (Subbaramaiah *et al*., 1998). Taken together, resveratrol inhibits tumour growth, in part, through its anti-inflammatory action.

Intense research on resveratrol has been triggered by its famous link to the "French paradox". Individuals who consume red wine apparently have lower incidence of cardiovascular-related death, suggesting that resveratrol exerts cardioprotective effect (Fremont *et al.*, 2000). In recent years, this remarkable molecule has been extensively studied and shown to contribute beneficial effects in cancer, inflammation, cardiovascular and age-related diseases (Baur and Sinclair, 2006). A link to life span and aging was revealed when several plant polyphenols having similar structural moieties (e.g. piceatannol and resveratrol) activated human deacetylase (SIRT 1) *in vitro* and sirtuin 2 (SIR 2) *in vivo* in yeast (Howitz *et al*., 2003). At low micromolar concentration, resveratrol induced a two-fold increase in SIRT1 activity and stimulated SIR 2 to improve the life span of *Saccharomyces cerevisiae* by 70% (Howitz *et al*., 2003). The life-extending effect of resveratrol has been attributed to activation of SIR 2 that stabilises rDNA repeats rather than its anti-oxidant activity (Howitz *et al*., 2003). In a following report, resveratrol extended the life span of a roundworm, *Caenorhabditis elegans* indicating an evolutionary conserved mechanism of sirtuins in regulation of ageing and the role of resveratrol in regulating the activity of sirtuin under stress (Wood *et al*., 2004). Furthermore, resveratrol was able to extend the life span and alleviated the deleterious effects of a high calorie diet in mice through activation of known longevity pathways (Baur *et al*., 2006). To this end, these sirtuin-activating compounds seemingly belong to a small subset of chemicals which have low molecular weights and similar stilbene scaffolds (Howitz *et al*., 2003). However, none of the oligomers of resveratrol have been investigated in this aspect. Therefore, it will be of interest to investigate whether resveratrol oligomers can act on similar pathways.

Most of the compounds isolated from *Hopea dryobalanoides* and related species were cytotoxic. However, the exact mechanisms of action and possible molecular targets are poorly understood. The fact that *Hopea dryobalanoides* extract inhibited SK1 activity (see chapter 3, Fig. 3.12) suggests that one or more of the compounds isolated from *Hopea dryobalanoides* could act as SK1 inhibitors. Hence, the *Hopea dryobalanoides* extract was fractionated for further investigation. The structures of these active compounds were elucidated and confirmed to be resveratrol dimers, ampelopsin A and balanocarpol. Importantly, resveratrol and its dimers inhibited cancer cell growth and induced apoptosis by inhibiting SK1 expression/activity.

5.3 Results

5.3.1 *Hopea dryobalanoides* extract inhibits SK1 and down-regulates its expression

As shown in Chapter 3, *Hopea dryobalanoides* extract was active in inhibiting both DNA synthesis and SK1 activity, indicating that SK1 inhibitors are produced by the plant. To verify the effect of potential inhibitors acting on SK1, a cell-based functional assay was used to compare the isolated inhibitors with established SK1 inhibitors. In this regard, SKi has been shown to down-regulate SK1 expression in mammalian cells due to inhibitor-induced proteasomal degradation (Loveridge *et al*., 2010). In agreement, SKi induced down-regulation of SK1 with concomitant PARP cleavage in MCF-7 Neo cells (Fig 5.3). Down-regulation of SK1 was up-stream of apoptosis since pre-treatment of the cells with the caspase3/7 inhibitor (Ac-DEVD-CHO) did not reduce the down-regulation of SK1. To compare whether the plant extract exhibits a similar functional profile as other SK1 inhibitors, MCF-7 Neo cells were treated with DMS, SKi or *Hopea dryobalanoides* extract. Significant down-regulation of SK1 by the plant extract was observed at treatment for 48 hrs (Fig 5.3D). These data suggest the presence of SK1 inhibitors in *Hopea dryobalanoides* extract. Therefore, the extract was fractionated to identify the active compounds from purer fractions.

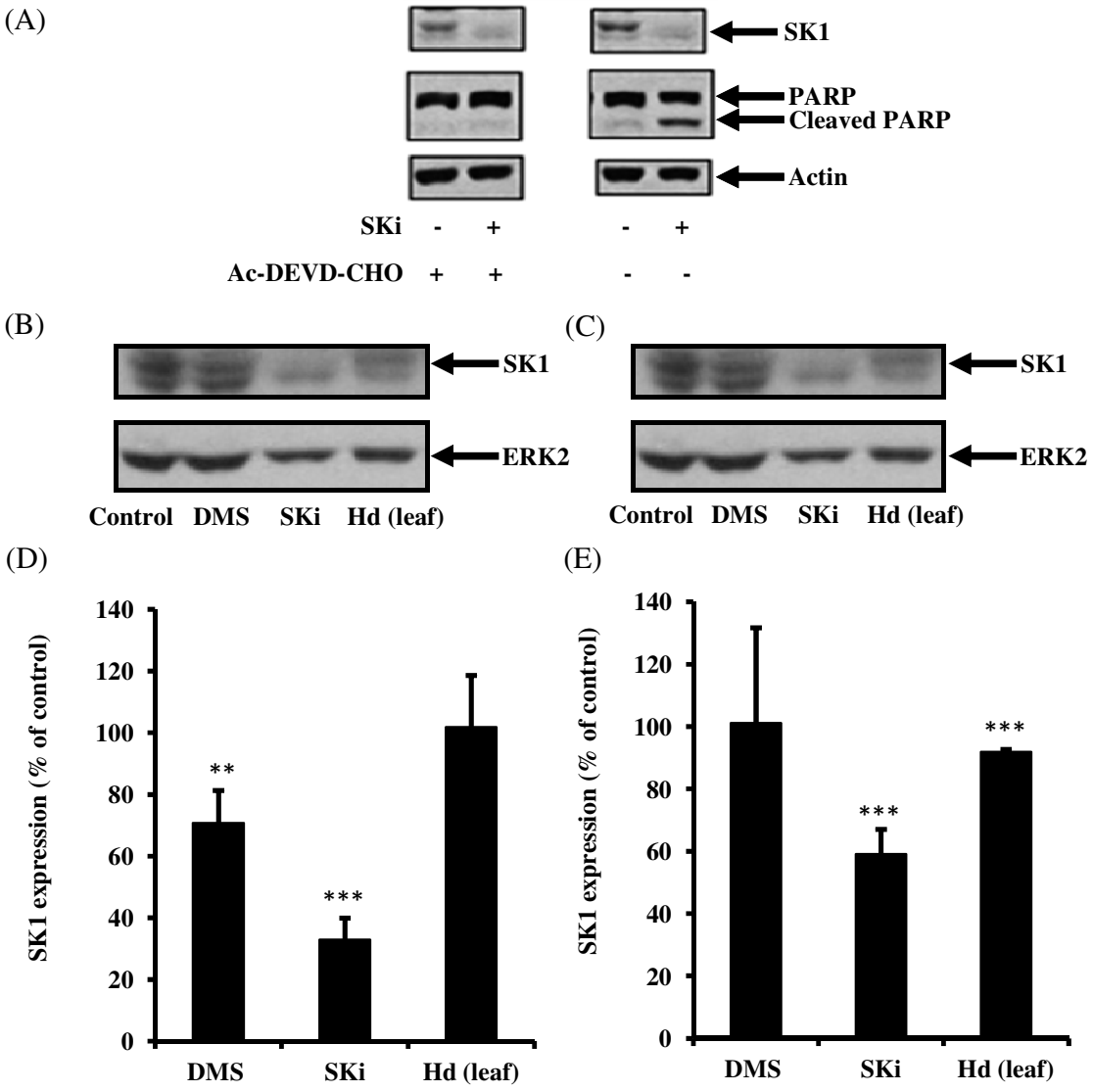


Figure 5.3 Inhibitor-induced down-regulation of SK1. (A) MCF-7 Neo cells were pre-treated with Ac-DEVD-CHO (100 µM) or vehicle control (0.2% DMSO) for 30 mins and then treated with 10 µM SKi or control (0.1% DMSO) for another 24 hrs. **(B)** and **(C)** Quiescent MCF-7 Neo cells were incubated with control 10 µM DMS, 10 µM SKi or 500 µg/ml Hopea dryobalanoides leaf, Hd (leaf) extract for **(B)** 24 hrs and **(C)** 48 hrs. Cells were harvested and analysed by Western blotting using anti-SK1 and anti-PARP antibodies. Blots were then stripped and reprobed with anti-actin or anti-ERK2 antibodies to ensure comparable protein loading. Bar graphs showing quantification of the effects of inhibitors on SK1 expression by quantifying the SK1: ERK2 ratio for cells treated for **(D)** 24 hrs and **(E)** 48 hrs. Data are expressed as a percentage of control (**P<0.01, **p<0.001 for inhibitor-treated cells versus control, n=3). Results are representative of three independent experiments.

5.3.2 Fractionation of *Hopea dryobalanoides* extract

500 mg of *Hopea dryobalanoides* detannified methanolic extract was fractionated by Flash chromatography to produce 12 different fractions. Upon freeze-drying to remove residual solvents and water, the fractions were reconstituted with DMSO before being tested in other assays. The weights of these fractions are shown in Table 5.2. ~ 170 mg of fractions were obtained after Flash chromatography, suggesting that some compounds were retained in the column.

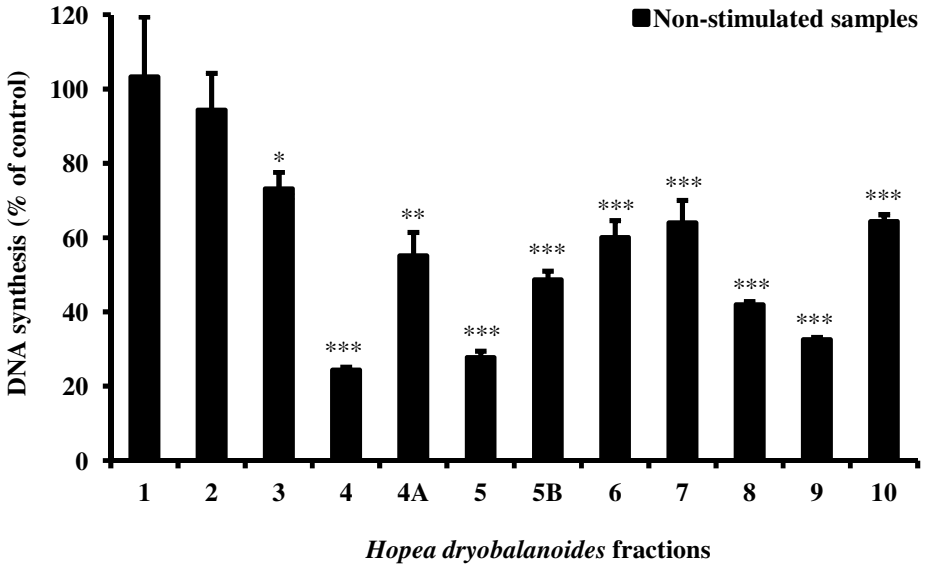
Table 5.2 Weights of different fractions collected after Flash chromatography

Fractions	Weight (mg)
1	2
2	5
3	3
4	3
4A	15
5	11
5B	2.5
6	7.7
7	46
8	68
9	3.4
10	2

5.3.3 *Hopea dryobalanoides* fractions inhibit DNA synthesis and/or SK1 activity

It was not known at this stage which fractions contained the active compounds. Therefore, the fractions were tested in [^{3}H]-thymidine incorporation assays to assess their activities on cell proliferation. As shown in Fig 5.4, Fractions 4, 5 and 9 were the most active fractions in reducing DNA synthesis in MCF-7 Neo cells. When these fractions were screened in SK1 activity assay, Fraction 5 was the most active fraction and inhibited almost 90% of SK1 activity when compared to control (Fig. 5.5).

(A)



(B)

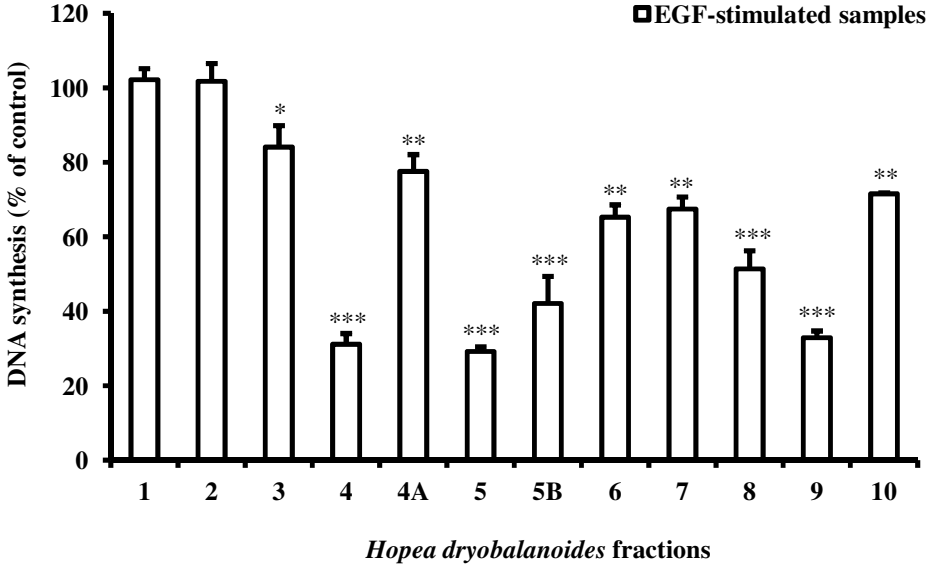


Figure 5.4 Effects of *Hopea dryobalanoides* fractions on DNA synthesis. Inhibition of DNA synthesis**.** Quiescent MCF-7 Neo cells were treated with 5 µg/ml of *Hopea dryobalanoides* fractions (1-10) or vehicle control (0.05% DMSO) for 15 mins without stimulation **(A)** or stimulated with 25 ng/ml EGF **(B)**. Cells were then incubated for 15 hrs and then with [^{3}H]-thymidine (0.5 µCi/ml) added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent means +/- standard deviations of triplicate determinations, *p<0.05, **p<0.01, ***p<0.001 *versus* control. Similar results were obtained in three independent experiments.

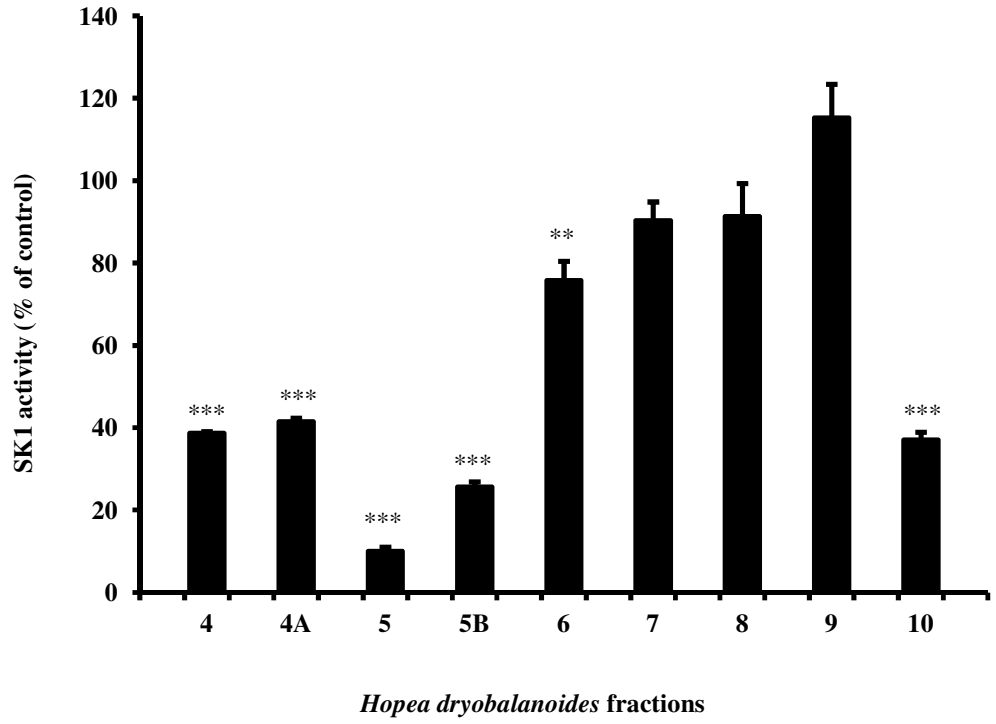


Figure 5.5 Effects of *Hopea dryobalanoides* fractions on SK1 activity. SK1 activity was assayed as described under Methods (section 2.2.15) using 10 μM sphingosine and 250 μM [^{32}P]-ATP as the substrates and HEK 293 cell lysates containing over-expressed recombinant SK1 in the presence of plant fractions (500 μg/ml). Data are expressed as percentage of control and represent means +/- standard deviations of triplicate determinations, **p<0.01, ***p<0.001 *versus* control.

To investigate whether these fractions could mimic SKi in down-regulating SK1 expression, MCF-7 Neo cells were used as a model. Previously, EGF was shown to induce ERK1/2 activation which was inhibited by treatment with SKi and *Hopea dryobalanoides* extract (see Chapter 3, Fig. 3.14). In agreement, the treatment with SKi reduced SK1 expression and inhibited EGF-induced ERK1/2 activation (Fig. 5.6). Fraction 5 closely mimicked the actions of SKi in down-regulating SK1 and suppressed ERK1/2 activation. Apart from that, some fractions showed different actions in regulating SK1 expression *versus* activity. For example, despite inhibiting SK1 activity (Fig. 5.5), Fraction 4 and 5B did not down-regulate SK1 expression. Fraction 9 reduces ERK1/2 activation but did not affect SK1 expression whereas Fraction 10 reduced SK1 activity (Fig.5.5) and expression but had less effect on ERK1/2 activation. The total plant extract also reduced SK1 expression, used as a positive control (Fig. 5.6). Collectively, these results show that most of the anti-SK1 activity of *Hopea dryobalanoides* extract resided in Fraction 5.

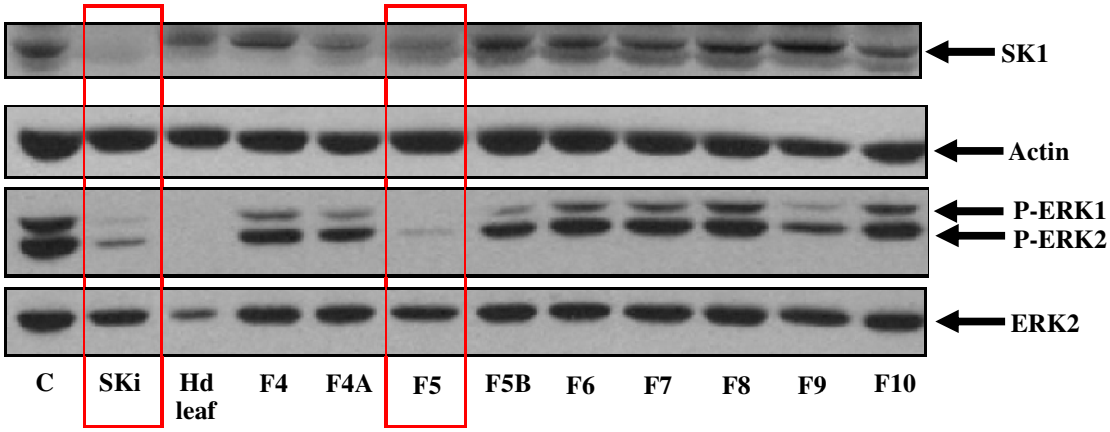


Figure 5.6 Effects of *Hopea dryobalanoides* fractions on SK1 expression. MCF-7 cells were treated with control (DMSO 0.2%), C; SKi (10 µM), *Hopea dryobalanoides* leaf extract (200 µg/ml), Hd leaf; Fractions 4-10 (20 µg/ml), F4-F10 for 48 hrs (inhibitors were replenished after 24 hrs). EGF 25 ng/ml was added for 5 mins before harvesting the cells. Cells lysates were analysed by Western blotting using anti-SK1 antibody. Blots were also probed with anti-phospho ERK1/2 antibody to assess effect of fractions on the activation status of these enzymes. Blots were then stripped and reprobed with anti-actin and anti-ERK2 to ensure comparable protein loading. Results are representative of three independent experiments.

5.3.4 Structure elucidation of ampelopsin A and balanocarpol

Structure elucidation of the active compound in Fraction 5 was achieved using NMR and LC-MS. Due to the low amount of the sample after freeze-drying (~5mg), the NMR spectra were obtained through the use of a Shigemi NMR tube to improve signal acquisition. One and two dimensional NMR experiments confirmed the structure of the active compound in Fraction 5 to be ampelopsin A, previously isolated from *Hopea parviflora* (Tanaka *et al*., 2000). As shown in Fig 5.7, ampelopsin A was present in high quantity—the peaks were sharp with little background noise. The proposed structure was included in all following figures for illustration. Integrating the peaks of these protons revealed that there are 22 protons in the molecule. Five of these protons were highly de-shielded and appear at much lower field (>9ppm) in the spectrum, indicating the presence of exchangeable protons such as phenolic hydroxyls (OH). Indeed, pre-saturating (suppressing) the water signal in the spectrum reduced the intensity of these dissociable protons (Fig. 5.8). Additionally, another hydroxyl was identified (8a-OH) since its intensity was also reduced during pre-saturation of the water signal, suggesting that the proton is exchangeable. In fact, this was an aliphatic hydroxyl because it resonated at much higher field of the spectrum (δ 4.88). Besides, there are 2 pairs of aromatic protons δ 6.98/6.68 and δ 6.72/6.54 (Fig. 5.7). Each of these peaks integrated as two protons and had a coupling constant of 8.4 Hz indicating ortho-coupling of aromatic protons belonging to two para-disubstituted rings, A_1 and B_1. Likewise, two pairs of meta-coupled aromatic protons δ 6.40/6.03 and 6.26/5.97 which had smaller coupling constant of about 2 Hz were assigned to two tetra-substituted rings, A_2 and B_2. A large deshielding proton attached to a benzylic carbon bearing an oxygen atom was assigned as H7b (δ 5.66) which coupled to H8b (δ 3.94) with a coupling constant of 11.2 Hz.

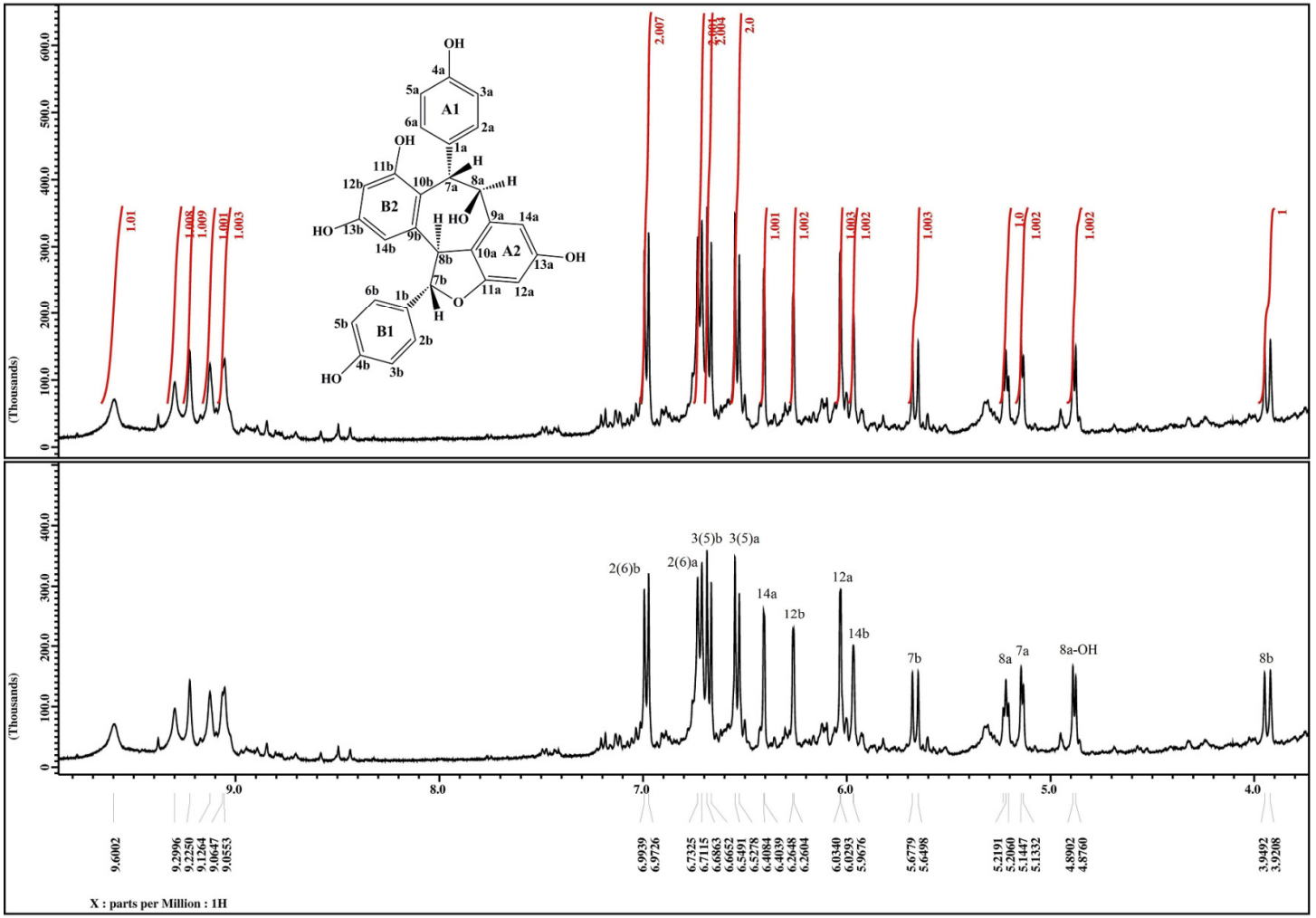


Figure 5.7 ^{1}H-NMR spectrum of ampelopsin A measured at 400 MHz in DMSO-d_6. Top panel: ^{1}H-NMR with integration to show the number of protons present in ampelopsin A. Bottom panel: ^{1}H-NMR with corresponding protons labelled.

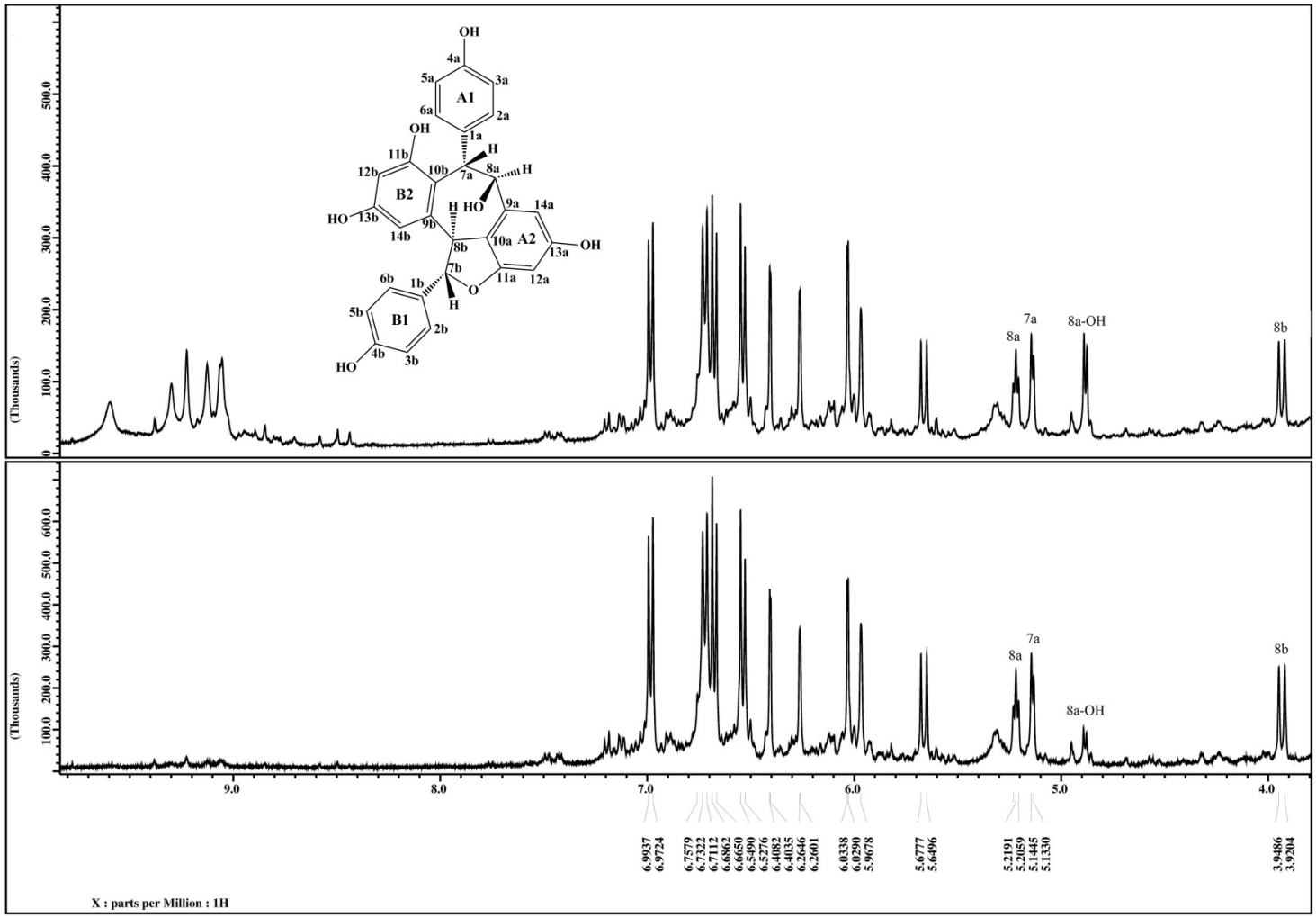


Figure 5.8 Pre-saturation ^{1}H-NMR spectra of ampelopsin A measured at 400 MHz in DMSO-d_6. Top panel: ^{1}H-NMR before pre-saturation. Bottom panel: ^{1}H-NMR with pre-saturation

The proposed structure was further supported by ^{13}C-NMR experiments. The ^{13}C-NMR spectrum also shows that ampelopsin A is relatively pure since all carbon peaks were sharp with little background noise (Fig. 5.9). ^{13}C-DEPT-135 NMR spectrum shows methine (CH) and methyl (CH_3) signals positive but shows methylene (CH_2) signals negative whereas signals from quaternary carbons are suppressed. As shown in Fig 5.9, there were no negative signals in the ^{13}C-DEPT-135-NMR spectrum, indicating that the molecule does not contain methylene groups. In addition, 12 carbons were identified as quaternary carbons with no hydrogen substitutions. The positions of these quaternary carbons at much lower field of the spectrum (>90ppm) also suggests the presence of polyphenolic rings in the molecule.

More spectroscopic evidence was obtained from other 2D-NMR experiments. In the COSY spectrum, cross peaks indicate spin-spin coupled protons which are next to each other (neighbouring protons). Protons bonded to the same carbon are usually identical with no coupling. However, two-bond coupling or geminal coupling ($^2J_{HH}$) can be observed when two protons bonded to the same carbon are not identical (also known as diastereotopic). These protons are not in the same chemical environment because of other substituents on the carbon atom. A different phenomenon was observed in this molecule where 8a-OH (shown as a doublet) was coupled to H8a (a triplet or a doublet of doublet) (Fig. 5.10). Due to the dissociable nature of 8a-OH, there would have been no coupling from neighbouring protons. However, 8a-OH for some reason (e.g. ring constraint or solvent effect) was not as strongly dissociable as other phenolic hydroxyls. Additionally, 8a-OH is an aliphatic hydroxyl which coupled to methine proton (H7a and H8a). Other neighbouring protons were confirmed as H8b/H7b, which had large coupling constant indicating strong correlation. Two pairs of ortho-coupled aromatic protons 2(6)a/3(5)a and 2(6)b/3(5)b were also identified (Fig 5.10).

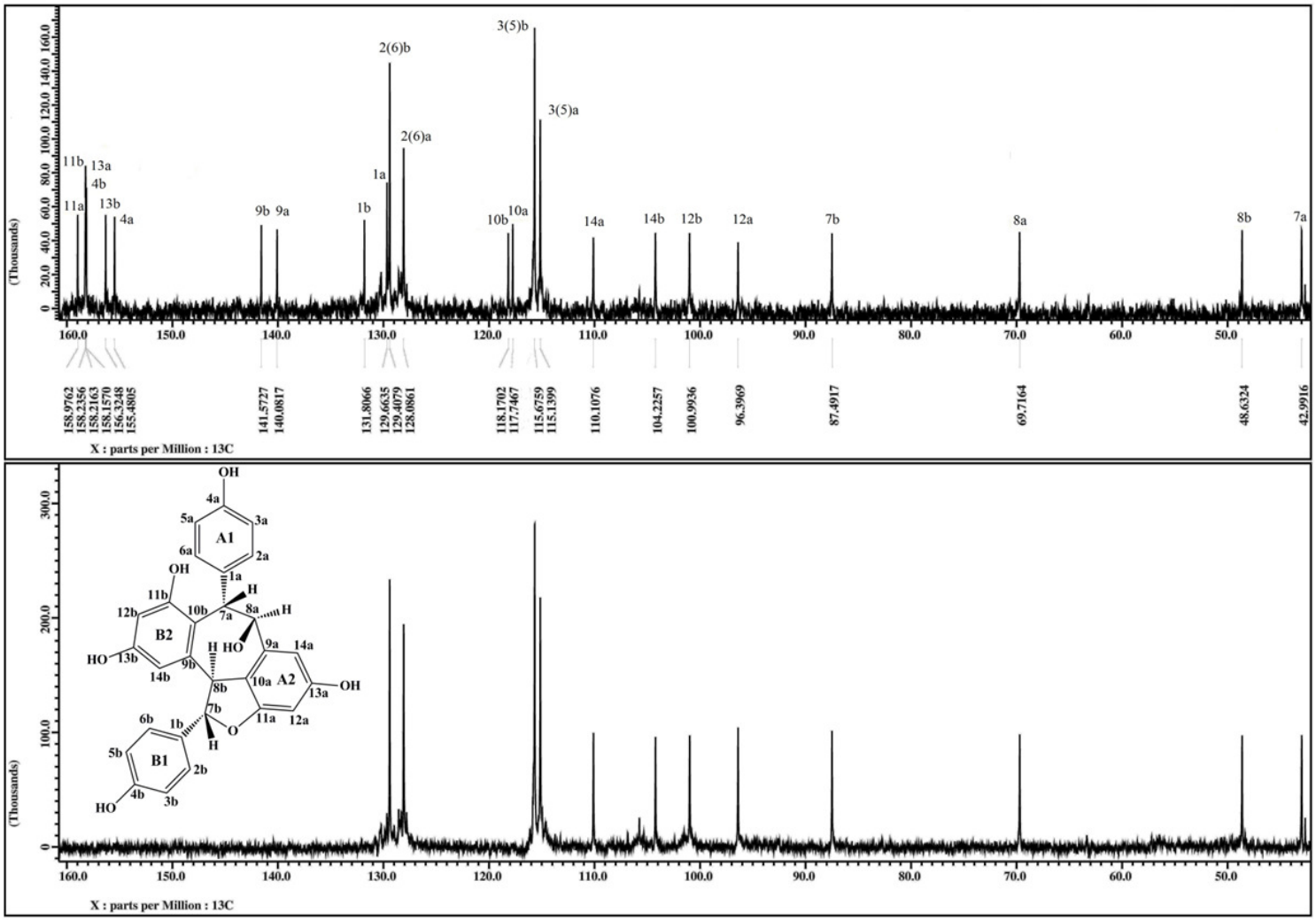


Figure 5.9 ^{13}C-NMR spectra of ampelopsin A measured in DMSO-d_6. Top panel: ^{13}C-NMR spectrum. Bottom panel: ^{13}C-DEPT-135 NMR spectrum.

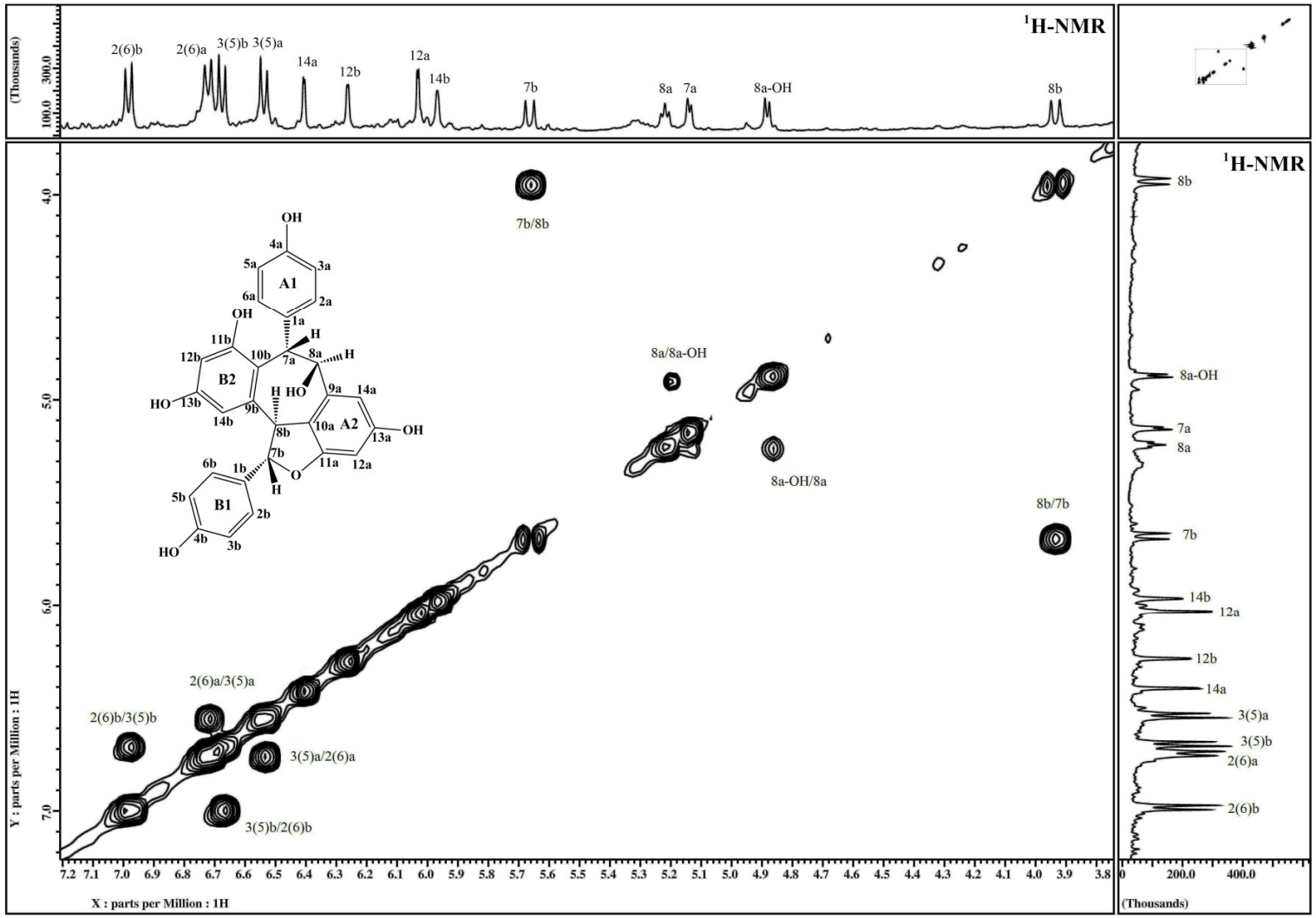


Figure 5.10 COSY spectrum of ampelopsin A measured at 400 MHz in DMSO-d_6.

The connections between each proton and carbon present in the molecule were obtained with two dimensional HMQC and HMBC spectra. All protons were appropriately assigned to each corresponding carbon based on the HMQC spectrum (Fig. 5.11). Of note, 8a-OH did not correlate with any carbon in the spectrum confirming its identity as an OH and not a CH. In addition, extensive correlations from protons with carbons in long distance were observed in the HMBC spectrum allowing the connection between different spin systems to be confirmed (Fig. 5.12). For example, H7a (δ 5.14) shows typical three ($^3J_{CH}$) and four ($^4J_{CH}$) bond couplings to neighbouring carbons: H7a/C11b, H7a/C9a, H7a/C9b, and H7a/C2(6)a. In addition, two bond couplings ($^2J_{CH}$) were also observed: H7a/C8a, H7a/C10b and H7a/C1a, allowing the connection between ring B2, A1 and A2 to be established. Other significant correlations included H7b/C2(6)b and H7b/C9b indicating that ring B1 is connected to the benzofuran ring. The connection of ring B1 at C7b is further supported by other correlations including H8b/C1b and H8b/C9b which connects the benzofuran ring to ring B2. In addition, the expanded HMBC spectrum (Fig. 5.13) allows the assignment of several carbons resonating closely to each other in the ^{13}C-NMR spectrum. H12b and H14b correlated strongly ($^2J_{CH}$) with C13b whereas H7a had three-bond ($^3J_{CH}$) correlation with C11b (δ 158.24). Based on the direction of the contour, C13a was assigned at δ 158.22 as it correlated with H12a. Selected long range correlations are shown in Fig. 5.14. Therefore, HMBC allows the linkage of the different spin systems in the molecule, supporting the proposed structure to be ampelopsin A.

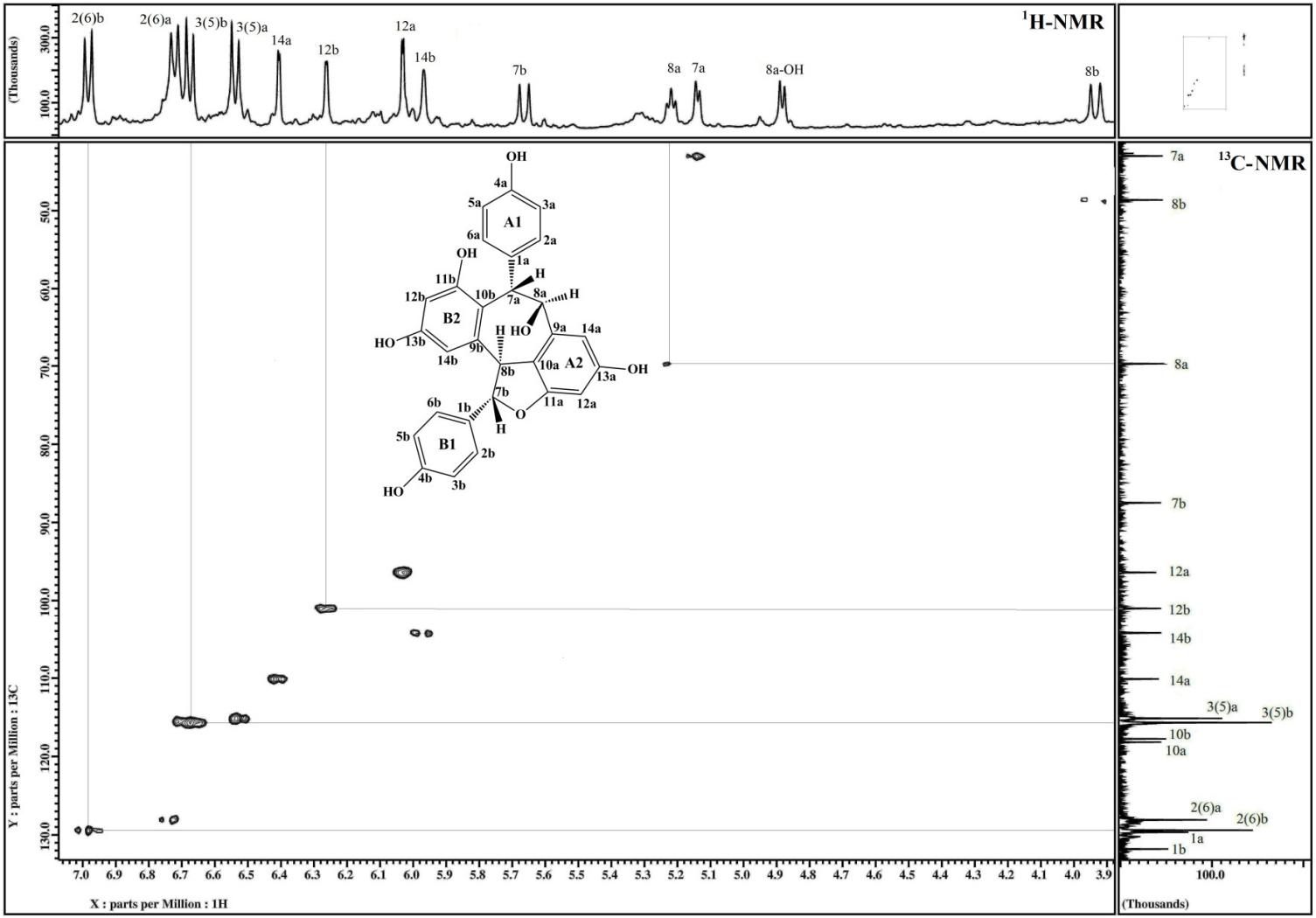


Figure 5.11 HMQC spectrum of ampelopsin A measured at 400 MHz in DMSO-d_6.

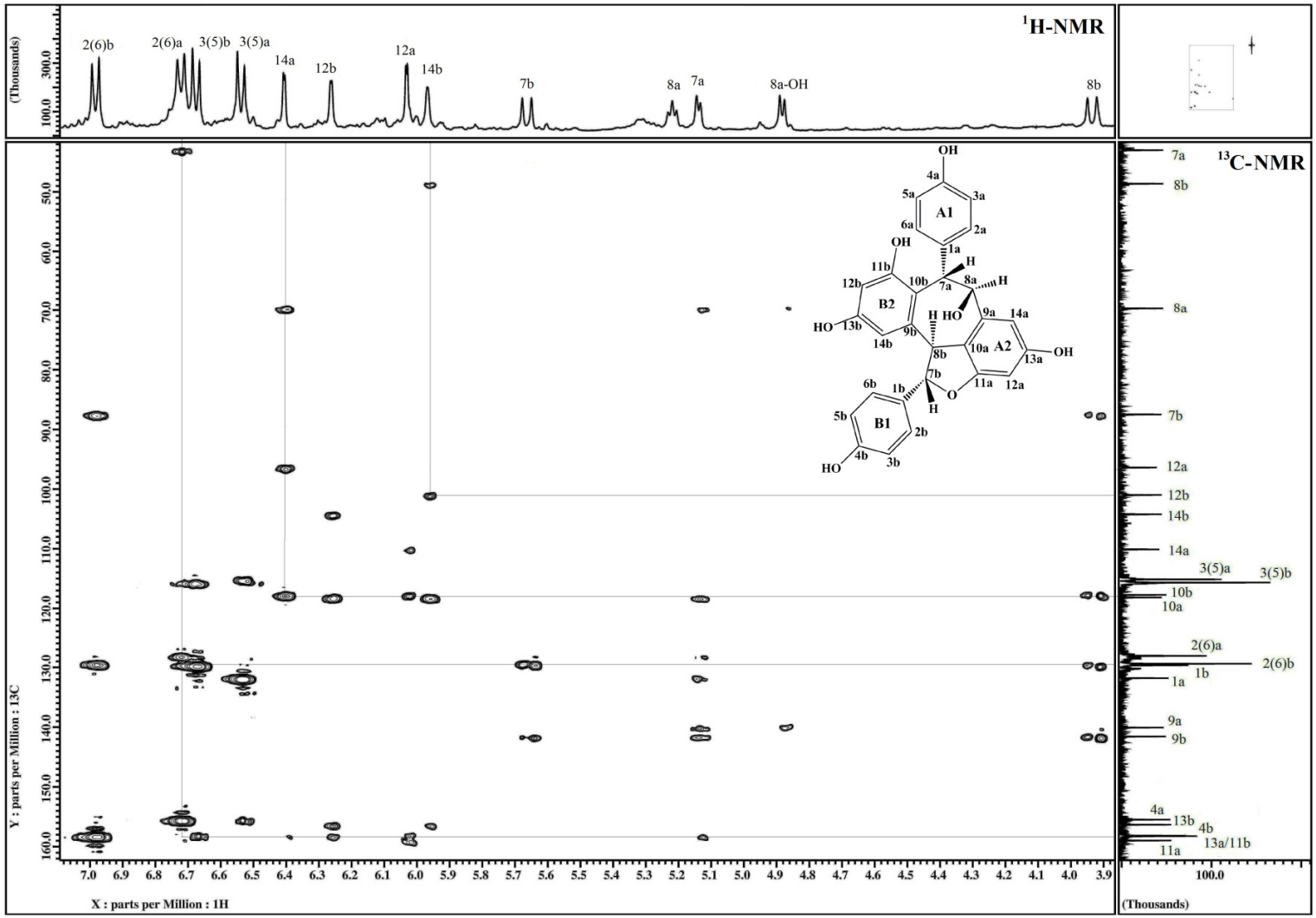


Figure 5.12 HMBC spectrum of ampelopsin A measured at 400 MHz in DMSO-d_6.

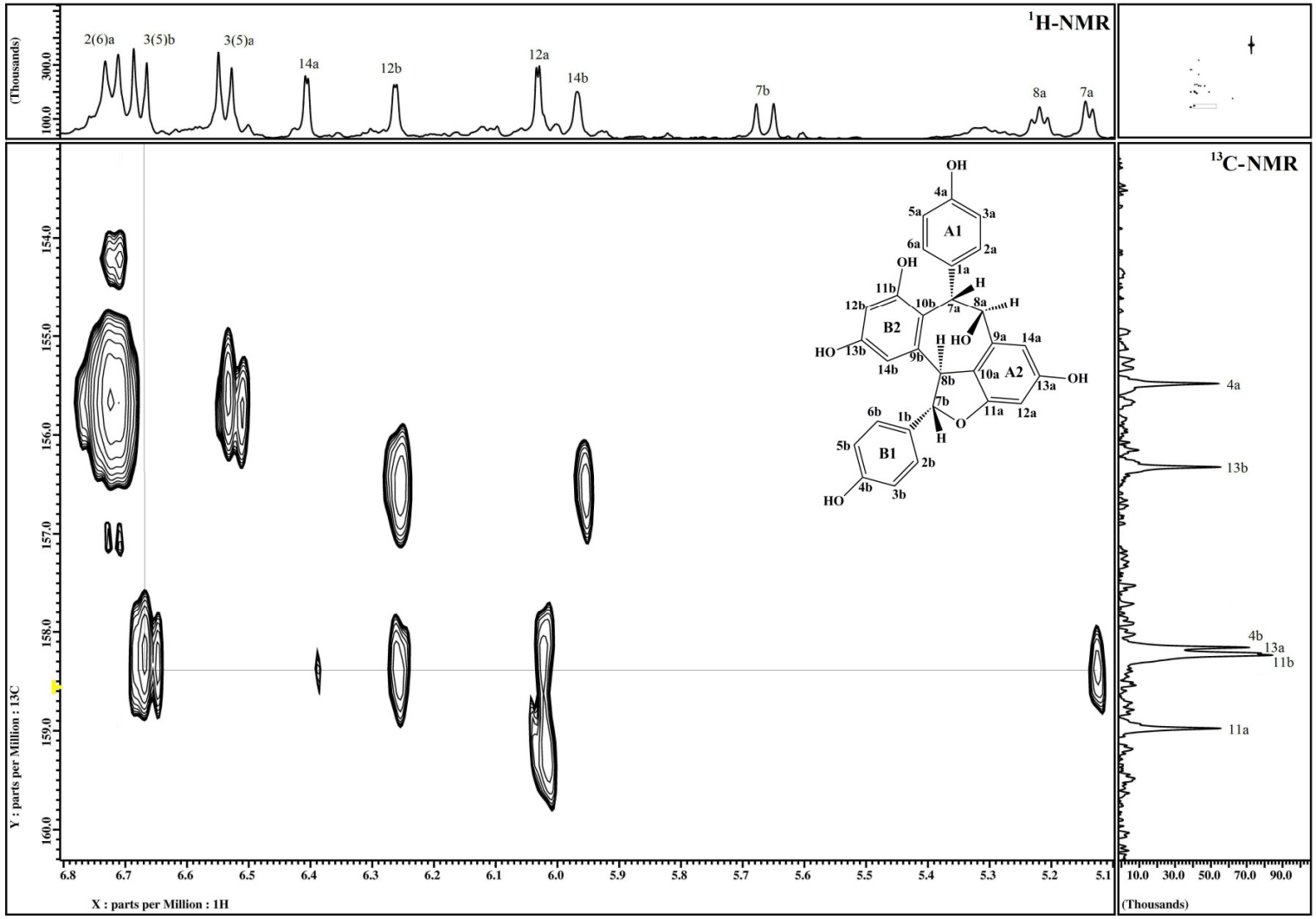


Figure 5.13 Expanded HMBC spectrum of ampelopsin A measured at 400 MHz in DMSO-d_6.

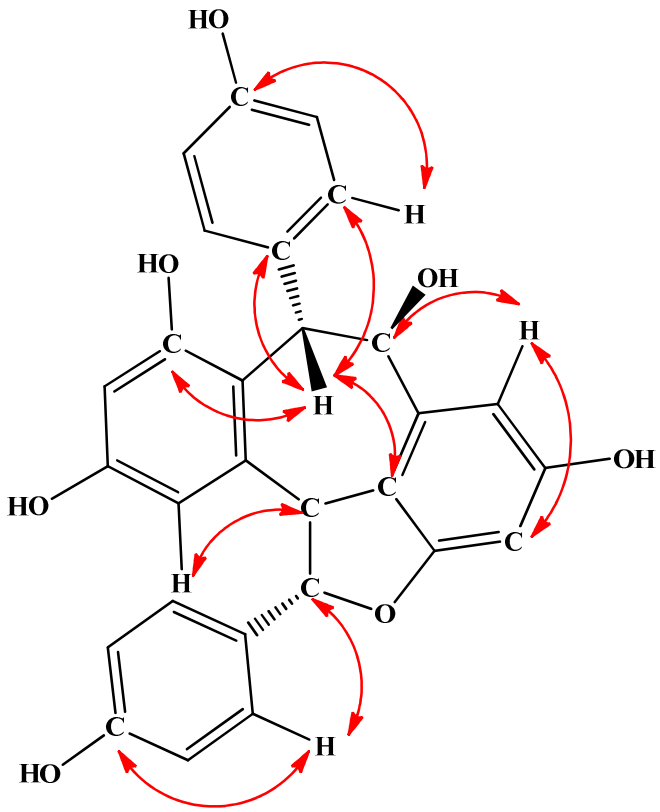


Figure 5.14 Long range correlations in ampelopsin A. Red arrows indicate long range correlations between carbons and protons in the molecule. Only selected correlations which establish the connection between different parts of the molecule are shown.

The structure of the ampelopsin A was supported by molecular weight obtained from LC-MS. Negative ion ESI-MS carried out in HPLC/ThermoFinnigan LCQ-Decaiontrap using water-acetonitrile as mobile phase in a C18-reverse phase column gave an $[M-H]^-$ ion at m/z 469.1 with λ_{max} at 282nm (MeOH). The exact mass was also obtained with Orbitrap HRESI mass spectrometer which gave an $[M-H]^-$ ion at m/z 469.1295 (Fig. 5.15), corresponding to the molecular formula $C_{28}H_{22}O_7$.

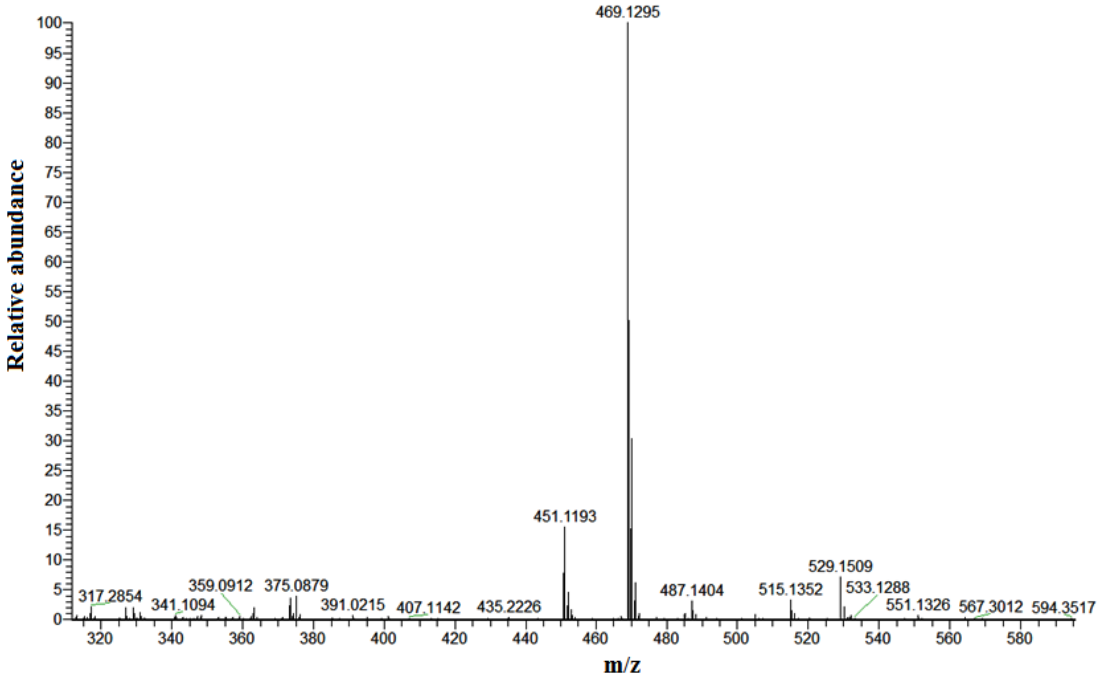


Figure 5.15 Mass spectrum of ampelopsin A. Negative ion ESI-MS was carried out in Orbitrap HRESI mass spectrometer using water-acetonitrile as mobile phase in a C18-reverse phase column.

All chemical shifts of proton and carbon in ampelopsin A were comparable to those reported by Tanaka and colleagues (2000) who measured the spectra in acetone-d_6 instead of DMSO-d_6 (Table 5.3). Differences of proton and carbon chemical shifts in these studies reflect the effect of different solvents used in the NMR experiments. DMSO is very miscible with water and usually contains a lot of water which can exchange with hydroxyls in the NMR sample. As a result, the chemical shifts of the hydroxyls are different from those measured in acetone-d_6.

Table 5.3 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for ampelopsin A

No.	Ampelopsin A (experimental, DMSO-d_6)		Ampelopsin A (Tanaka *et al*., 2000; acetone-d_6)	
	δ H ppm(J, Hz)	δ C ppm	δ H ppm (J, Hz)	δ C ppm
1a	-	129.7	-	130.9
2(6)a	6.72 *d* (8.4)	128.1	6.88 *d* (8.3)	128.7
3(5)a	6.54 *d* (8.4)	115.1	6.62 *d* (8.3)	115.4
4a	-	155.5		156.0
7a	5.14 *d* (4.6)	43.0	5.45 *d* (4.9)	43.8
8a	5.22 *dd* (4.6, 5.2)	69.7	5.42 *br d* (4.9)	71.2
9a	-	140.1	-	140.2
10a	-	117.7	-	118.9
11a	-	159.0	-	160.1
12a	6.03 *d* (1.8)	96.4	6.14 *br d* (1.9)	110.5
13a	-	158.2	-	158.8
14a	6.40 *d* (1.8)	110.1	6.64 *d* (1.9)	110.5
1b	-	131.8	-	132.5
2(6)b	6.98 *d* (8.4)	129.4	7.09 *d* (8.8)	129.9
3(5)b	6.68 *d* (8.4)	115.7	6.75 *d* (8.8)	116.0
4b	-	158.1	-	158.4
7b	5.66 *d* (11.2)	87.5	5.42 *d* (11.3)	88.3
8b	3.94 *d* (11.2)	48.6	4.15*br d* (11.3)	49.5
9b	-	141.6	-	143.1
10b	-	118.2	-	118.2
11b	-	158.2	-	157.2
12b	6.26 *d* (1.8)	101.0	6.42 *d* (2.5)	101.6
13b	-	156.3	-	158.8
14b	5.97 *br s*	104.2	6.21 *br s*	105.5
OH	4.88 *d* (5.2, 8a-OH), 9.06, 9.13, 9.23, 9.30, 9.60 each *br s*		3.57 *br s* (8a-OH), 8.08, 8.17, 8.26, 8.32, 8.40 each *br s*	

A new batch of *Hopea dryobalanoides* was requested from FRIM in order to isolate more ampelopsin A. However, ^{1}H NMR of the leaves, bark and twig extracts did not reveal any signals for ampelopsin A (Fig. 5.16). These findings illustrate one of the difficulties in phytochemistry, i.e. variability may exist between different samples from the same species collected at different times and places. However, the aromatic region (δ 6-8) of ^{1}H-NMR of the bark and twig extracts of *Hopea dryobalanoides* showed the presence of some interesting phenolic compounds (Fig. 5.16). Upon VLC fractionation of the bark extract and subsequent purification using Sephadex LH-20, balanocarpol was isolated. Balanocarpol is an isomer (7-epimer) of ampelopsin A (Fig.5.2).

The molecular structure of balanocarpol was elucidated using NMR experiments. Balanocarpol was isolated in high purity (~95%) from the bark of *Hopea dryobalanoides*, as evidenced by high intensity of the proton signals compared to very low background noise (Fig. 5.17). Neighbouring protons such as H7a/H8a, H7b/H8b, H2(6)a/H3(5)a and H2(6)b/H3(5)b were identified from the COSY spectrum (Fig. 5.18). In contrast to ampelopsin A, 8a-OH in balanocarpol did not couple with neighbouring protons (8a or 7a). Indeed, the signal of 8a-OH was broad (possibly due to the use of acetone-d_6), indicating that it may be readily exchangeable with other dissociable protons (water or phenolic hydroxyls) (Fig. 5.17).

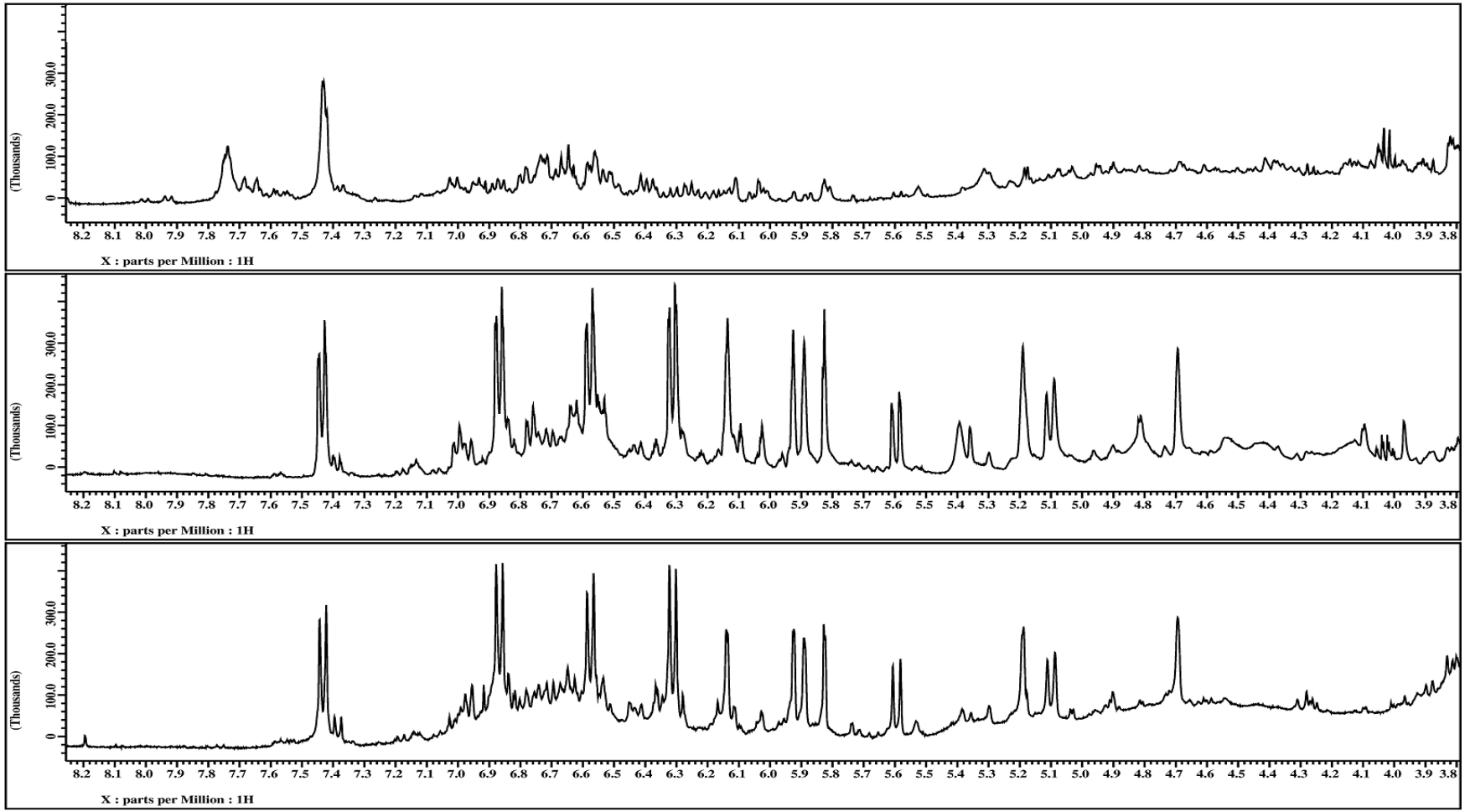


Figure 5.16 ^{1}H-NMR spectra of methanolic extracts of *Hopea Dryobalanoides*. The leave (top panel), bark (middle panel) and twig (bottom panel) extracts were measured at 400 MHz in acetone-d_6.

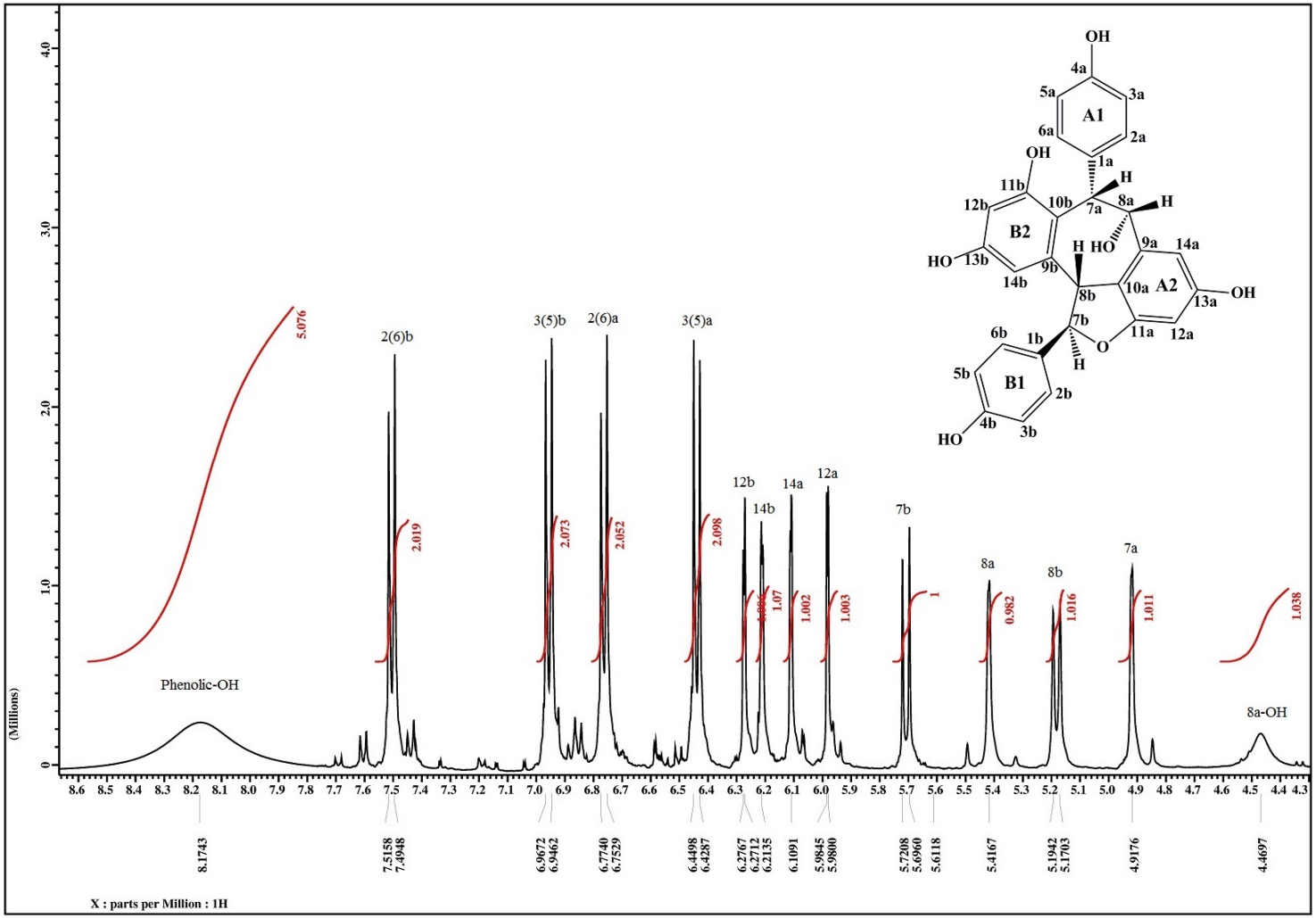


Figure 5.17 ^{1}H-NMR spectrum of balanocarpol measured at 400 MHz in acetone-d_6. Integration shows the number of protons present in balanocarpol.

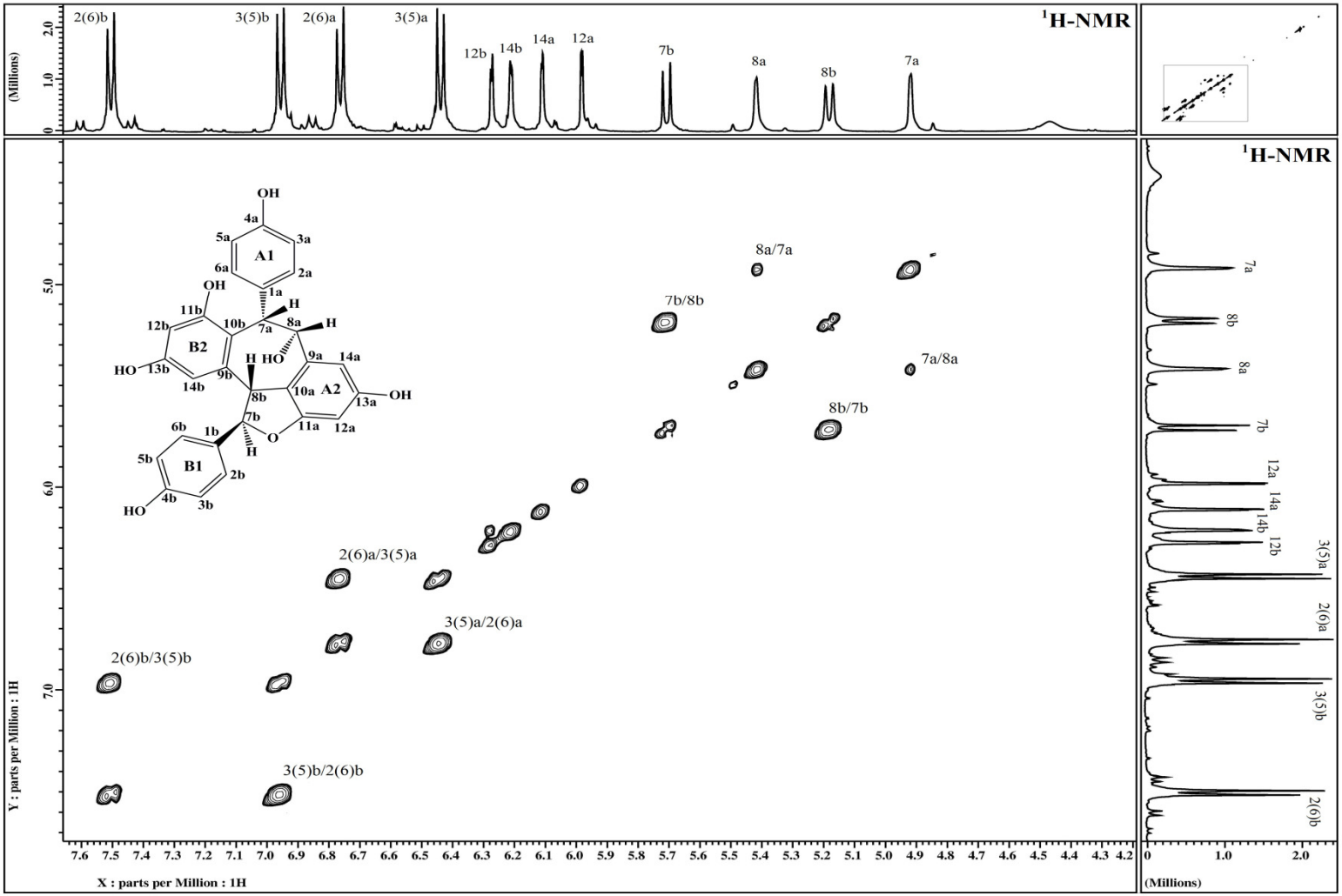


Figure 5.18 COSY spectrum of balanocarpol measured at 400 MHz in acetone-d_6

More spectroscopic evidence supporting the proposed structure was obtained from the ^{13}C-NMR spectrum. The purity of the sample was again confirmed to be high as evidenced by very low background noise (Fig. 5.19). In addition, the ^{13}C-DEPT-135 spectrum allowed the identification of quaternary carbons whose signals would be suppressed (Fig. 5.19). HMQC spectrum was acquired to identify the carbons to which the protons were attached. Intriguingly, the assignment of four protons (12a, 12b, 14a, 14b) in balanocarpol by Tanaka and colleagues (2000) was different from the present study (Fig. 5.20, Table 5.4).

The connections between each group of protons and neighbouring carbons were further established using HMBC. Since balanocarpol is an isomer (7-epimer) of ampelopsin A, the HMBC data showing the connection between ring A1, A2, B1, B2 and the benzofuran ring in balanocarpol are similar to those of ampelopsin A (Fig. 5.21). However, the relative stereochemistry of these two isomers is different. Magnetic nuclei can interact through nuclear Overhauser effect (NOE) if they are close to each other in space and a NOESY spectrum can provide such information. As shown in Fig. 5.22, H7a interacted with H8a indicating that they could "see" each other through space and were in a *cis* configuration. In addition, NOEs were also observed between H8b and H8a, indicating that H8b and H8a were also in a *cis* configuration. Moreover, NOE was observed between H8b and H2(6)b suggesting that they are also in a *cis* configuration. By contrast, H7a/H8a was in a *trans*-configuration in ampelopsin A (Tanaka *et al*., 2000). The proposed structure for balanocarpol was supported by LC-MS which gave an $[M-H]^-$ ion at m/z 469.0, corresponding to the molecular formula $C_{28}H_{22}O_7$ (Fig. 5.23).

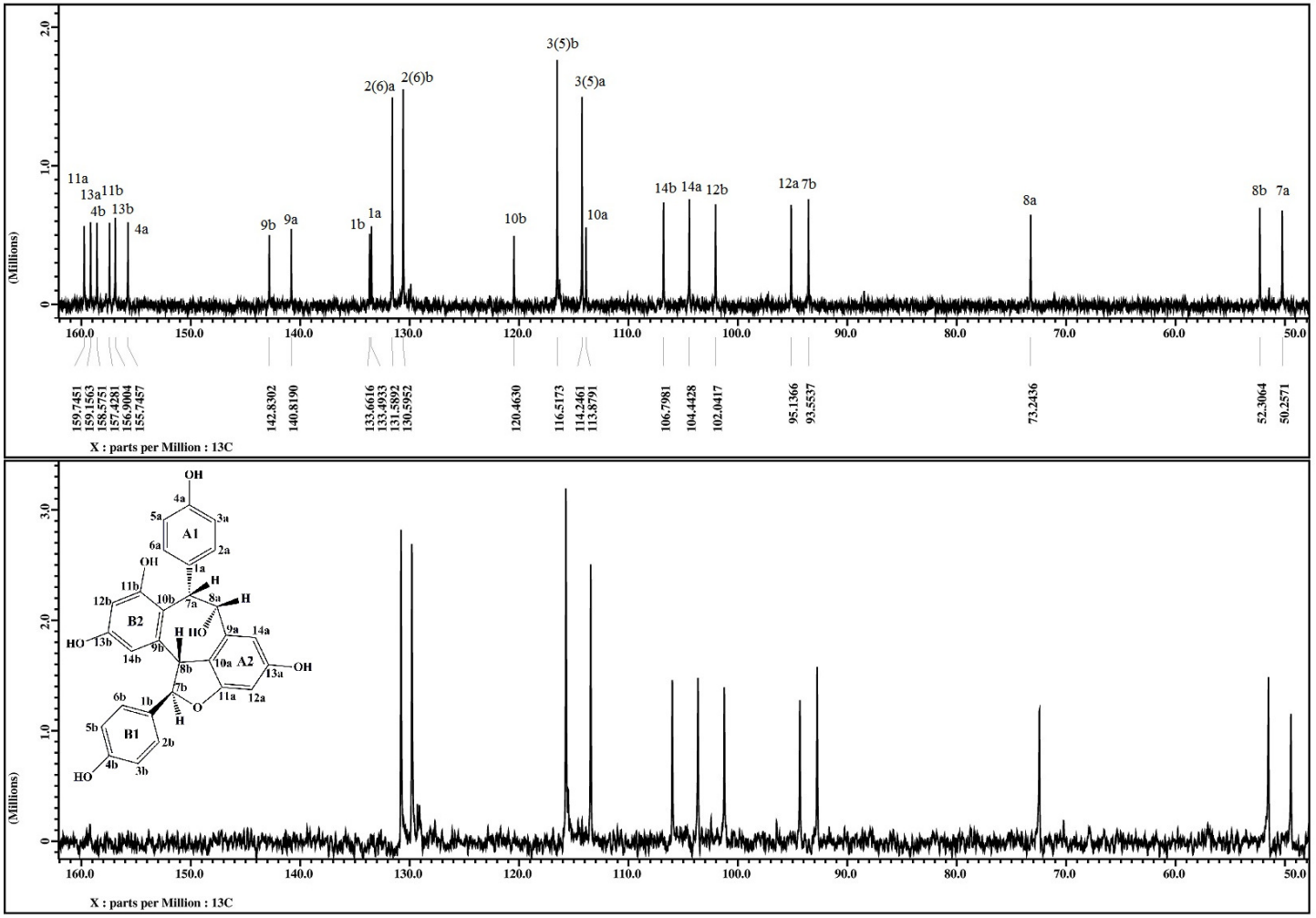


Figure 5.19 ^{13}C-NMR spectra of balanocarpol measured in acetone-d_6. Top panel: ^{13}C-NMR. Bottom panel: ^{13}C-DEPT-135 NMR

Table 5.4 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for balanocarpol

No.	Balanocarpol (experimental; acetone-d_6)		Balanocarpol (Tanaka *et al*., 2000; acetone-d_6)	
	δ H ppm (J, Hz)	δ C ppm	δ H ppm (J, Hz)	δ C ppm
1a	-	133.5	-	133.5
2(6)a	6.76 *d* (8.4)	131.6	6.75 *d* (8.3)	131.5
3(5)a	6.44 *d* (8.4)	114.2	6.42 *d* (8.3)	114.2
4a	-	155.7	-	155.8
7a	4.92 *br s*	50.3	4.90 *br s*	50.3
8a	5.42 *br s*	73.2	5.40 *br s*	73.2
9a	-	140.8	-	140.8
10a	-	113.9	-	113.8
11a	-	159.7	-	159.7
12a	5.98 *d* (1.8)	95.1	6.20 *br s*	95.1
13a	-	159.2	-	159.2
14a	6.10 *d* (1.8)	104.4	6.26 *d* (2.0)	104.4
1b	-	133.7	-	133.7
2(6)b	7.50 *d* (8.4)	130.6	7.50 *d* (8.3)	130.5
3(5)b	6.96 *d* (8.4)	116.5	6.95 *d* (8.3)	116.4
4b	-	158.6	-	158.6
7b	5.71 *d* (9.6)	93.6	5.69 *d* (9.3)	93.5
8b	5.18 *d* (9.6)	52.3	5.16 *br d* (9.3)	52.3
9b	-	142.8	-	142.8
10b	-	120.5	-	120.4
11b	-	157.4	-	157.4
12b	6.27 *d* (1.8)	102.0	6.09 *br s*	102.0
13b	-	156.9	-	156.9
14b	6.21 *br s*	106.8	5.96 *d* (2.3)	106.8
OH	4.47 *br s* (8a-OH) 7.74-8.56, *br s* (5 phenolic-OH)		4.36 *d* (4.4, 8a-OH), 7.74, 7.85, 7.97, 8.04, 8.56 each *br s*	

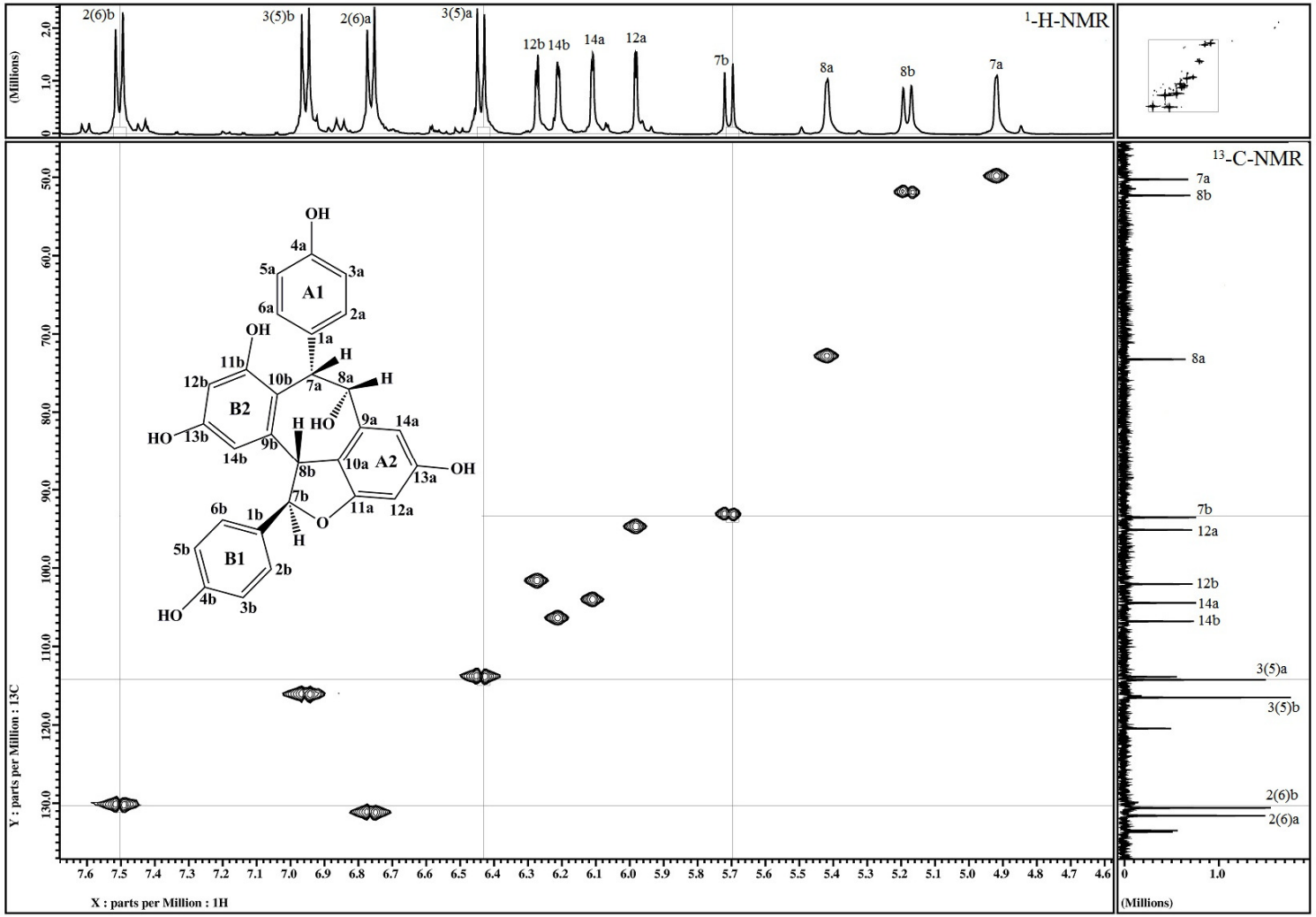


Figure 5.20 HMQC spectrum of balanocarpol measured at 400 MHz in acetone-d_6.

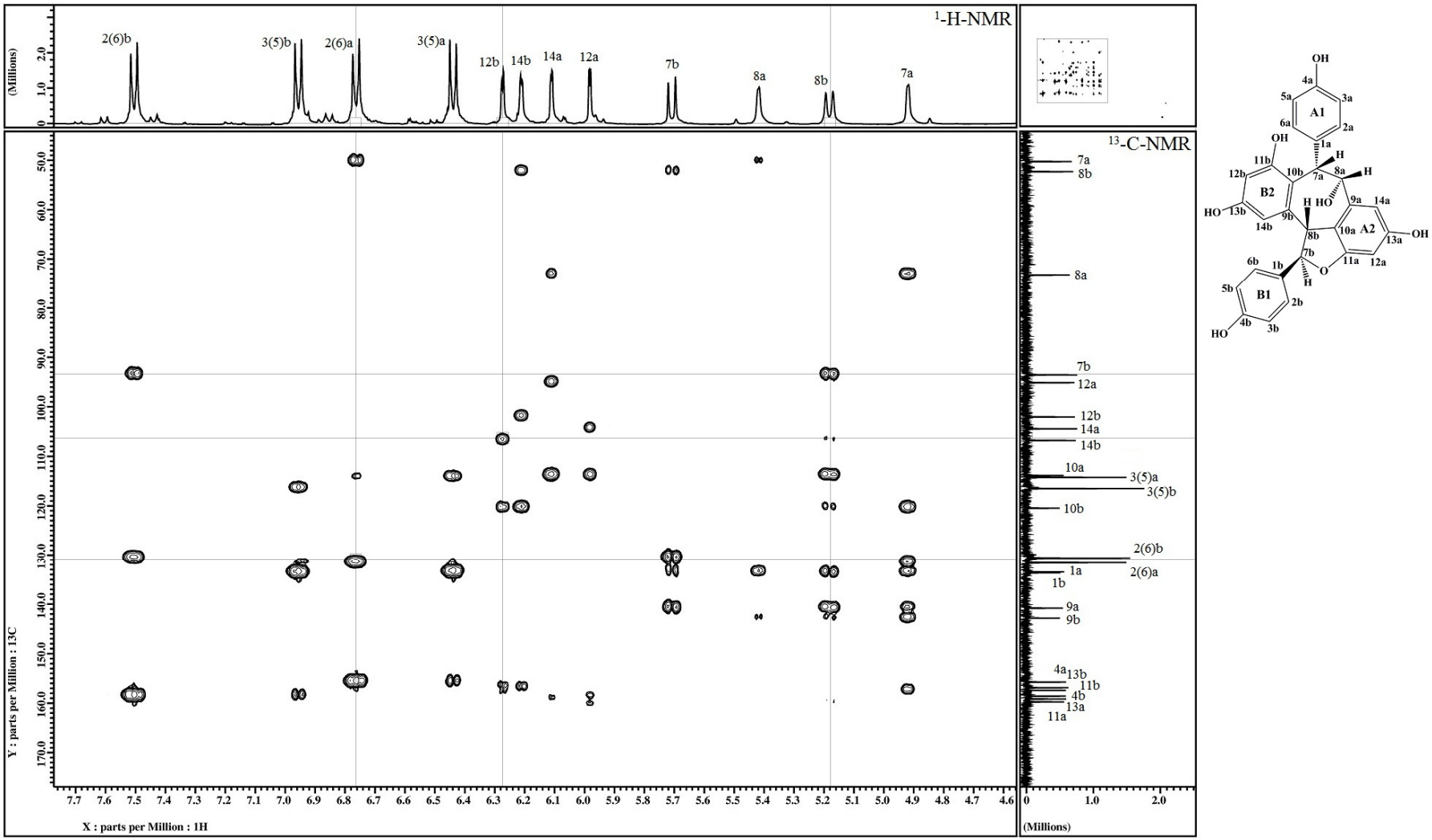


Figure 5.21 HMBC spectrum of balanocarpol measured at 400 MHz in acetone-d_6.

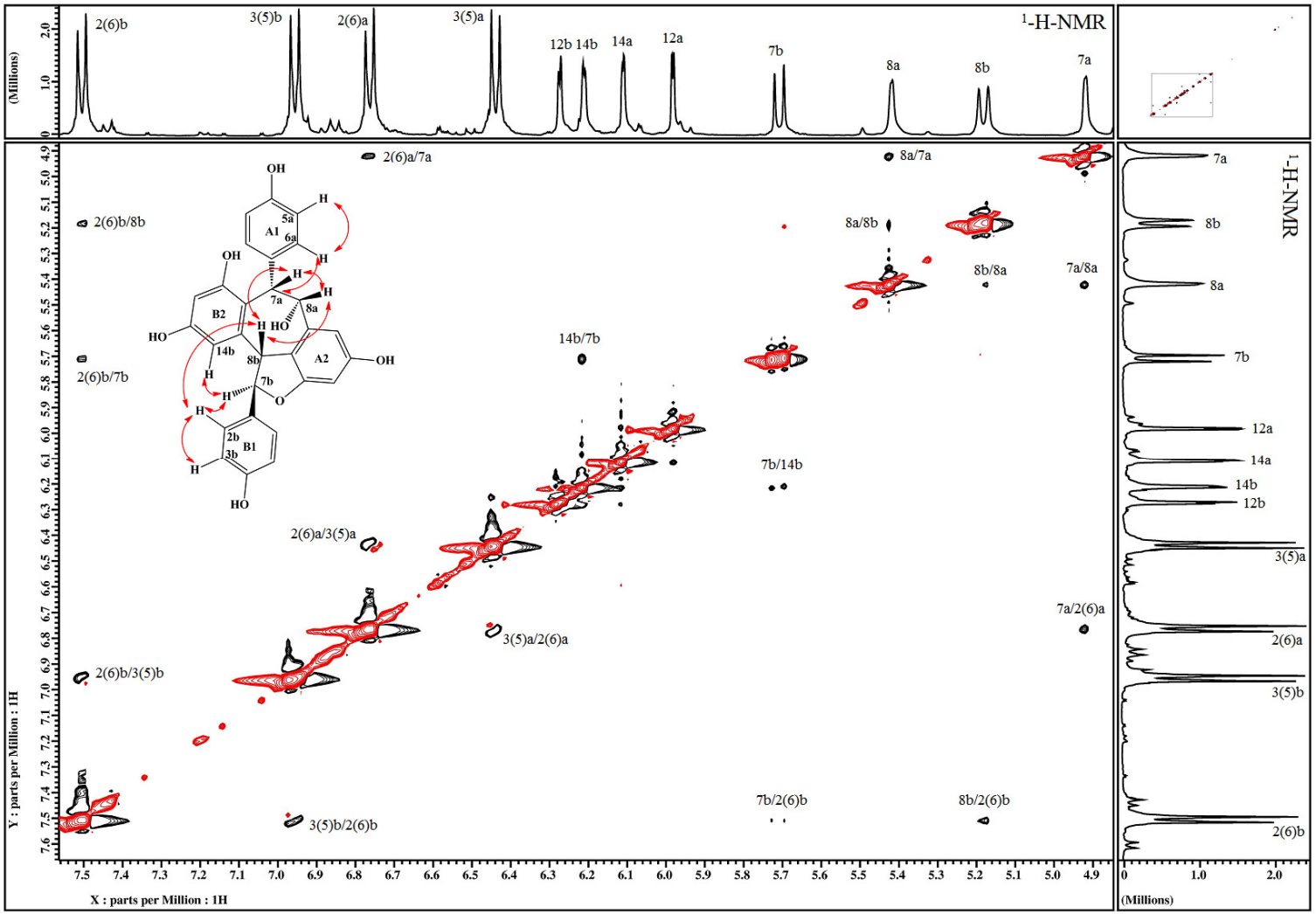


Figure 5.22 NOESY spectrum of balanocarpol measured at 400 MHz in acetone-d_6

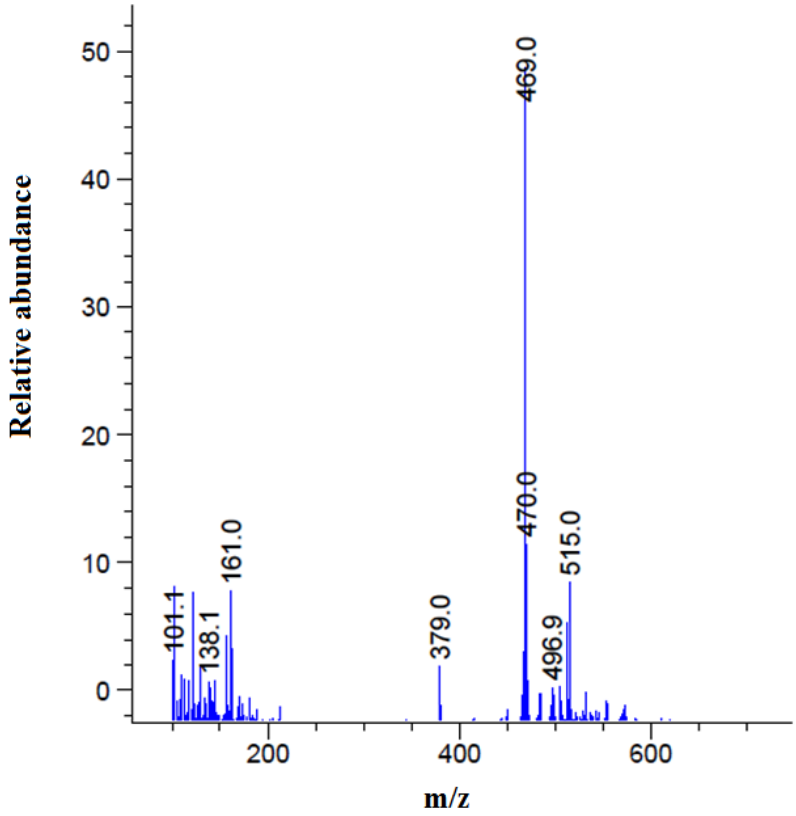


Figure 5.23 Mass spectrum of balanocarpol. Negative ion APCI-MS was carried out (Agilent Technology 6130, Quadrupole LC/MS) using water-acetonitrile as mobile phase in a C18-reverse phase column.

5.3.5 Biological effects of resveratrol

Resveratrol has been linked to a myriad of biological activities including anticancer effect (Jang *et al*., 1997). Since ampelopsin A and balanocarpol are resveratrol dimers formed by the fusion of one *cis*- and one *trans*-isomer of resveratrol (Fig. 5.24), it was of interest to investigate whether *cis* or *trans*-resveratrol can inhibit SK. As shown in Fig. 5.25A, both *cis*- and *trans*-resveratrol inhibited purified SK1 but not purified SK2 activity. Moreover, *trans*-resveratrol was more effective than the *cis*-isomer in inhibiting SK1 activity. Therefore, the following studies focus on the biological effects of *trans*-resveratrol (hereafter referred as resveratrol). Resveratrol was found to inhibit the proliferation of MCF-7 cells in both time and concentration-dependent manners (Fig. 5.25B). Resveratrol also induced apoptosis as evidenced by cleavage of PARP in MCF-7 cells (Fig. 5.25C). Kinetic inhibition studies show that resveratrol acted in a competitive manner with sphingosine for SK1, exhibiting K_{ic}= 160 +/- 40 µM (Fig. 5.26).

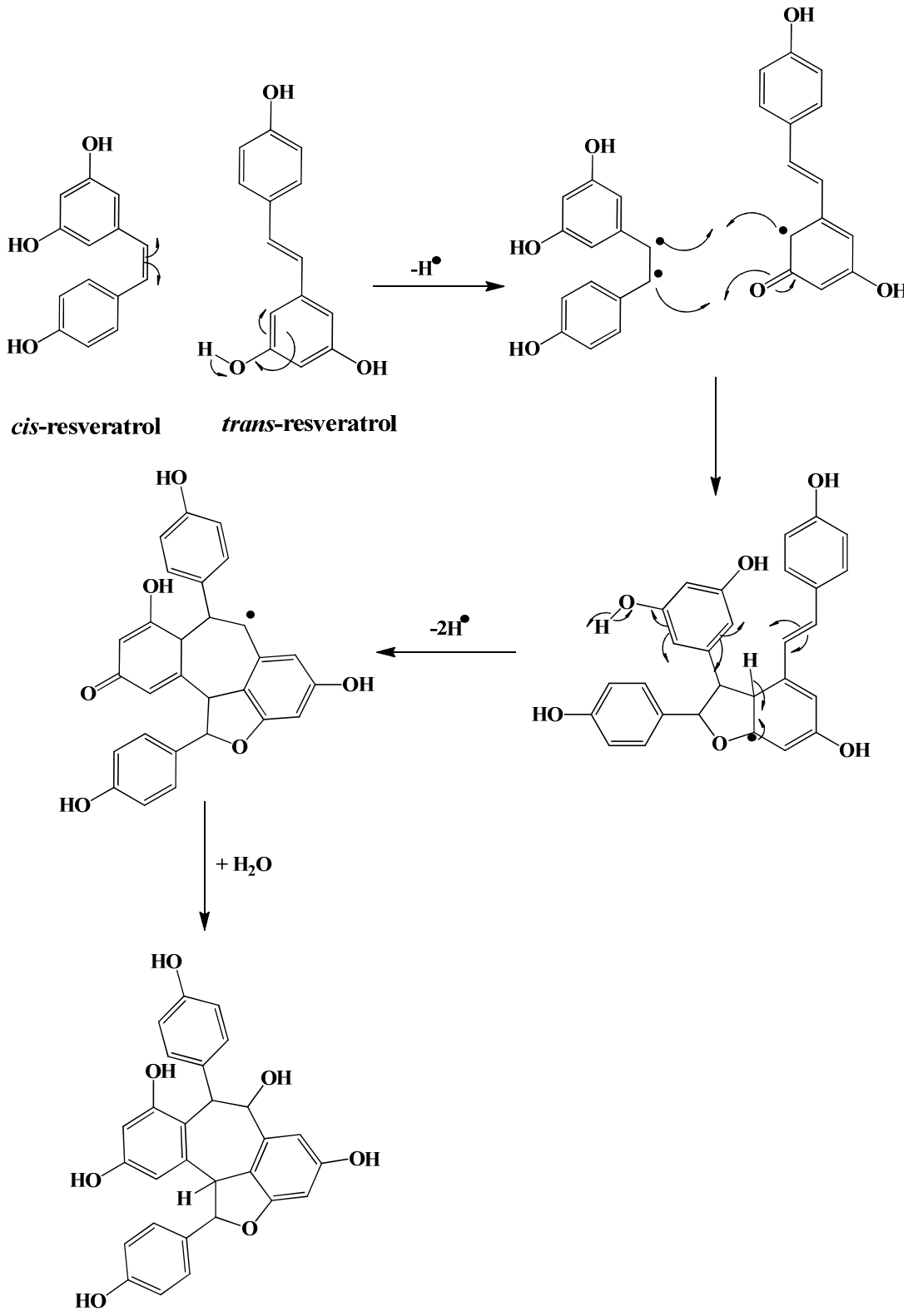


Figure 5.24 Proposed biosynthetic route of ampelopsin A or balanocarpol. Adapted from Sotheeswaran *et al*., (1993).

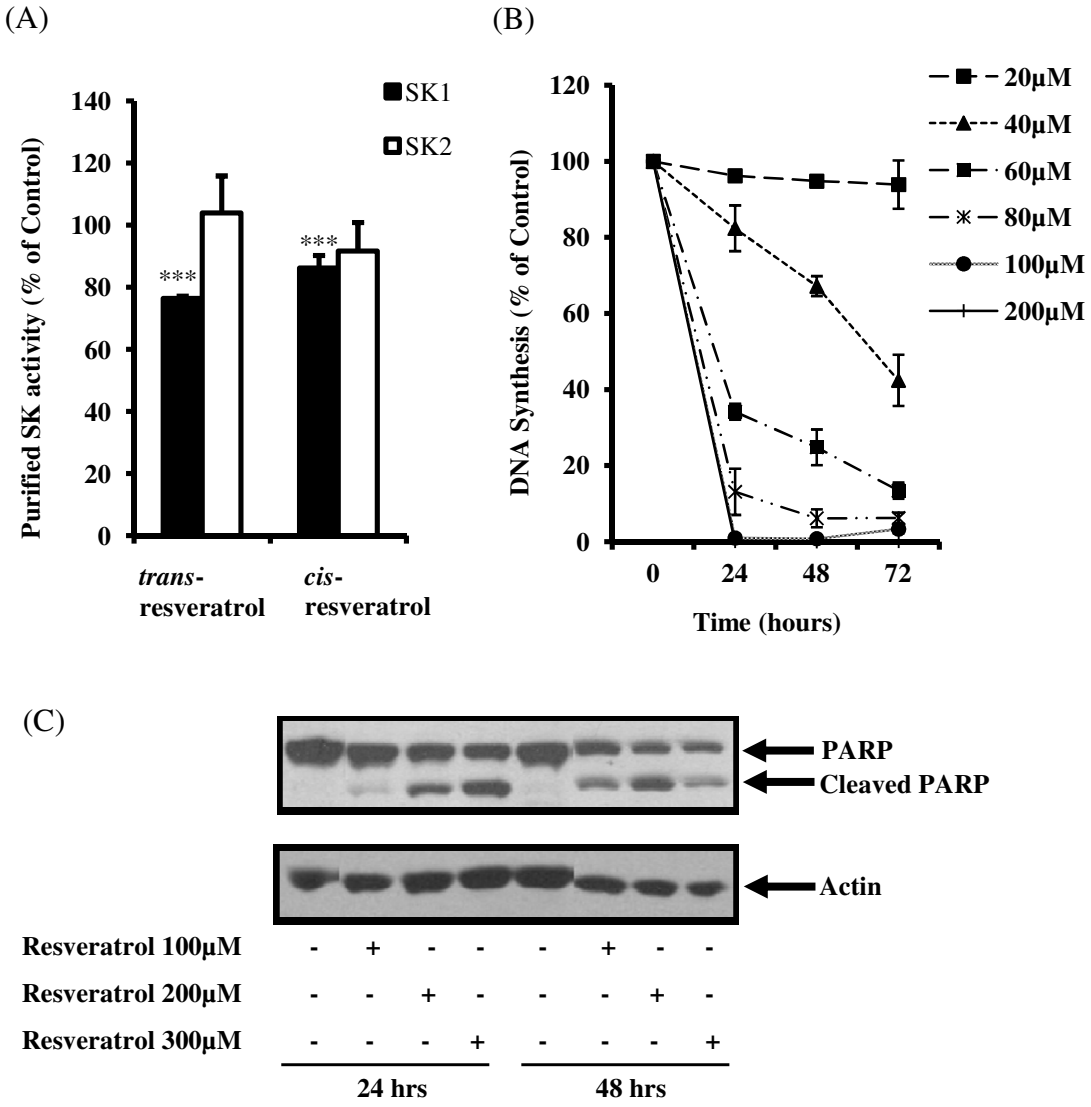
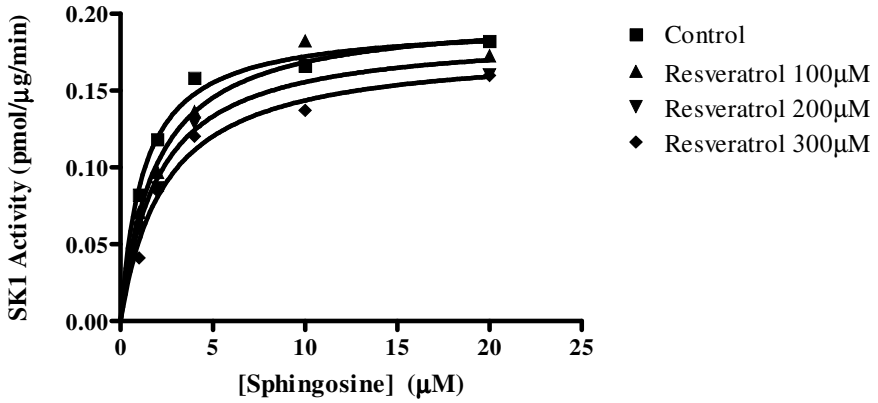


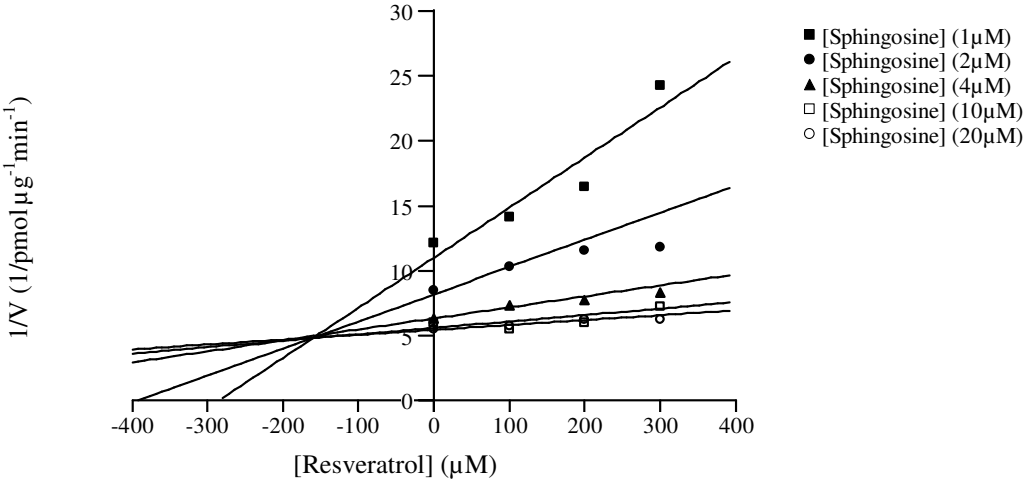
Figure 5.25 Biological effects of resveratrol. (A) Effect of *trans*- and *cis*-resveratrol (both at 500 µM) on purified SK1 or SK2 activity assayed using 10 µM sphingosine and 250 µM [^{32}P]-ATP as the substrates. Data are expressed as percentage of control and represent mean and standard deviations of six determinations, *** p<0.001 *versus* control. **(B)** MCF-7 cells were treated with *trans*-resveratrol at indicated concentrations or vehicle control (0.05% DMSO) for 24-72 hrs and then with 0.5 µCi/ml of [^{3}H]-thymidine added for 5 hrs. Uptake of [^{3}H]-thymidine by cells was measured as described under Methods (section 2.2.14). Data are expressed as percentage of vehicle control and represent means and standard deviations of triplicate determinations. **(C)** MCF-7 cells were treated with *trans*-resveratrol (100, 200, 300 µM) or control (DMSO 0.3%) for 24 and 48 hrs. Cell lysates were prepared and analysed by Western blotting using anti-PARP antibody and then stripped and reprobed with anti-actin antibody to ensure comparable protein loading. Results are representative of three independent experiments.

(A)



Resveratrol (µM)	0	100	200	300
V_{max} (pmol/µg/min)	0.19	0.20	0.19	0.18
K_m (µM)	1.25	1.90	2.03	2.42

(B)



(C)

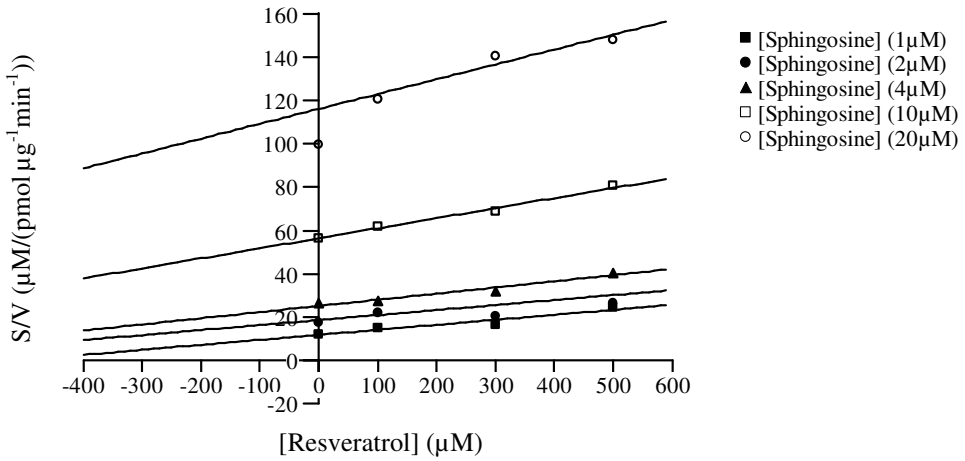


Figure 5.26 Inhibitor kinetic analysis of resveratrol for stably expressed SK1 in HEK 293 cells. **(A)** Non-linear regression analysis. **(B)** Dixon plot. **(C)** S/V *versus* [Resveratrol] plot. Similar results were obtained in three independent experiments.

In addition, the effect of resveratrol on SK expression was also investigated. Resveratrol did not reduce SK1 expression but instead increased it (at 40 μM and 100 μM) in HEK 293 cells (transiently transfected with myc-tagged SK1 plasmid) (Fig. 5.27A). Interestingly, even though resveratrol did not inhibit purified SK2 activity (Fig. 5.25A), it reduced SK2 expression in HEK 293 cells (transiently transfected with myc-tagged SK2 plasmid) in a concentration and time-dependent manners (Fig. 5.27B). To investigate whether the effect of resveratrol is cell-type specific, a cancer cell line was used for comparison. As shown in Fig. 5.27C, resveratrol reduced SK1 expression in MCF-7 cells (transiently transfected with FLAG-tagged SK1 plasmid). In agreement to previous observations, SK1 expression was increased by treating HEK 293 cells with resveratrol (Fig. 5.27C).

The present study also established that balanocarpol inhibited SK1 activity. In common with resveratrol, balanocarpol inhibited SK1 competitively (with sphingosine) but was more potent, exhibiting K_{ic}= 90 +/- 10 μM (Fig. 5.28A, B). Moreover, at high concentration, balanocarpol might exhibit uncompetitive inhibition, as evidenced by exhibiting a K_{iu} ~ 500 μM (Fig. 5.28C). Therefore, in contrast to resveratrol, balanocarpol is a mixed competitive SK1 inhibitor.

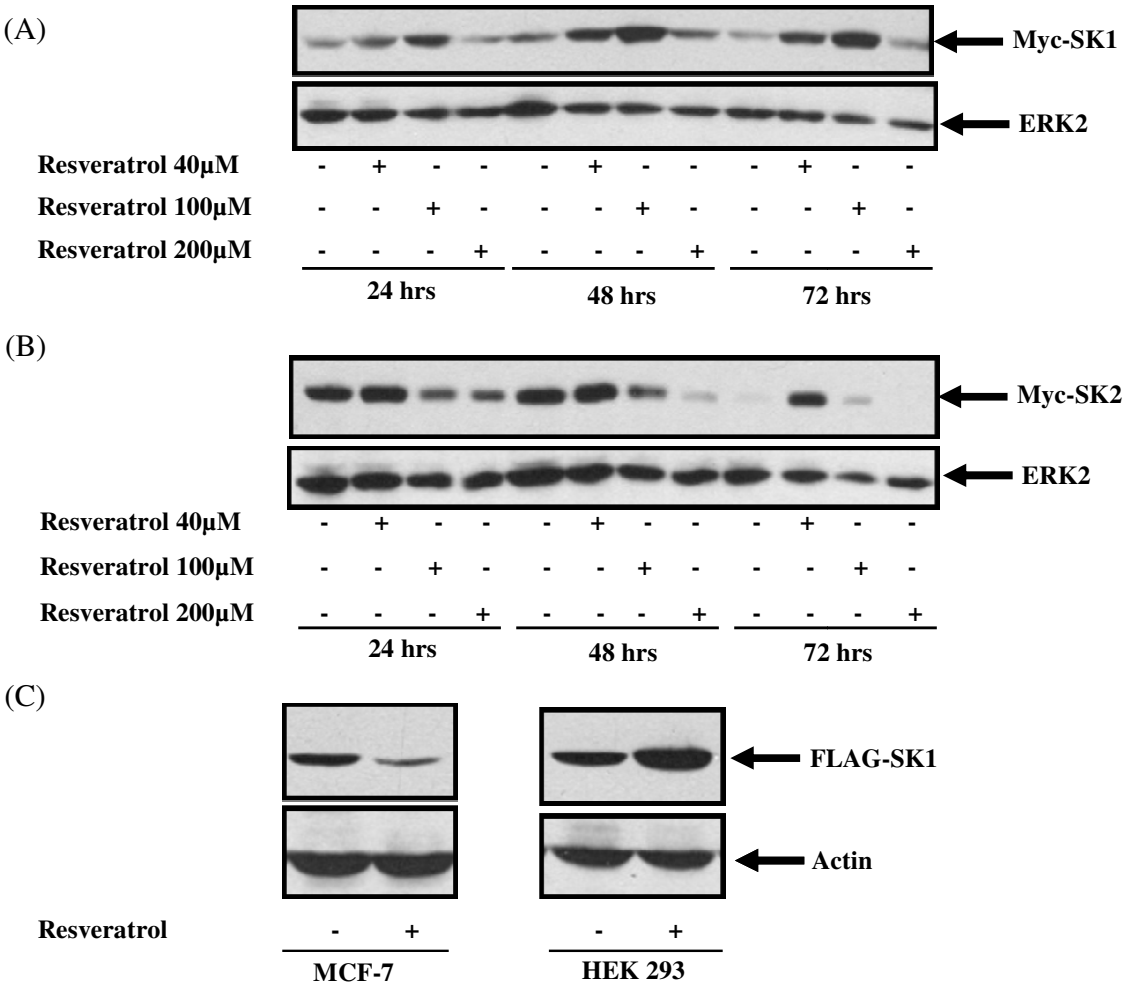
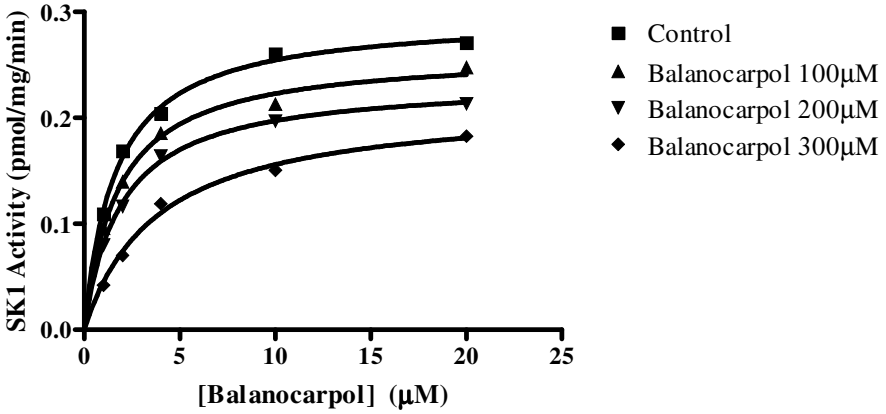


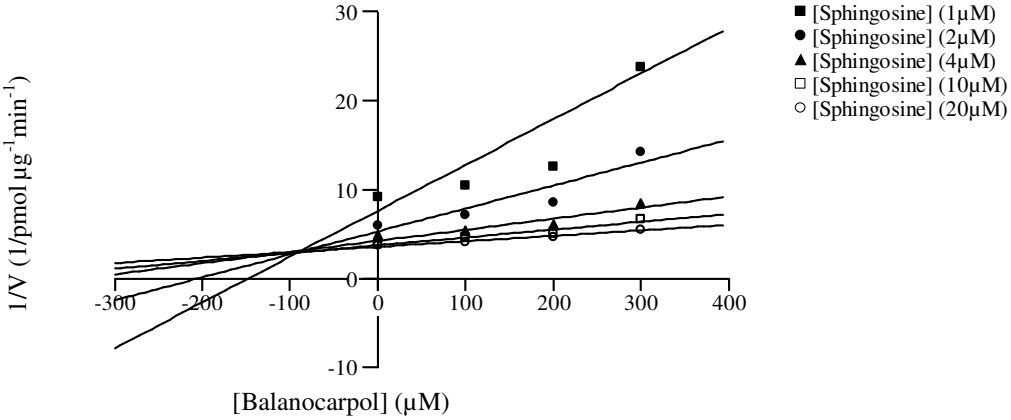
Figure 5.27 Effects of resveratrol on SK expression. HEK 293 cells transiently over-expressing Myc-SK1 **(A)** or Myc-SK2 **(B)** in the presence of resveratrol (40, 100, 200 μM) for 24, 48 and 72 hrs. **(C)** MCF-7 cells or HEK 293 cells over-expressing FLAG-SK1 in the presence of 200 μM resveratrol for 24 hrs. Cell lysates were prepared and analysed by Western blotting using anti-myc or anti-FLAG antibodies. Blots were then stripped and reprobed with anti-ERK2 or anti-actin antibodies to ensure comparable protein loading. Results are representative of three independent experiments.

(A)



Balanocarpol (μM)	0	100	200	300
V_{max} (pmol/μg/min)	0.29	0.26	0.24	0.22
K_m (μM)	1.64	1.75	1.93	3.84

(B)



(C)

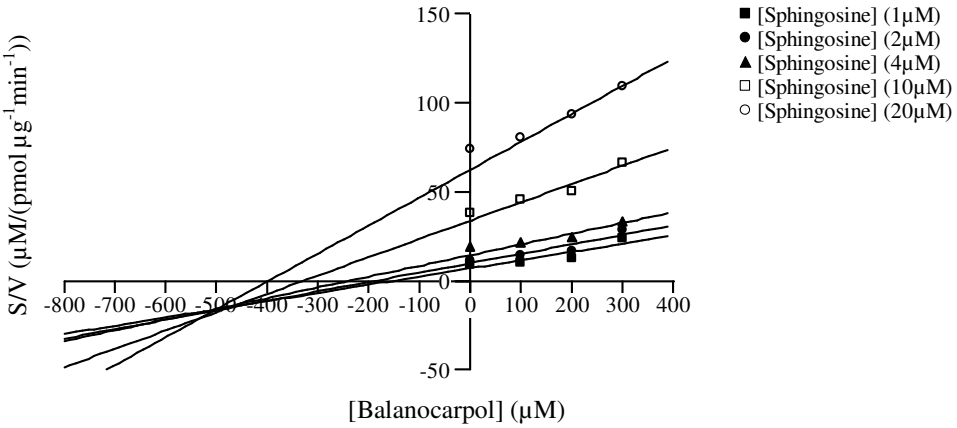


Figure 5.28 Inhibitor kinetic analysis of balanocarpol for stably expressed SK1 in HEK 293 cells. (A) Non-linear regression analysis. **(B)** Dixon plot. **(C)** S/V *versus* [Balanocarpol] plot. Similar results were obtained in three independent experiments.

5.4 Discussion

Plants are known to produce a myriad of bioactive compounds in response to stress and invasion. These compounds are classified as secondary metabolites which can be induced by initial oxidative bursts, a rapid defence mechanism launched against infectious organisms (Wojtaszek, 1997). Acting in parallel, these chemicals inhibited the growth of invading organisms as well as some of the host cells at the site of infection. Thus, it is possible to discover cell growth inhibitors/ apoptotic enhancers from secondary metabolites synthesised by plants. In fact, several anticancer compounds have been developed from plant leads including a highly active compound, paclitaxel isolated from *Taxus brevifolia*. Through millions of years of evolution, different plants have developed distinct biochemical pathways to synthesize bioactive compounds acting on different targets. A lot of these cytotoxic molecules have been isolated from plants but their cellular targets are poorly understood. In the present study, ampelopsin A and its isomer, balanocarpol have been successfully isolated from *Hopea dryobalanoides* (see section 5.3.4). The building blocks for ampelopsin A is resveratrol which has been linked to a multitude of biological effects including anticancer activity. These bioactive compounds (e.g. ampelopsin A and resveratrol) inhibited proliferation of MCF-7 cells. These findings will be discussed in relation to their effects on SK1 activity and expression.

5.4.1 Isolation and structural elucidation of Ampelopsin A and balanocarpol

NMR is a powerful tool for structural elucidation of complex organic compounds. NMR uses radiofrequency to excite atoms in a molecule and collect the signals, i.e. "energy" released by the atoms when they relax. In addition, the detection is dependent on the natural abundance of the isotopes of interest (e.g. ^{1}H and ^{13}C). Therefore, NMR is also quantitative. Ampelopsin A is relatively pure as evidenced by its intense signals in the NMR spectra (both ^{1}H-NMR and ^{13}C-NMR). On the other hand, balanocarpol has been established as a 7-epimer (differing in configuration at only one chiral centre) of ampelopsin A (Fig. 5.2). Intriguingly, the chemical shifts of four protons (12a, 12b, 14a,

14b) in balanocarpol were different than that published by Tanaka and colleagues (2000). In addition, the chemical shifts for all carbons are identical to the literature values. This suggests that previous assignments for these protons were incorrect. Indeed, 2D-NMR experiments (HMQC, HMBC and NOESY) supported the assignments of all protons and carbons for balanocarpol in the present study. Of note, only relative stereochemistry can be established using NOESY. Formal investigation using X-ray crystallography can determine the absolute configurations for both ampelopsin A and balanocarpol. This method has been used with success in the elucidation of the absolute structure of hopeaphenol (Coggon *et al*., 1970).

Determining the absolute structure of resveratrol oligomers will enable the study of their SAR since inhibitor binding is stereospecific. However, isolation of these structural analogues from natural products can be a daunting task, given that the yield of these compounds may be very low. An alternative approach is to synthesise these compounds but the major stumbling block has been the complexity associated with the structures of these oligomers. Despite this, a possible route of synthesis was developed for malibatol A (Kraus and Kim, 2003), a derivative of ampelopsin A. In many cases, controlled chemical synthesis was still not feasible, especially for higher-order oligomers. More recently, Snyder and colleagues (2011) successfully developed a programmable and scalable synthetic approach which allows controlled synthesis of possibly the entire resveratrol family. Formal investigation of these structural analogues may lead to the identification of important pharmacophores for different targets, aiding lead optimisation efforts.

5.4.2 Resveratrol oligomers as direct sphingosine kinase inhibitors

A study on red grape skin polyphenolic extracts (SGE) linked the anti-angiogenic effects of resveratrol to perturbation of S1P and VEGF signalling (Barthomeuf *et al*., 2006). Endothelial cells pre-treated with SGE showed reduced S1P-induced migration, ERK1/2 activation and platelet-activating factor synthesis (Barthomeuf *et al*., 2006). However,

SGE contains other polyphenolic compounds including resveratrol oligomers in significant quantities. Therefore, direct association of resveratrol with these biological effects should not be made without careful consideration of the presence of other polyphenolic compounds. Dipterocarps such as *Hopea dryobalanoides* typically produces resveratrol oligomers. The fact that these secondary metabolites are produced by biogenesis of resveratrol monomers raises the question whether oligomerisation produces compounds which better serve as specific regulators in different signalling pathways. Indeed, a resveratrol tetramer, vaticanol C has been shown to inhibit the growth of colon cancer cells by inducing apoptosis through perturbation of mitochondria membrane potential leading to caspase activation (Ito *et al*., 2003). Therefore, formal investigations are warranted to delineate specific biological actions of resveratrol oligomers.

Several signalling pathways have been implicated as the target for resveratrol-induced cancer cell death. Particularly, multiple lines of evidence indicate that resveratrol perturbs the SK1/S1P signalling pathway. First, resveratrol has been reported to induce apoptosis in MDA-MB-231 cells by increasing *de novo* synthesis of ceramide through indirect activation of serine palmitoyltransferase and neutral sphingomyelinase (Scarlatti *et al*., 2003). Second, Issuree and colleagues (2009) showed that resveratrol inhibits the activation of human neutrophils through inhibition of SK1-induced inflammatory response triggered by C5a (a chemoattractant cytokine). Resveratrol also inhibited activation of PLD which is an upstream activator of SK1 (Issuree *et al*., 2009). Third, dietary polyphenols including resveratrol have also been shown to down-regulate SK1 expression and activities in prostate cancer cells (Brizuela *et al*., 2010). However, these studies link the effect of resveratrol to inhibition of ERK/PLD pathway which is upstream of SK1 activation. As a result, these studies have attributed the observed biological effects of resveratrol to inhibition of SK1 activation rather than SK1 activity *per se*.

The present study provides a novel insight into the role of resveratrol in the inhibition of the SK1/S1P signalling pathway (see section 5.3.3 and 5.3.5). This is the first evidence to show that resveratrol can inhibit SK1 directly. Kinetic inhibition studies establish that resveratrol is a competitive inhibitor (with sphingosine) of SK1. This is correlated with the findings that resveratrol perturbs the balance of ceramide/S1P ratio by increasing pro-apoptotic ceramide levels (Scarlatti *et al*., 2003). There are two points regarding the moderate affinity of resveratrol for SK1. First, resveratrol competes with sphingosine to bind to SK1; inhibition of SK1 activity may be very efficient in cells because the level of sphingosine is low intracellularly. Second, potent cellular activity can also be achieved if resveratrol is actively taken up and accumulates in cells. Of note, low (1-40μM) to high micromolar (>100 μM) concentrations are usually employed to study the biological activities of resveratrol, especially its anticancer effect (see next two paragraphs). Therefore, resveratrol may exhibit high cellular activity, albeit with moderate affinity for SK1.

Suppression of cell growth and induction of apoptosis are desirable biological effects for the treatment of cancer. Resveratrol has been shown to inhibit proliferation and induce apoptosis in breast cancer cells (Nakagawa *et al*., 2001; Pozo-Guisado *et al*., 2002; Scarlatti *et al*., 2003). In agreement, the present study shows that resveratrol inhibits MCF-7 cell growth (time and concentration-dependently) and induces apoptosis by stimulating PARP cleavage (Fig. 5.25). In addition, resveratrol can act as a partial agonist of the oestrogen receptor. However, in the presence of oestrogen, it reduces oestrogen-induced MCF-7 cell growth, thereby acting as an oestrogen receptor antagonist (Lu and Serrero, 1999). Interestingly, SKi bears structural similarity to resveratrol and it has recently been demonstrated to bind to the antagonistic domain of an oestrogen receptor (Antoon *et al*., 2011). Resveratrol also induces apoptosis through activation of caspase 9 resulting in depolarisation of mitochondrial membranes in several lymphoblastic cell lines (Dörrie *et al*., 2001). Furthermore, resveratrol acts synergistically with other polyphenols such as ellagic acid and induces apoptosis or cell cycle arrest in MOLT-4 human leukemia cells (Mertens-Talcot and Percival, 2005).

Taken together, resveratrol shows promising potential for the treatment of cancer by inducing apoptosis.

High concentration (usually >50 μM) of resveratrol is required for the induction of apoptosis whereas low concentration (10-40 μM) is sufficient to suppress cancer cell growth. For example, significant increase in the number of apoptotic MCF-7 cells could only be observed with the treatment of 100 μM or 150 μM resveratrol (Pozo-Guisado *et al*., 2002). The present study found that the IC_{50} for inhibition of MCF-7 cell growth by resveratrol was around 30μM whereas apoptosis was induced by 100-300 μM of resveratrol (Fig. 5.25). These findings suggest that resveratrol acts on multiple cellular targets depending on its intracellular bioavailability. Concentrations of resveratrol needed to achieve half-maximal response for a range of targets are usually in the range of 1-40μM: COX-1 inhibitor (15μM), COX-2 (32.2μM) anti-oxidant/free radical scavenging effect (27μM), oestrogen receptor antagonist (5 μM), activation of SIRT1 (11μM) and Sir2 (2-5μM) (Jang *et al*., 1997; Subbaramaiah *et al*., 1998; Lu and Serrero, 1999; Fremont *et al.*, 2000; Howitz *et al*., 2003). Therefore, inhibition of cell growth by resveratrol may be associated with inhibition of COX-1, COX-2, oestrogen receptor or other targets depending on cell-type. The present study shows that resveratrol can also inhibit SK1 directly, and this is correlated with the induction of apoptosis. Indeed, resveratrol induces growth arrest and apoptosis in MDA-MB-231 cells through increase formation and accumulation of pro-apoptotic ceramide (Scarlatti *et al*., 2003). Resveratrol also down-regulates expression of NF-κB which is downstream of SK1 activation (Xia *et al*., 1999; Xia *et al*., 2002; Pozo-Guisado *et al*., 2005).

SK1 inhibitors have been shown to induce proteasomal degradation of SK1 in MCF-7 cells (Lim *et al*., 2011b). Down-regulation of SK1 expression by resveratrol has also been observed. Brizuela and colleagues (2010) noted down-regulation of SK1 expression in PC-3 cells after prolonged treatment with resveratrol. Of note, resveratrol-induced SK1 down-regulation occurs at late time points, usually >48hrs (Brizuela *et al*., 2010). It remains to be determined whether resveratrol-induced SK1 down-regulation is

also mediated through the ubiquitin-proteasome system in a fashion similar to other SK1 inhibitors. Formal investigation is also needed to assess whether SK1 down-regulation induced by resveratrol is at the post-transcriptional (mRNA) or post-translational (protein) level. In the present study, resveratrol did not reduce ectopically over-expressed SK1 in HEK 293 cells. Interestingly, enforced over-expression of SK1 in PC-3 cells could reverse down-regulation of SK1 activity and expression by resveratrol (Brizuela *et al*., 2010). By contrast, the expression level of ectopically expressed SK1 in HEK 293 cells was increased in the presence of resveratrol (Fig. 5.27A, C). This is reminiscent of the findings by Gude and colleagues (2008) who observed an up-regulation of SK1 expression in apoptotic leukemia cells treated with DMS—interpreted as a trigger for inflammatory response by releasing more S1P to attract leukocytes. This suggests that different types of cells may respond differently to the effects of SK1 inhibitors on SK1 expression. Indeed, when SK1 is over-expressed in MCF-7 cells in the presence of resveratrol, SK1 expression was down-regulated (Fig. 5.27C). Besides, resveratrol also reduced SK2 expression in HEK 293 cells (Fig. 5.27B). More work is needed to characterise this phenomenon since resveratrol is an inducer of apoptosis. Additionally, SK2 expression can be affected by different culturing conditions such as serum starvation which reduced SK2 expression (Maceyka *et al*., 2005). Therefore, pro-growth and pro-apoptotic factors may play a role in regulating SK2 expression.

There are two models which describe the evolution of conserved biosynthetic pathways to produce structurally-related secondary metabolites: the target-based and diversity-based models (Fischbach and Clardy, 2007). The first model is well supported by the identification of natural compounds which have high affinity for their respective biological targets. These types of molecules have evolved to improve the survival of their hosts. For example, rapamycin (a polyketide) is an immunosuppressant binding specifically to the FK-binding protein (FKBP-12), forming a FKBP-12-rapamycin complex which binds mTOR and inhibits its signalling (Choi *et al*., 1996). This work elegantly shows that rapamycin can act like a "molecular glue" to promote protein dimerisation by binding to a distinct hydrophobic region in each protein concurrently,

preventing down-stream signalling of these proteins. The second model proposes that evolution favours the survival of organisms that can maximise diversity of their protective metabolites. Various examples in natural product synthesis support this model. For instance, terpene consists of a large group of organic compounds, all derived from a simple unit, isoprene (C_5H_8). There are at least 136 distinct gibberellin-family diterpenes formed in Nature but only a few of them have potent biological effects; others are regarded as side products which may have different activity or no activity at all (Fischbach and Clardy, 2007). Therefore, some secondary metabolites might be synthesised in a diversity-oriented approach which aims to maximise diversity and minimise metabolic cost by utilising a common building block.

A novel finding in the present study is that a resveratrol dimer such as ampelopsin A, inhibits SK1 activity and induces down-regulation of SK1 expression in MCF-7 cells (Fig. 5.4 and Fig. 5.5). Moreover, an isomer of ampelopsin A (balanocarpol) has been successfully isolated and shown to inhibit SK1 activity (Fig. 5.28). The significance of these findings is highlighted by the potency of balanocarpol in reducing SK1 activity. In contrast to resveratrol, balanocarpol is a mixed competitive SK1 inhibitor (with sphingosine). Furthermore, it has a lower K_{ic} (90 µM, nearly two fold more potent than resveratrol), indicating that it has higher affinity for SK1. It is attractive to speculate that a single molecule of balanocarpol may bind at least two SK1 molecules, acting like a chelator to block the catalytic activity of each enzyme simultaneously. This observation is reminiscent of the action of rapamycin, a natural product which possesses a unique scaffold that binds two different proteins (FKBP-12 and mTOR), capable of forming inactive protein-ligand complexes (Choi *et al*., 1996). Biosynthesis of resveratrol oligomers can also be regarded as a diversity-oriented approach in a fashion similar to the biosynthesis of other natural compounds such as terpenes. For example, hopeaphenol also known as dibalanocarpol (a resveratrol tetramer) was the most potent compound in inhibiting proliferation of P-388 cells (hopeaphenol: IC_{50}=5.7µM; balanocarpol: IC_{50}=33.6µM) (Sahidin *et al*., 2005). It will be important to investigate whether hopeaphenol also inhibits SK1 and to evaluate its potency. Also, a high content of

resveratrol in red wine has been famously linked to the “French paradox”. However, other resveratrol oligomers are also present in red wine and they may act on distinct signalling pathways to mediate different biological effects. The study of resveratrol oligomers has been hampered by difficulty in isolation from natural sources and intractable chemical synthesis. Very recently, controlled synthesis of resveratrol oligomers has been achieved (Snyder *et al*., 2011). This significant development not only indicates that there is still a lot of interest in the study of resveratrol and its oligomers but also highlights their tremendous potential for the development of novel therapeutics. The present study provides the first evidence that resveratrol dimer (balanocarpol) is more potent than resveratrol monomer in inhibiting SK1 activity. Hence, further studies are needed to assess the cellular activity of balanocarpol. If there is a correlation between cellular activity and affinity for the targets, resveratrol oligomerisation as a way to improve potency or selectivity may represent a new paradigm in phytochemistry.

Finally, it has been suggested that polyphenols produced by plants could be used by heterotrophs such as animals as a signalling cue to improve survival in response to environmental stress (Howitz and Sinclair, 2008). This hypothesis, also termed as xenohormesis was largely based on the fact that a wide range of phytochemicals are able to interact with different signalling pathways in animals by either inhibiting or activating more than one target. In fact, the similarity between many signalling molecules in plants and animals suggest that common biosynthetic pathway existed before the two kingdoms diverged (Kushiro *et al*., 2003). Therefore, it is possible that resveratrol oligomers may have specialised functions—acting on selective targets. One of these targets is SK which catalyses the formation of S1P, a potent bioactive molecule involved in the regulation of mammalian cell growth and plant signalling (e.g. transpiration and seed germination). Thus, future studies should focus on determining the biological activities and cellular targets of other resveratrol oligomers in the resveratrol family.

Chapter 6 General Discussion

Drug discovery has long been an interdisciplinary endeavour that is both lengthy and expensive (Drews, 2000). The time taken for a drug to reach the market since its discovery takes an average of 10 years and costs at least a billion dollars (Balunas and Kinghorn, 2005). Early drug discovery efforts focus on target validation, hits discovery and lead optimisation. Then, preclinical studies are carried out to establish *in vitro* and *in vivo* efficacy (pharmacodynamics) in various disease models and to gain a better understanding on pharmacokinetics and toxicology of the lead compounds. These initial drug discovery endeavours are increasingly being conducted by biotechnology industry and academia whereas big pharmaceutical companies focus on late stage drug developments which are most costly (Drews, 2000). Upon sufficient demonstration of the safety and efficacy of lead compounds in preclinical studies, an investigational new drug status will be granted by regulatory agencies for clinical studies. Early clinical studies aim to carry out proof of concept human trials. Pharmaceutical industry plays a major role in establishing clinical safety and efficacy in large clinical trials and drug marketing. Therefore, it is obvious that drug discovery is not a simple process but involves multidisciplinary efforts.

Sphingosine kinase (SK1 and SK2) as well as the cognate receptors for S1P are emerging as important targets for drug discovery. To put this into perspective, development of SK inhibitors is still in pre-clinical studies or early clinical trials whereas FTY720 (a $S1P_1$ functional antagonist) has recently been approved for the treatment of multiple sclerosis. Not surprisingly, there is immense demand to develop compounds that can act on SK/S1P signalling pathway, either as SK inhibitors or S1P receptor ligands. In this regard, there is substantial evidence demonstrating a role for SK1 and/or SK2 in driving tumour development (see chapter 1, section 1.3). Therefore, this project was initiated with the aims of discovering and characterising novel compounds which can be eventually developed into effective anticancer drugs that target SK.

With a focus on early stages in drug discovery, this project contributes to hit discovery, compound profiling and target validation. First, hit discovery was carried out to identify cytotoxic compounds with activities against SK1/SK2. One of the main findings is the discovery of several novel synthetic analogues and natural compounds as promising anticancer compounds. These include synthetic analogues of the QAB compound series, FTY720 analogues and resveratrol oligomers. To this end, compound profiling has established selectivity of FTY720 analogues against different SK isoforms (SK1 *versus* SK2). Particularly, (*R*)-FTY720-OMe has been discovered as a selective SK2 inhibitor while SKi and (*S*)-FTY720 vinylphosphonate show activities against both SK1 and SK2. Future studies are needed to establish their specificity against other protein targets and to investigate the selectivity and biological activity of other promising hits such as QAB801A. A critical assessment at the compound profiling stage includes drug-likeness and toxicological evaluations. The former is related to assessing the bio-physicochemical properties (e.g. solubility, permeability and metabolic stability) and pharmacokinetics (absorption, distribution, metabolism and elimination) of hit/lead compounds (Bleicher *et al*., 2003). This type of characterisation is part of the study of SAR, which should be considered for future work.

Target validation is one of the most important stages in drug discovery. Rigorous *in vitro* and *in vivo* tests are needed to establish that the identified target is indeed responsible for the pathogenesis of a disease. For example, changes in the expression of a target between normal and clinical samples could implicate a pathological role for the target. In addition, this suggests that modulation of the target activity/expression should ameliorate disease progression or reverse a disease phenotype (Lindsay, 2003). There are various means to achieve such validation using genetic (e.g. gene knockout, siRNA) or chemical tools (e.g. inhibitor, antibody). Insufficient validation of such a causal relationship between a target and a disease can often lead to costly failure in drug development (Smith, 2003). *In vitro* target validation aims to establish the cellular and molecular mechanisms of a disease process. This can be achieved by studying clinical samples from patients and cell models—the molecular approach (Lindsay, 2003). On the

other hand, *in vivo* target validation requires the use of animal models or clinical study to establish the role of a target and the efficacy of inhibiting the target in a disease—the system approach (Lindsay, 2003). More resources are needed to perform *in vivo* validation, especially when clinical studies are conducted on a large number of patients. Accordingly, target validation is a crucial step which is performed throughout the drug discovery cycle.

SK1 is a promising target for the development of novel anticancer therapeutics. Accumulating evidence has shown that SK1 is over-expressed in many tumours, especially in breast cancers where over-expression of SK1, $S1P_1$ and $S1P_3$ contribute to decreased patient survival and increased recurrence to tamoxifen resistance (Watson, *et al*., 2010). In addition, Long *et al*., (2010a) recapitulated the phenotypes of these patient cohorts and showed that targeting SK1 in patients whose tumours are $ER^+/Her2^-$ may be an effective treatment strategy. In agreement to previous studies, compounds that perturb SK/S1P signalling pathway such as DMS and SKi (Chapter 3), FTY720 and (*S*)-FTY720 vinylphosphonate (Chapter 4), resveratrol and its oligomers (Chapter 5) commonly inhibit cell proliferation and induce apoptosis, thereby exhibiting favourable profiles for cancer treatment. Moreover, SK1 inhibitors also induce down-regulation of SK1 expression and reduce S1P-induced actin rearrangement, thereby acting potentially as anti-metastatic agents. These inhibitors might also have higher therapeutic efficacy by inducing the removal of SK1 from cancer cells compared with compounds which simply inhibit SK1 reversibly. (*R*)-FTY720-OMe also demonstrates efficacy in reducing proliferation, S1P-induced actin rearrangement in MCF-7 cell. It also induces PARP cleavage and down-regulation of SK2 expression in HEK 293 cells. By discovering and characterising novel compounds as SK1/SK2 inhibitors in the present study, this project not only expands the repertoire of SK inhibitors but also provides additional chemical tools for target validation. Future studies should include animal models (e.g. xenograft over-expressing SK1/SK2) to establish the *in vivo* efficacy of these inhibitors. Ultimately, full target validation should be assessed in clinical studies.

Another important aspect of drug discovery is lead optimisation, an iterative process aiming to improve potency, selectivity and bio-physicochemical properties of hits to develop effective drug leads. Successful lead optimisation depends largely on chemical synthesis, computer-assisted modelling and robust functional assays (Bleicher *et al*., 2003). A critical requirement for this process is knowledge of the protein structure which can be elucidated using X-ray crystallography. Structural information obtained from crystallography can provide a high-resolution image of the binding clefts (including ligand binding and allosteric sites) in which compounds can be docked *in silico* using various docking methods (Blundell *et al*., 2002). However, proteins are not static and may display significantly different conformations resulting from post-translational modifications (e.g. activation through protein phosphorylation) or ligand binding. Successful computer-assisted modelling is also dependent on the quality of the structural information. For enzyme targets, it is thus important to determine which conformational state has been adopted by the enzyme during catalysis and whether this can be crystallised. Once a three-dimensional model has been established, structure-based virtual compound screening can then be conducted (Blundell *et al*., 2002). A series of compounds which fits well into the model can be synthesised and assessed in functional bioassays to rank their potency, as part of a SAR study. Not surprisingly, the development of highly potent SK inhibitors has been hampered by the lack of a crystal structure for any SK isoforms. In this case, inhibitors of SK may be used to facilitate the crystallisation of an enzyme-inhibitor complex. Alternatively, medicinal chemists have recently utilised the crystal structure of DgkB to create a homology model of SK1 in the pursuit of highly potent and selective SK inhibitors (Kennedy *et al*., 2011). These amidine-based SK inhibitors exhibit nanomolar potency and high selectivity against SK1. Further studies are warranted to evaluate the efficacy of these inhibitors in cancer models and to optimise their physicochemical and pharmacokinetic properties.

In relation to lead optimisation, significant contributions from the current project include the discovery of a putative allosteric site on SK1 which is only accessible when SK1 is bound to the substrate, forming the SK1-sphingosine complex. Knowing the inhibition

modality for SK1 inhibitors is important for subsequent structure-based lead optimisation. For instance, to obtain a crystal structure for a SK1-inhibitor complex with an uncompetitive inhibitor such as (*S*)-FTY720 vinylphosphonate which only binds to the enzyme-substrate complex, crystallisation conditions must be optimised to include sphingosine. Any attempts to crystallise enzyme-inhibitor complexes in the absence of the substrate to which the inhibitor displays uncompetitive inhibition will not be successful. Discovering a diverse compound series with different inhibition modalities is also essential in subsequent lead optimisation for two reasons. First, it is difficult to predict cellular activity or *in vivo* efficacy for enzyme inhibitors in part, due to intrinsic difficulties associated with the measurement of *in vivo* substrate concentration. Substrate concentration can also differ in physiological and pathological states. Only assessment of inhibitor efficacy in cell-based assay or *in vivo* disease models can determine whether a competitive inhibitor is superior to an uncompetitive or a mixed inhibitor. For example, FTY720 is a competitive SK1 inhibitor with the lowest K_i, yet SKi (a mixed inhibitor) induces more SK1 down-regulation and PARP cleavage in MCF-7 cells. Other factors such as differential bioavailability of the inhibitors can also affect cellular activity. Second, compounds with different inhibition modalities often have distinct chemical structures which can improve the diversity of the lead compound series. Based on the inhibition modality, medicinal chemists can then synthesise chemical analogues for SAR study. Therefore, the study of enzyme inhibitor kinetics provides important information for subsequent lead optimisation efforts.

One of the biggest challenges in drug discovery is the race for the development of first-in-class medicine. It is not uncommon to find that once a "druggable" target is identified, multiple organisations may be in a race to develop the first-in-class drug by pursuing the same target and develop compounds with similar chemical structures and/or mechanisms of action (DiMasi and Faden, 2011). As a result, there is limited scope to compete if the chemical space of the drug is protected by patenting. One way to improve success is to increase the diversity of chemical compounds by exploring novel scaffolds. It has been suggested that chemical space of all small organic molecules (< 500 Da) is vast with a

staggering number of compounds (10^{60}) (Dobson, 2004). However, only a small fraction of chemical space is occupied by biologically active compounds—the biologically relevant chemical space. Advances in medicinal chemistry e.g. combinatorial chemistry (where large chemical libraries can be generated from a few simple molecules) has made the production of many complex organic molecules possible. The diversity of these synthetic compounds, however, does not match the expectation that they can cover the biologically relevant chemical space. Conversely, natural products share the same degree of chemical diversity as successful drug molecules, highlighting their immense potential for drug discovery (Dobson, 2004). Moreover, natural products are carefully "crafted" by Nature to facilitate the attainment of vast diversity and potent biological activity with optimum bio-physicochemical properties through dedicated enzymatic reactions (Clardy and Walsh, 2004). Pharmaceutical output can thus be improved by charting novel chemical space covered by natural products. The current project addressed the need for novel scaffolds by screening plant extracts against SK1 activity. The successful isolation of ampelopsin A and balanocarpol has led to the discovery that their common building block, resveratrol, is also active in inhibiting SK1 activity. Moreover, a resveratrol dimer (balanocarpol) is twice as potent as resveratrol on a molar basis, indicating that resveratrol oligomerisation improves inhibitor potency. Therefore, resveratrol oligomers contain novel scaffolds that can be developed into effective anticancer drug leads.

In conclusion, several hits have been discovered from the current project, which serve as a good starting point for the development of selective SK inhibitors. These compounds can be used as chemical probes for target validation and/or leads for drug development. Through the use of these compounds, novel insights have been gained into allosteric regulation of SK1 and inhibitor-induced down-regulation of SK1. A selective SK2 inhibitor with anticancer activity has also been identified, substantiating the notion that inhibiting SK2 may have beneficial effects in cancer treatment. Unique chemical scaffolds present in isolated natural compounds provide opportunities for lead optimisation studies and patents. Besides, evaluation of the SAR between resveratrol and

its dimer gives an illuminating insight into the evolution of important biosynthetic pathways in plants. The findings from this project thereby provide an impetus for the development of effective inhibitors acting on SK1 and/or SK2, paving the way for the advancement of cancer chemotherapy.

References

Adams, J. A. (2003). Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model? *Biochemistry, 42*(3), 601-607.

Agrawal, A. A. (2007). Macroevolution of plant defense strategies. *Trends Ecol Evol, 22*(2), 103-109.

Albertson, D. G., & Pinkel, D. (2003). Genomic microarrays in human genetic disease and cancer. *Hum Mol Genet, 12*(Spec No 2), R145-152.

Alderton, F., Rakhit, S., Kong, K.C., Palmer, T., Sambi, B., Pyne, S., & Pyne, N. J. (2001). Tethering of the platelet-derived growth factor β receptor to G-protein-coupled receptors. A novel platform for integrative signaling by these receptor classes in mammalian cells. *J Biol Chem, 276*(30), 28578-28585.

Alemany, R., van Koppen, C. J., Danneberg, K., Ter Braak, M., & Meyer Zu Heringdorf, D. (2007). Regulation and functional roles of sphingosine kinases. *Naunyn Schmiedebergs Arch Pharmacol, 374*(5-6), 413-428.

Alvarez, S. E., Harikumar, K. B., Hait, N. C., Allegood, J., Strub, G. M., Kim, E. Y., Maceyka, M., Jiang, H., Luo, C., Kordula, T., Milstien, S., & Spiegel, S. (2010). Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature, 465*(7301), 1084-1088.

Alvarez, S. E., Milstien, S., & Spiegel, S. (2007). Autocrine and paracrine roles of sphingosine-1-phosphate. *Trends Endocrinol Metab, 18*(8), 300-307.

An, S., Zheng, Y., & Bleu, T. (2000). Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg3 and Edg5. *J Biol Chem, 275*(1), 288-296.

Antoon, J. W., Meacham, W. D., Bratton, M. R., Slaughter, E. M., Rhodes, L. V., Ashe, H. B., Wiese, T. E., Burow, M. E., & Beckman, B. S. (2011). Pharmacological inhibition of sphingosine kinase isoforms alters estrogen receptor signaling in human breast cancer. *J Mol Endocrinol, 46*(3), 205-216.

Arikawa, K., Takuwa, N., Yamaguchi, H., Sugimoto, N., Kitayama, J., Nagawa, H., Takehara, K., & Takuwa, Y. (2003). Ligand-dependent inhibition of B16 melanoma cell migration and invasion via endogenous $S1P_2$ G protein-coupled receptor. Requirement of inhibition of cellular Rac activity. *J Biol Chem, 278*(35), 32841-32851.

Arimura, G., Kost, C., & Boland, W. (2005). Herbivore-induced, indirect plant defences. *Biochim Biophys Acta, 1734*(2), 91-111.

Athar, M., Back, J. H., Tang, X., Kim, K. H., Kopelovich, L., Bickers, D. R., & Kim, A. L. (2007). Resveratrol: a review of preclinical studies for human cancer prevention. *Toxicol Appl Pharmacol*, *224*(3), 274-283.

Baehrecke, E. H. (2005). Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol, 6*(6), 505-510.

Bakali, H. M., Herman, M. D., Johnson, K. A., Kelly, A. A., Wieslander, A., Hallberg, B. M., & Nordlund, P. (2007). Crystal structure of YegS, a homologue to the mammalian diacylglycerol kinases, reveals a novel regulatory metal binding site. *J Biol Chem, 282*(27), 19644-19652.

Balunas, M. J., & Kinghorn, A. D. (2005). Drug discovery from medicinal plants. *Life Sci, 78*(5), 431-441.

Bandhuvula, P., Tam, Y. Y., Oskouian, B., & Saba, J. D. (2005). The immune modulator FTY720 inhibits sphingosine-1-phosphate lyase activity. *J Biol Chem*, *280*(40), 33697-33700.

Banno, Y., Kato, M., Hara, A., & Nozawa, Y. (1998). Evidence for the presence of multiple forms of Sph kinase in human platelets. *Biochem J, 335 (Pt 2)*, 301-304.

Barr, R. K., Lynn, H. E., Moretti, P. A., Khew-Goodall, Y., & Pitson, S. M. (2008). Deactivation of sphingosine kinase 1 by protein phosphatase 2A. *J Biol Chem, 283*(50), 34994-35002.

Barthomeuf, C., Lamy, S., Blanchette, M., Boivin, D., Gingras, D., & Beliveau, R. (2006). Inhibition of sphingosine-1-phosphate- and vascular endothelial growth factor-induced endothelial cell chemotaxis by red grape skin polyphenols correlates with a decrease in early platelet-activating factor synthesis. *Free Radic Biol Med, 40*(4), 581-590.

Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K., Pistell, P. J., Poosala, S., Becker, K. G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K. W., Spencer, R. G., Lakatta, E. G., Le Couteur, D., Shaw, R. J., Navas, P., Puigserver, P., Ingram, D. K., de Cabo, R., & Sinclair, D. A. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*, *444*(7117), 337-342.

Baur, J. A., & Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat Rev Drug Discov, 5*(6), 493-506.

Beljanski, V., Knaak, C., & Smith, C. D. (2010). A novel sphingosine kinase inhibitor induces autophagy in tumor cells. *J Pharmacol Exp Ther, 333*(2), 454-464.

Benson, C., White, J., De Bono, J., O'Donnell, A., Raynaud, F., Cruickshank, C., McGrath, H., Walton, M., Workman, P., Kaye, S., Cassidy, J., Gianella-Borradori, A., Judson, I., & Twelves, C. (2007). A phase I trial of the selective oral cyclin-dependent kinase inhibitor seliciclib (CYC202; *R*-Roscovitine), administered twice daily for 7 days every 21 days. *Br J Cancer, 96*(1), 29-37.

Berdyshev, E. V., Gorshkova, I., Skobeleva, A., Bittman, R., Lu, X., Dudek, S. M., Mirzapoiazova, T., Garcia, J. G., & Natarajan, V. (2009). FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J Biol Chem, 284*(9), 5467-5477.

Beutler, J. A., Kashman, Y., Pannell, L. K., Cardellina, J. H., 2nd, Alexander, M. R., Balaschak, M. S., Prather, T. R., Shoemaker, R. H., & Boyd, M. R. (1997). Isolation and characterization of novel cytotoxic saponins from *Archidendron ellipticum*. *Bioorg Med Chem, 5*(8), 1509-1517.

Billich, A., Bornancin, F., Devay, P., Mechtcheriakova, D., Urtz, N., & Baumruker, T. (2003). Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem*, *278*(48): 47408-47415.

Bleicher, K. H., Böhm, H. J., Müller, K., & Alanine, A. I. (2003). Hit and lead generation: beyond high-throughput screening. *Nat Rev Drug Discov, 2*(5), 369-378.

Blundell, T. L., Jhoti, H., & Abell, C. (2002). High-throughput crystallography for lead discovery in drug design. *Nat Rev Drug Discov, 1*(1), 45-54.

Bonhoure, E., Pchejetski, D., Aouali, N., Morjani, H., Levade, T., Kohama, T., & Cuvillier, O. (2006). Overcoming MDR-associated chemoresistance in HL-60 acute myeloid leukemia cells by targeting sphingosine kinase-1. *Leukemia, 20*(1), 95-102.

Boutros, T., Chevet, E., & Metrakos, P. (2008). Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacol Rev, 60*(3), 261-310.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*, 248-254.

Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S., & Burtin, P. (2010). Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov, 9*(11), 883-897.

Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C. A., Zollinger, M., & Lynch, K. R. (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem, 277*(24), 21453-21457.

Brizuela, L., Dayon, A., Doumerc, N., Ader, I., Golzio, M., Izard, J. C., Hara, Y., Malavaud, B., & Cuvillier, O. (2010). The sphingosine kinase-1 survival pathway is a molecular target for the tumor-suppressive tea and wine polyphenols in prostate cancer. *FASEB J, 24*(10), 3882-3894.

Buehrer, B. M., & Bell, R. M. (1992). Inhibition of sphingosine kinase *in vitro* and in platelets. Implications for signal transduction pathways. *J Biol Chem, 267*(5), 3154-3159.

Burden, D., & Osheroff, N. (1998). Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim Biophys Acta, 1400*(1-3), 139-154.

Chae, S. S., Paik, J. H., Furneaux, H., & Hla, T. (2004). Requirement for sphingosine 1-phosphate receptor-1 in tumor angiogenesis demonstrated by *in vivo* RNA interference. *J Clin Invest, 114*(8), 1082-1089.

Chalfant, C. E., & Spiegel, S. (2005). Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *J Cell Sci, 118*(Pt 20), 4605-4612.

Chin, Y. W., Balunas, M. J., Chai, H. B., & Kinghorn, A. D. (2006). Drug discovery from natural sources. *AAPS J, 8*(2), E239-253.

Choi, J., Chen, J., Schreiber, S. L., & Clardy, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science, 273*(5272), 239-242.

Choong, C. Y., Wickneswari, R., Norwati, M., & Abbott, R. J. (2008). Phylogeny of *Hopea* (Dipterocarpaceae) inferred from chloroplast DNA and nuclear PgiC sequences. *Mol Phylogenet Evol, 48*(3), 1238-1243.

Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., De Placido, S., Bianco, A. R., & Tortora, G. (2000). Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res, 6*(5), 2053-2063.

Clardy, J., & Walsh, C. (2004). Lessons from natural molecules. *Nature, 432*(7019), 829-837.

Coggon, P., Janes, N. F., King, F. E., King, T. J., Molyneux, R. J., Morgan, J. W. W., & Sellars, K. (1965). Hopeaphenol an extractive of heartwood of *Hopea odorata* and *Balanocarpus heimii*. *J Chem Soc*, 406-409.

Coggon, P., Mcphail, A. T., & Wallwork, S. C. (1970). Structure of hopeaphenol: X-ray analysis of benzene solvate of dibromodeca-o-methylhopeaphenol. *J Chem Soc B*(5), 884-897.

Coll, J. C., & Bowden, B. F. (1986). The application of vacuum liquid-chromatography to the separation of terpene mixtures. *J Nat Prod, 49*(5), 934-936.

Copeland, R. A. (2003). Mechanistic considerations in high-throughput screening. *Anal Biochem, 320*(1), 1-12.

Cortés, A., Cascante, M., Cárdenas, M. L., & Cornish-Bowden, A. (2001). Relationships between inhibition constants, inhibitor concentrations for 50% inhibition and types of inhibition: new ways of analysing data. *Biochem J, 357*(Pt 1), 263-268.

Coursol, S., Fan, L. M., Le Stunff, H., Spiegel, S., Gilroy, S., & Assmann, S. M. (2003). Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. *Nature, 423*(6940), 651-654.

Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., & Spiegel, S. (1996). Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature, 381*(6585), 800-803.

Dai, J. R., Hallock, Y. F., Cardellina, J. H., & Boyd, M. R. (1998). HIV-inhibitory and cytotoxic oligostilbenes from the leaves of *Hopea malibato*. *J Nat Prod*, *61*(3), 351-353.

Davis, M. D., Clemens, J. J., Macdonald, T. L., & Lynch, K. R. (2005). Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem, 280*(11), 9833-9841.

Dayanandan, S., Ashton, P. S., Williams, S. M., & Primack, R. B. (1999). Phylogeny of the tropical tree family Dipterocarpaceae based on nucleotide sequences of the chloroplast RBCL gene. *Am J Bot*, *86*(8), 1182-1190.

Delon, C., Manifava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S., & Ktistakis, N. T. (2004). Sphingosine kinase 1 is an intracellular effector of phosphatidic acid. *J Biol Chem, 279*(43), 44763-44774.

Dickson, M. A., Carvajal, R. D., Merrill, A. H., Gonen, M., Cane, L. M., & Schwartz, G. K. (2011). A phase I clinical trial of safingol in combination with cisplatin in advanced solid tumors. *Clin Cancer Res*, *17*(8):2484-92.

DiMasi, J. A., & Faden, L. B. (2011). Competitiveness in follow-on drug R&D: a race or imitation? *Nat Rev Drug Discov, 10*(1), 23-27.

Ding, G., Sonoda, H., Yu, H., Kajimoto, T., Goparaju, S. K., Jahangeer, S., Okada, T., & Nakamura, S. (2007). Protein kinase D-mediated phosphorylation and nuclear export of sphingosine kinase 2. *J Biol Chem, 282*(37), 27493-27502.

Dobson, C. M. (2004). Chemical space and biology. *Nature, 432*(7019), 824-828.

Döll, F., Pfeilschifter, J., & Huwiler, A. (2007). Prolactin upregulates sphingosine kinase-1 expression and activity in the human breast cancer cell line MCF7 and triggers enhanced proliferation and migration. *Endocr Relat Cancer, 14*(2), 325-335.

Don, A. S., & Rosen, H. (2009). A lipid binding domain in sphingosine kinase 2. *Biochem Biophys Res Commun, 380*(1), 87-92.

Dörrie, J., Gerauer, H., Wachter, Y., & Zunino, S. J. (2001). Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. *Cancer Res, 61*(12), 4731-4739.

Drews, J. (2000). Drug discovery: a historical perspective. *Science, 287*(5460), 1960-1964.

Edsall, L. C., Van Brocklyn, J. R., Cuvillier, O., Kleuser, B., & Spiegel, S. (1998). N,N-Dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide. *Biochemistry, 37*(37), 12892-12898.

Fischbach, M., & Clardy, J. (2007). One pathway, many products. *Nat Chem Biol, 3*(7), 353-355.

Fremont, L. (2000). Biological effects of resveratrol. *Life Sci*, *66*(8), 663-673.

French, K. J., Schrecengost, R. S., Lee, B. D., Zhuang, Y., Smith, S. N., Eberly, J. L., Yun, J. K., & Smith, C. D. (2003). Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res, 63*(18), 5962-5969.

French, K. J., Upson, J. J., Keller, S. N., Zhuang, Y., Yun, J. K., & Smith, C. D. (2006). Antitumor activity of sphingosine kinase inhibitors. *J Pharmacol Exp Ther, 318*(2), 596-603.

French, K. J., Zhuang, Y., Maines, L. W., Gao, P., Wang, W., Beljanski, V., Upson, J. J., Green, C. L., Keller, S. N., & Smith, C. D. (2010). Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. *J Pharmacol Exp Ther, 333*(1), 129-139.

Fukuda, Y., Aoyama, Y., Wada, A., & Igarashi, Y. (2004). Identification of PECAM-1 association with sphingosine kinase 1 and its regulation by agonist-induced phosphorylation. *Biochim Biophys Acta, 1636*(1), 12-21.

Gamble, J. R., Xia, P., Hahn, C. N., Drew, J. J., Drogemuller, C. J., Brown, D., & Vadas, M. A. (2006). Phenoxodiol, an experimental anticancer drug, shows potent antiangiogenic properties in addition to its antitumour effects. *Int J Cancer, 118*(10), 2412-2420.

Garcia M., Jemal A., Ward E. M., Center M. M., Hao Y., Siegel R. L., Thun M. J. (2007). *Global Cancer Facts & Figures 2007*. Atlanta, GA: American Cancer Society.

Garcia-Calvo, M., Peterson, E. P., Leiting, B., Ruel, R., Nicholson, D. W., & Thornberry, N. A. (1998). Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem, 273*(49), 32608-32613.

Gillies, L., Lee, S. C., Long, J. S., Ktistakis, N., Pyne, N. J., & Pyne, S. (2009). The sphingosine 1-phosphate receptor 5 and sphingosine kinases 1 and 2 are localised in centrosomes: possible role in regulating cell division. *Cell Signal, 21*(5), 675-684.

Goetzl, E. J., & Rosen, H. (2004). Regulation of immunity by lysosphingolipids and their G protein-coupled receptors. *J Clin Invest, 114*(11), 1531-1537.

Gonda, K., Okamoto, H., Takuwa, N., Yatomi, Y., Okazaki, H., Sakurai, T., Kimura, S., Sillard, R., Harii, K., & Takuwa, Y. (1999). The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem J, 337* (Pt 1), 67-75.

Goparaju, S. K., Jolly, P. S., Watterson, K. R., Bektas, M., Alvarez, S., Sarkar, S., Mel, L., Ishii, I., Chun, J., Milstien, S., & Spiegel, S. (2005). The $S1P_2$ receptor negatively regulates platelet-derived growth factor-induced motility and proliferation. *Mol Cell Biol, 25*(10), 4237-4249.

Gräler, M. H., & Goetzl, E. (2004). The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G protein-coupled receptors. *FASEB J*, *18*(3):551-553.

Gräler, M. H., Bernhardt, G., & Lipp, M. (1998). EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics, 53*(2), 164-169.

Gräler, M. H., Grosse, R., Kusch, A., Kremmer, E., Gudermann, T., & Lipp, M. (2003). The sphingosine 1-phosphate receptor $S1P_4$ regulates cell shape and motility via coupling to G_i and $G_{12/13}$. *J Cell Biochem, 89*(3), 507-519.

Gude, D. R., Alvarez, S. E., Paugh, S. W., Mitra, P., Yu, J., Griffiths, R., Barbour, S. E., Milstien, S., & Spiegel, S. (2008). Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB J*, *22*(8), 2629-2638.

Gunasekera, S. P., Kinghorn, A. D., Cordell, G. A., & Farnsworth, N. R. (1981). Plant anticancer agents. XIX constituents of *Aquilaria malaccensis*. *J Nat Prod, 44*(5), 569-572.

Hait, N. C., Allegood, J., Maceyka, M., Strub, G. M., Harikumar, K. B., Singh, S. K., Luo, C., Marmorstein, R., Kordula, T., Milstien, S., & Spiegel, S. (2009). Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science, 325*(5945), 1254-1257.

Hait, N. C., Bellamy, A., Milstien, S., Kordula, T., & Spiegel, S. (2007). Sphingosine kinase type 2 activation by ERK-mediated phosphorylation. *J Biol Chem, 282*(16), 12058-12065.

Hait, N. C., Sarkar, S., Le Stunff, H., Mikami, A., Maceyka, M., Milstien, S., & Spiegel, S. (2005). Role of sphingosine kinase 2 in cell migration toward epidermal growth factor. *J Biol Chem, 280*(33), 29462-29469.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hannun, Y. A., & Bell, R. M. (1989). Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science, 243*(4890), 500-507.

Hartwell, L. H., & Kastan, M. B. (1994). Cell cycle control and cancer. *Science, 266*(5192), 1821-1828.

Harvey, A. L. (2008). Natural products in drug discovery. *Drug Discov Today, 13*(19-20), 894-901.

Hengst, J. A., Guilford, J. M., Conroy, E. J., Wang, X., & Yun, J. K. (2010a). Enhancement of sphingosine kinase 1 catalytic activity by deletion of 21 amino acids from the COOH-terminus. *Arch Biochem Biophys, 494*(1), 23-31.

Hengst, J. A., Guilford, J. M., Fox, T. E., Wang, X., Conroy, E. J., & Yun, J. K. (2009). Sphingosine kinase 1 localized to the plasma membrane lipid raft microdomain overcomes serum deprivation induced growth inhibition. *Arch Biochem Biophys, 492*(1-2), 62-73.

Hengst, J. A., Wang, X., Sk, U. H., Sharma, A. K., Amin, S., & Yun, J. K. (2010b). Development of a sphingosine kinase 1 specific small-molecule inhibitor. *Bioorg Med Chem Lett, 20*(24), 7498-7502.

Hla, T., & Maciag, T. (1990). An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem, 265*(16), 9308-9313.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. L., Scherer, B., & Sinclair, D. A. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature*, *425*(6954), 191-196.

Howitz, K. T., & Sinclair, D. A. (2008). Xenohormesis: sensing the chemical cues of other species. *Cell*, *133*(3), 387-391.

Huwiler, A., Doll, F., Ren, S., Klawitter, S., Greening, A., Romer, I., Bubnova, S., Reinsberg, L., & Pfeilschifter, J. (2006). Histamine increases sphingosine kinase-1 expression and activity in the human arterial endothelial cell line EA.hy 926 by a PKC-α-dependent mechanism. *Biochim Biophys Acta, 1761*(3), 367-376.

Igarashi, N., Okada, T., Hayashi, S., Fujita, T., Jahangeer, S., & Nakamura, S. (2003). Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. *J Biol Chem, 278*(47), 46832-46839.

Iliya, I., Akao, Y., Matsumoto, K., Nakagawa, Y., Zulfiqar, A., Ito, T., Oyama, M., Murata, H., Tanaka, T., Nozawa, Y., & Iinuma, M. (2006). Growth inhibition of stilbenoids in Welwitschiaceae and Gnetaceae through induction of apoptosis in human leukemia HL60 cells. *Biol Pharm Bull, 29*(7), 1490-1492.

Im, D. S., Heise, C. E., Ancellin, N., O'Dowd, B. F., Shei, G. J., Heavens, R. P., Rigby, M. R., Hla, T., Mandala, S., McAllister, G., George, S. R., & Lynch, K. R. (2000). Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem, 275*(19), 14281-14286.

Ishii, I., Friedman, B., Ye, X., Kawamura, S., McGiffert, C., Contos, J. J., Kingsbury, M. A., Zhang, G., Brown, J. H., & Chun, J. (2001). Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP_{B3}/EDG-3. *J Biol Chem, 276*(36), 33697-33704.

Ishii, I., Ye, X., Friedman, B., Kawamura, S., Contos, J. J., Kingsbury, M. A., Yang, A. H., Zhang, G., Brown, J. H., & Chun, J. (2002). Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, $S1P_2$/LP_{B2}/EDG-5 and $S1P_3$/LP_{B3}/EDG-3. *J Biol Chem, 277*(28), 25152-25159.

Issuree, P. D., Pushparaj, P. N., Pervaiz, S., & Melendez, A. J. (2009). Resveratrol attenuates C5a-induced inflammatory responses *in vitro* and *in vivo* by inhibiting phospholipase D and sphingosine kinase activities. *FASEB J*, *23*(8), 2412-2424.

Ito, A., Cui, B., Chávez, D., Chai, H. B., Shin, Y. G., Kawanishi, K., Kardono, L. B., Riswan, S., Farnsworth, N. R., Cordell, G. A., Pezzuto, J. M., & Kinghorn, A. D. (2001a). Cytotoxic polyacetylenes from the twigs of *Ochanostachys amentacea*. *J Nat Prod, 64*(2), 246-248.

Ito, T., Akao, Y., Yi, H., Ohguchi, K., Matsumoto, K., Tanaka, T., Iinuma, M., & Nozawa, Y. (2003). Antitumor effect of resveratrol oligomers against human cancer cell lines and the molecular mechanism of apoptosis induced by Vaticanol C. *Carcinogenesis, 24*(9), 1489-1497.

Ito, T., Tanaka, T., Nakaya, K., Iinuma, M., Takahashi, Y., Naganawa, H., Ohyama, M., Nakanishi, Y., Bastow, K. F., & Lee, K. H. (2001b). A novel bridged stilbenoid trimer and four highly condensed stilbenoid oligomers in *Vatica rassak*. *Tetrahedron*, *57*(34), 7309-7321.

Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., & Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, *275*(5297), 218-220.

Jarman, K. E., Moretti, P. A., Zebol, J. R., & Pitson, S. M. (2010). Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium- and integrin-binding protein 1. *J Biol Chem, 285*(1), 483-492.

Jo, E., Sanna, M. G., Gonzalez-Cabrera, P. J., Thangada, S., Tigyi, G., Osborne, D. A., Hla, T., Parrill, A. L., & Rosen, H. (2005). $S1P_1$-selective *in vivo*-active agonists from high-throughput screening: off-the-shelf chemical probes of receptor interactions, signaling, and fate. *Chem Biol, 12*(6), 703-715.

Johnson, K. R., Becker, K. P., Facchinetti, M. M., Hannun, Y. A., & Obeid, L. M. (2002). PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA). *J Biol Chem, 277*(38), 35257-35262.

Johnson, K. R., Johnson, K. Y., Crellin, H. G., Ogretmen, B., Boylan, A. M., Harley, R. A., & Obeid, L. M. (2005). Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. *J Histochem Cytochem, 53*(9), 1159-1166.

Jones, D. (2008). Pathways to cancer therapy. *Nat Rev Drug Discov, 7*(11), 875-876.

Kennedy, A. J., Mathews, T. P., Kharel, Y., Field, S. D., Moyer, M. L., East, J. E., Houck, J. D., Lynch, K. R., & Macdonald, T. L. (2011). Development of amidine-based sphingosine kinase 1 nanomolar inhibitors and reduction of sphingosine 1-phosphate in human leukemia cells. *J Med Chem*, *54*(10), 3524-3548.

Kihara, A., Anada, Y., & Igarashi, Y. (2006). Mouse sphingosine kinase isoforms SPHK1a and SPHK1b differ in enzymatic traits including stability, localization, modification, and oligomerization. *J Biol Chem, 281*(7), 4532-4539.

Kim, J. W., Kim, Y. W., Inagaki, Y., Hwang, Y. A., Mitsutake, S., Ryu, Y. W., Lee, W. K., Ha, H. J., Park, C. S., & Igarashi, Y. (2005). Synthesis and evaluation of sphingoid analogs as inhibitors of sphingosine kinases. *Bioorg Med Chem, 13*(10), 3475-3485.

Kinghorn, A. D., Farnsworth, N. R., Soejarto, D. D., Cordell, G. A., Pezzuto, J. M., Udeani, G. O., Wani, M. C., Wall, M. E., Navarro, H. A., Kramer, R. A., Menendez, A. T., Fairchild, C. R., Lane, K. E., Forenza, S., Vyas, D. M., Lam, K. S., & Shu, Y. Z. (1999). Novel strategies for the discovery of plant-derived anticancer agents. *Pure Appl Chem, 71*(9), 1611-1618.

Kinghorn, A. D., Su, B. N., Jang, D. S., Chang, L. C., Lee, D., Gu, J. Q., Carcache-Blanco, E. J., Pawlus, A. D., Lee, S. K., Park, E. J., Cuendet, M., Gills, J. J., Bhat, K., Park, H. S., Mata-Greenwood, E., Song, L. L., Jang, M., & Pezzuto, J. M. (2004). Natural inhibitors of carcinogenesis. *Planta Med, 70*(8), 691-705.

Kiuchi, M., Adachi, K., Tomatsu, A., Chino, M., Takeda, S., Tanaka, Y., Maeda, Y., Sato, N., Mitsutomi, N., Sugahara, K., & Chiba, K. (2005). Asymmetric synthesis and biological evaluation of the enantiomeric isomers of the immunosuppressive FTY720-phosphate. *Bioorganic & Medicinal Chemistry*, *13*(2), 425-432.

Kluk, M. J., & Hla, T. (2002). Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim Biophys Acta, 1582*(1-3), 72-80.

Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., & Spiegel, S. (1998). Molecular cloning and functional characterization of murine sphingosine kinase. *J Biol Chem, 273*(37), 23722-23728.

Kohno, T., Matsuyuki, H., Inagaki, Y., & Igarashi, Y. (2003). Sphingosine 1-phosphate promotes cell migration through the activation of Cdc42 in Edg-6/$S1P_4$-expressing cells. *Genes Cells, 8*(8), 685-697.

Koide, Y., Hasegawa, T., Takahashi, A., Endo, A., Mochizuki, N., Nakagawa, M., & Nishida, A. (2002). Development of novel EDG3 antagonists using a 3D database search and their structure-activity relationships. *J Med Chem, 45*(21), 4629-4638.

Kono, K., Sugiura, M., & Kohama, T. (2002). Inhibition of recombinant sphingosine kinases by novel inhibitors of microbial origin, F-12509a and B-5354c. *J Antibiot (Tokyo), 55*(1), 99-103.

Kono, K., Tanaka, M., Mizuno, T., Kodama, K., Ogita, T., & Kohama, T. (2000a). B-535a, b and c, new sphingosine kinase inhibitors, produced by a marine bacterium; taxonomy, fermentation, isolation, physico-chemical properties and structure determination. *J Antibiot (Tokyo), 53*(8), 753-758.

Kono, K., Tanaka, M., Ogita, T., Hosoya, T., & Kohama, T. (2000b). F-12509a, a new sphingosine kinase inhibitor, produced by a discomycete. *J Antibiot (Tokyo), 53*(5), 459-466.

Kono, K., Tanaka, M., Ogita, T., & Kohama, T. (2000c). Characterization of B-5354c, a new sphingosine kinase inhibitor, produced by a marine bacterium. *J Antibiot (Tokyo), 53*(8), 759-764.

Kono, K., Tanaka, M., Ono, Y., Hosoya, T., Ogita, T., & Kohama, T. (2001). S-15183a and b, new sphingosine kinase inhibitors, produced by a fungus. *J Antibiot (Tokyo), 54*(5), 415-420.

Kono, M., Belyantseva, I. A., Skoura, A., Frolenkov, G. I., Starost, M. F., Dreier, J. L., Lidington, D., Bolz, S. S., Friedman, T. B., Hla, T., & Proia, R. L. (2007). Deafness and stria vascularis defects in $S1P_2$ receptor-null mice. *J Biol Chem, 282*(14), 10690-10696.

Kono, M., Mi, Y., Liu, Y., Sasaki, T., Allende, M. L., Wu, Y. P., Yamashita, T., & Proia, R. L. (2004). The sphingosine-1-phosphate receptors $S1P_1$, $S1P_2$, and $S1P_3$ function coordinately during embryonic angiogenesis. *J Biol Chem, 279*(28), 29367-29373.

Kraus, G. A., & Kim, I. (2003). Synthetic approach to malibatol A. *Org Lett, 5*(8), 1191-1192.

Kubbutat, M. H., Jones, S. N., & Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. *Nature, 387*(6630), 299-303.

Kushiro, T., Nambara, E., & McCourt, P. (2003). Hormone evolution: The key to signalling. *Nature*, *422*(6928), 122-122.

Labesse, G., Douguet, D., Assairi, L., & Gilles, A. M. (2002). Diacylglyceride kinases, sphingosine kinases and NAD kinases: distant relatives of 6-phosphofructokinases. *Trends Biochem Sci, 27*(6), 273-275.

Lacaná, E., Maceyka, M., Milstien, S., & Spiegel, S. (2002). Cloning and characterization of a protein kinase A anchoring protein (AKAP)-related protein that interacts with and regulates sphingosine kinase 1 activity. *J Biol Chem, 277*(36), 32947-32953.

LaMontagne, K., Littlewood-Evans, A., Schnell, C., O'Reilly, T., Wyder, L., Sanchez, T., Probst, B., Butler, J., Wood, A., Liau, G., Billy, E., Theuer, A., Hla, T., & Wood, J. (2006). Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization. *Cancer Res, 66*(1), 221-231.

Langcake, P., & Pryce, R. J. (1977a). New class of phytoalexins from grapevines. *Experientia*, *33*(2), 151-152.

Langcake, P., & Pryce, R. J. (1977b). Production of resveratrol and viniferins by grapevines in response to UV irradiation. *Phytochemistry*, *16*(8) 1193-1196.

Lavieu, G., Scarlatti, F., Sala, G., Carpentier, S., Levade, T., Ghidoni, R., Botti, J., & Codogno, P. (2006). Regulation of autophagy by sphingosine kinase 1 and its role in cell survival during nutrient starvation. *J Biol Chem, 281*(13), 8518-8527.

Le Stunff, H., Mikami, A., Giussani, P., Hobson, J. P., Jolly, P. S., Milstien, S., & Spiegel, S. (2004). Role of sphingosine-1-phosphate phosphatase 1 in epidermal growth factor-induced chemotaxis. *J Biol Chem, 279*(33), 34290-34297.

Lebman, D. A., & Spiegel, S. (2008). Cross-talk at the crossroads of sphingosine-1-phosphate, growth factors, and cytokine signaling. *J Lipid Res, 49*(7), 1388-1394.

Lee, M. J., Evans, M., & Hla, T. (1996). The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. *J Biol Chem, 271*(19), 11272-11279.

Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., & Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell, 99*(3), 301-312.

Lee, M. J., Thangada, S., Paik, J. H., Sapkota, G. P., Ancellin, N., Chae, S. S., Wu, M., Morales-Ruiz, M., Sessa, W. C., Alessi, D. R., & Hla, T. (2001). Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol Cell, 8*(3), 693-704.

Lee, M. J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., & Hla, T. (1998). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science, 279*(5356), 1552-1555.

Lee, W. J., Yoo, H. S., Suh, P. G., Oh, S., Lim, J. S., & Lee, Y. M. (2004). Sphingosine mediates FTY720-induced apoptosis in LLC-PK1 cells. *Exp Mol Med, 36*(5), 420-427.

Leonard, S. S., Xia, C., Jiang, B. H., Stinefelt, B., Klandorf, H., Harris, G. K., & Shi, X. (2003). Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem Biophys Res Commun*, *309*(4), 1017-1026.

Lim, K. G., Sun, C., Bittman, R., Pyne, N. J., & Pyne, S. (2011a). (*R*)-FTY720 methyl ether is a specific sphingosine kinase 2 inhibitor: effect on sphingosine kinase 2 expression in HEK 293 cells and actin rearrangement and survival of MCF-7 breast cancer cells. *Cell Signal, 23*(10), 1590-1595.

Lim, K. G., Tonelli, F., Li, Z., Lu, X., Bittman, R., Pyne, S., & Pyne, N. J. (2011b). FTY720 analogues as sphingosine kinase 1 inhibitors: enzyme inhibition kinetics, allosterism, proteasomal degradation and actin rearrangement in MCF-7 breast cancer cells. *J Biol Chem*, *286*(21), 18633-18640.

Lim, S. H., Mahmood, K., Komiyama, K., & Kam, T. S. (2008). A cycloartane incorporating a fused tetrahydrofuran ring and a cytotoxic lactam from *Monocarpia marginalis*. *J Nat Prod, 71*(6), 1104-1106.

Lindsay, M. A. (2003). Target discovery. *Nat Rev Drug Discov, 2*(10), 831-838.

Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., & Spiegel, S. (2000a). Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem, 275*(26), 19513-19520.

Liu, H., Toman, R. E., Goparaju, S. K., Maceyka, M., Nava, V. E., Sankala, H., Payne, S. G., Bektas, M., Ishii, I., Chun, J., Milstien, S., & Spiegel, S. (2003). Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J Biol Chem, 278*(41), 40330-40336.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., Liu, C. H., Hla, T., Spiegel, S., & Proia, R. L. (2000b). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest, 106*(8), 951-961.

Long, J. S., Edwards, J., Watson, C., Tovey, S., Mair, K. M., Schiff, R., Natarajan, V., Pyne, N. J., & Pyne, S. (2010a). Sphingosine kinase 1 induces tolerance to human epidermal growth factor receptor 2 and prevents formation of a migratory phenotype in response to sphingosine 1-phosphate in estrogen receptor-positive breast cancer cells. *Mol Cell Biol, 30*(15), 3827-3841.

Long, J. S., Fujiwara, Y., Edwards, J., Tannahill, C. L., Tigyi, G., Pyne, S., & Pyne, N. J. (2010b). Sphingosine 1-phosphate receptor 4 uses HER2 (ERBB2) to regulate extracellular signal regulated kinase-1/2 in MDA-MB-453 breast cancer cells. *J Biol Chem, 285*(46), 35957-35966.

Loveridge, C., Tonelli, F., Leclercq, T., Lim, K. G., Long, J. S., Berdyshev, E., Tate, R. J., Natarajan, V., Pitson, S. M., Pyne, N. J., & Pyne, S. (2010). The sphingosine kinase 1 inhibitor 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole induces proteasomal degradation of sphingosine kinase 1 in mammalian cells. *J Biol Chem, 285*(50), 38841-38852.

Lu, R., & Serrero, G. (1999). Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *J Cell Physiol, 179*(3), 297-304.

Lu, X., Sun, C., Valentine, W. J., Shuyu, E., Liu, J., Tigyi, G., & Bittman, R. (2009). Chiral vinylphosphonate and phosphonate analogues of the immunosuppressive agent FTY720. *J Org Chem, 74*(8), 3192-3195.

Maceyka, M., Nava, V. E., Milstien, S., & Spiegel, S. (2004). Aminoacylase 1 is a sphingosine kinase 1-interacting protein. *FEBS Lett, 568*(1-3), 30-34.

Maceyka, M., Sankala, H., Hait, N. C., Le Stunff, H., Liu, H., Toman, R., Collier, C., Zhang, M., Satin, L. S., Merrill, A. H., Jr., Milstien, S., & Spiegel, S. (2005). SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. *J Biol Chem, 280*(44), 37118-37129.

MacLennan, A. J., Carney, P. R., Zhu, W. J., Chaves, A. H., Garcia, J., Grimes, J. R., Anderson, K. J., Roper, S. N., & Lee, N. (2001). An essential role for the H218/AGR16/EDG-5/LP_{B2} sphingosine 1-phosphate receptor in neuronal excitability. *Eur J Neurosci, 14*(2), 203-209.

Malek, R. L., Toman, R. E., Edsall, L. C., Wong, S., Chiu, J., Letterle, C. A., Van Brocklyn, J. R., Milstien, S., Spiegel, S., & Lee, N. H. (2001). Nrg-1 belongs to the endothelial differentiation gene family of G protein-coupled sphingosine-1-phosphate receptors. *J Biol Chem, 276*(8), 5692-5699.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., & Rosen, H. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science*, *296*(5566), 346-349.

Mathews, T. P., Kennedy, A. J., Kharel, Y., Kennedy, P. C., Nicoara, O., Sunkara, M., Morris, A. J., Wamhoff, B. R., Lynch, K. R., & Macdonald, T. L. (2010). Discovery, biological evaluation, and structure-activity relationship of amidine based sphingosine kinase inhibitors. *J Med Chem, 53*(7), 2766-2778.

Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L., & Cyster, J. G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature, 427*(6972), 355-360.

Melendez, A., Floto, R. A., Gillooly, D. J., Harnett, M. M., & Allen, J. M. (1998). FcγRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J Biol Chem, 273*(16), 9393-9402.

Melendez, A. J., Carlos-Dias, E., Gosink, M., Allen, J. M., & Takacs, L. (2000). Human sphingosine kinase: molecular cloning, functional characterization and tissue distribution. *Gene, 251*(1), 19-26.

Mertens-Talcott, S. U., & Percival, S. S. (2005). Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells. *Cancer Lett, 218*(2), 141-151.

Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K. T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K. H., & van Koppen, C. J. (1998). Sphingosine kinase-mediated Ca^{2+} signalling by G-protein-coupled receptors. *EMBO J, 17*(10), 2830-2837.

Meyer zu Heringdorf, D., Lass, H., Kuchar, I., Alemany, R., Guo, Y., Schmidt, M., & Jakobs, K. H. (1999). Role of sphingosine kinase in Ca^{2+} signalling by epidermal growth factor receptor. *FEBS Lett, 461*(3), 217-222.

Miller, D. J., Jerga, A., Rock, C. O., & White, S. W. (2008). Analysis of the *Staphylococcus aureus* DgkB structure reveals a common catalytic mechanism for the soluble diacylglycerol kinases. *Structure, 16*(7), 1036-1046.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G. F., Spiegel, S., & Proia, R. L. (2005). Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol, 25*(24), 11113-11121.

Molina, M. A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., & Baselga, J. (2001). Trastuzumab (Herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res, 61*(12), 4744-4749.

Muhtadi, Hakim, E. H., Juliawaty, L. D., Syah, Y. M., Achmad, S. A., Latip, J., & Ghisalberti, E. L. (2006). Cytotoxic resveratrol oligomers from the tree bark of *Dipterocarpus hasseltii*. *Fitoterapia, 77*(7-8), 550-555.

Nagaoka, Y., Otsuki, K., Fujita, T., & Uesato, S. (2008). Effects of phosphorylation of immunomodulatory agent FTY720 (fingolimod) on antiproliferative activity against breast and colon cancer cells. *Biol Pharm Bull, 31*(6), 1177-1181.

Nakagawa, H., Kiyozuka, Y., Uemura, Y., Senzaki, H., Shikata, N., Hioki, K., & Tsubura, A. (2001). Resveratrol inhibits human breast cancer cell growth and may mitigate the effect of linoleic acid, a potent breast cancer cell stimulator. *J Cancer Res Clin Oncol, 127*(4), 258-264.

Nava, V. E., Cuvillier, O., Edsall, L. C., Kimura, K., Milstien, S., Gelmann, E. P., & Spiegel, S. (2000a). Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer Res, 60*(16), 4468-4474.

Nava, V. E., Hobson, J. P., Murthy, S., Milstien, S., & Spiegel, S. (2002). Sphingosine kinase type 1 promotes estrogen-dependent tumorigenesis of breast cancer MCF-7 cells. *Exp Cell Res, 281*(1), 115-127.

Nava, V. E., Lacana, E., Poulton, S., Liu, H., Sugiura, M., Kono, K., Milstien, S., Kohama, T., & Spiegel, S. (2000b). Functional characterization of human sphingosine kinase-1. *FEBS Lett, 473*(1), 81-84.

Newman, D. J. (2008). Natural products as leads to potential drugs: an old process or the new hope for drug discovery? *J Med Chem, 51*(9), 2589-2599.

Ng, C. K., Carr, K., McAinsh, M. R., Powell, B., & Hetherington, A. M. (2001). Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. *Nature, 410*(6828), 596-599.

Nichols, C. E., Lamb, H. K., Lockyer, M., Charles, I. G., Pyne, S., Hawkins, A. R., & Stammers, D. K. (2007). Characterization of *Salmonella typhimurium* YegS, a putative lipid kinase homologous to eukaryotic sphingosine and diacylglycerol kinases. *Proteins, 68*(1), 13-25.

Niedernberg, A., Blaukat, A., Schöneberg, T., & Kostenis, E. (2003). Regulated and constitutive activation of specific signalling pathways by the human $S1P_5$ receptor. *Br J Pharmacol, 138*(3), 481-493.

Niedernberg, A., Scherer, C. R., Busch, A. E., & Kostenis, E. (2002). Comparative analysis of human and rat $S1P_5$ (edg8): Differential expression profiles and sensitivities to antagonists. *Biochem Pharmacol, 64*(8), 1243-1250.

O'Brien, N., Jones, S. T., Williams, D. G., Cunningham, H. B., Moreno, K., Visentin, B., Gentile, A., Vekich, J., Shestowsky, W., Hiraiwa, M., Matteo, R., Cavalli, A., Grotjahn, D., Grant, M., Hansen, G., Campbell, M. A., & Sabbadini, R. (2009). Production and characterization of monoclonal anti-sphingosine-1-phosphate antibodies. *J Lipid Res, 50*(11), 2245-2257.

Ohyama, M., Tanaka, T., Ito, T., Iinuma, M., Bastow, K. F., & Lee, K. H. (1999). Antitumor agents 200. Cytotoxicity of naturally occurring resveratrol oligomers and their acetate derivatives. *Bioorg Med Chem Lett, 9*(20), 3057-3060.

Okada, T., Ding, G., Sonoda, H., Kajimoto, T., Haga, Y., Khosrowbeygi, A., Gao, S., Miwa, N., Jahangeer, S., & Nakamura, S. (2005). Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. *J Biol Chem, 280*(43), 36318-36325.

Okamoto, H., Takuwa, N., Yatomi, Y., Gonda, K., Shigematsu, H., & Takuwa, Y. (1999). EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16. *Biochem Biophys Res Commun, 260*(1), 203-208.

Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., & Takuwa, Y. (2000). Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol Cell Biol, 20*(24), 9247-9261.

Oliver, F. J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M. C., de Murcia, G., & Menissier-de Murcia, J. M. (1998). Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis - Lesson from an uncleavable mutant. *J Biol Chem*, *273*(50), 33533-33539.

Olivera, A., Kohama, T., Tu, Z., Milstien, S., & Spiegel, S. (1998). Purification and characterization of rat kidney sphingosine kinase. *J Biol Chem, 273*(20), 12576-12583.

Olivera, A., & Spiegel, S. (1993). Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature, 365*(6446), 557-560.

Olivera, A., Urtz, N., Mizugishi, K., Yamashita, Y., Gilfillan, A. M., Furumoto, Y., Gu, H., Proia, R. L., Baumruker, T., & Rivera, J. (2006). IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses. *J Biol Chem, 281*(5), 2515-2525.

Oo, M. L., Thangada, S., Wu, M. T., Liu, C. H., Macdonald, T. L., Lynch, K. R., Lin, C. Y., & Hla, T. (2007). Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. *J Biol Chem*, *282*(12), 9082-9089.

Osada, M., Yatomi, Y., Ohmori, T., Ikeda, H., & Ozaki, Y. (2002). Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem Biophys Res Commun, 299*(3), 483-487.

Paugh, S. W., Cassidy, M. P., He, H. J., Milstien, S., Sim-Selley, L. J., Spiegel, S., & Selley, D. E. (2006). Sphingosine and its analog, the immunosuppressant 2-amino-2-(2-[4-octylphenyl] ethyl)-1,3-propanediol, interact with the CB1 cannabinoid receptor. *Mol Pharmacol*, *70*(1), 41-50.

Paugh, S. W., Paugh, B. S., Rahmani, M., Kapitonov, D., Almenara, J. A., Kordula, T., Milstien, S., Adams, J. K., Zipkin, R. E., Grant, S., & Spiegel, S. (2008). A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. *Blood, 112*(4), 1382-1391.

Payne, S. G., Oskeritzian, C. A., Griffiths, P., Subramanian, P., Barbour, S. E., Chalfant, C. E., Milstien, S., & Spiegel, S. (2007). The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors. *Blood*, *109*(3), 1077-1085.

Pchejetski, D., Golzio, M., Bonhoure, E., Calvet, C., Doumerc, N., Garcia, V., Mazerolles, C., Rischmann, P., Teissié, J., Malavaud, B., & Cuvillier, O. (2005). Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models. *Cancer Res, 65*(24), 11667-11675.

Phillipson, J. D. (2007). Phytochemistry and pharmacognosy. *Phytochemistry, 68*(22-24), 2960-2972.

Pitson, S. M. (2011). Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem Sci, 36*(2), 97-107.

Pitson, S. M., D'andrea, R. J., Vandeleur, L., Moretti, P. A., Xia, P., Gamble, J. R., Vadas, M. A., & Wattenberg, B. W. (2000a). Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem J, 350 Pt 2*, 429-441.

Pitson, S. M., Moretti, P. A., Zebol, J. R., Lynn, H. E., Xia, P., Vadas, M. A., & Wattenberg, B. W. (2003). Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J, 22*(20), 5491-5500.

Pitson, S. M., Moretti, P. A., Zebol, J. R., Vadas, M. A., D'Andrea, R. J., & Wattenberg, B. W. (2001). A point mutant of human sphingosine kinase 1 with increased catalytic activity. *FEBS Lett, 509*(2), 169-173.

Pitson, S. M., Moretti, P. A., Zebol, J. R., Xia, P., Gamble, J. R., Vadas, M. A., D'Andrea, R. J., & Wattenberg, B. W. (2000b). Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation. A dominant-negative sphingosine kinase. *J Biol Chem, 275*(43), 33945-33950.

Pitson, S. M., Moretti, P. A., Zebol, J. R., Zareie, R., Derian, C. K., Darrow, A. L., Qi, J., D'Andrea, R. J., Bagley, C. J., Vadas, M. A., & Wattenberg, B. W. (2002). The nucleotide-binding site of human sphingosine kinase 1. *J Biol Chem, 277*(51), 49545-49553.

Pitson, S. M., Xia, P., Leclercq, T. M., Moretti, P. A., Zebol, J. R., Lynn, H. E., Wattenberg, B. W., & Vadas, M. A. (2005). Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. *J Exp Med, 201*(1), 49-54.

Pommier, Y., Pourquier, P., Fan, Y., & Strumberg, D. (1998). Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta, 1400*(1-3), 83-105.

Pozo-Guisado, E., Alvarez-Barrientos, A., Mulero-Navarro, S., Santiago-Josefat, B., & Fernandez-Salguero, P. M. (2002). The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. *Biochem Pharmacol, 64*(9), 1375-1386.

Pozo-Guisado, E., Merino, J. M., Mulero-Navarro, S., Lorenzo-Benayas, M. J., Centeno, F., Alvarez-Barrientos, A., Fernandez-Salguero, P. M., & Salguero, P. M. (2005). Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-κB. *Int J Cancer, 115*(1), 74-84.

Pyne, N. J., & Pyne, S. (2010). Sphingosine 1-phosphate and cancer. *Nat Rev Cancer, 10*(7), 489-503.

Pyne, N. J., & Pyne, S. (2011). Receptor tyrosine kinase-G-protein-coupled receptor signalling platforms: out of the shadow? *Trends Pharmacol Sci 32*(8), 443-450.

Pyne, S., Chapman, J., Steele, L., & Pyne, N. J. (1996). Sphingomyelin-derived lipids differentially regulate the extracellular signal-regulated kinase 2 (ERK-2) and c-Jun N-terminal kinase (JNK) signal cascades in airway smooth muscle. *Eur J Biochem, 237*(3), 819-826.

Pyne, S., Lee, S. C., Long, J., & Pyne, N. J. (2009). Role of sphingosine kinases and lipid phosphate phosphatases in regulating spatial sphingosine 1-phosphate signalling in health and disease. *Cell Signal, 21*(1), 14-21.

Pyne, S., & Pyne, N. J. (2000). Sphingosine 1-phosphate signalling in mammalian cells. *Biochem J, 349*(Pt 2), 385-402.

Pyne, S., & Pyne, N. J. (2002). Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. *Biochim Biophys Acta, 1582*(1-3), 121-131.

Raben, D. M., & Wattenberg, B. W. (2009). Signaling at the membrane interface by the DGK/SK enzyme family. *J Lipid Res, 50 Suppl*, S35-39.

Ren, R. (2005). Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat Rev Cancer, 5*(3), 172-183.

Rosen, H., Sanna, M. G., Cahalan, S. M., & Gonzalez-Cabrera, P. J. (2007). Tipping the gatekeeper: S1P regulation of endothelial barrier function. *Trends Immunol, 28*(3), 102-107.

Rowinsky, E. K., Cazenave, L. A., & Donehower, R. C. (1990). Taxol: a novel investigational antimicrotubule agent. *J Natl Cancer Inst, 82*(15), 1247-1259.

Ruckhäberle, E., Rody, A., Engels, K., Gaetje, R., von Minckwitz, G., Schiffmann, S., Grösch, S., Geisslinger, G., Holtrich, U., Karn, T., & Kaufmann, M. (2008). Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. *Breast Cancer Res Treat, 112*(1), 41-52.

Sachs, C. W., Safa, A. R., Harrison, S. D., & Fine, R. L. (1995). Partial inhibition of multidrug resistance by safingol is independent of modulation of P-glycoprotein substrate activities and correlated with inhibition of protein kinase C. *J Biol Chem, 270*(44), 26639-26648.

Sahidin, Hakim, E. H., Juliawaty, L. D., Syah, Y. A., bin Din, L., Ghisalberti, E. L., Latip, J., Said, I. M., & Achmad, S. A. (2005). Cytotoxic properties of oligostilbenoids from the tree barks of *Hopea dryobalanoides*. *Z Naturforsch C*, *60*(9-10), 723-727.

Sakurai, N., Nakagawa-Goto, K., Ito, J., Sakurai, Y., Nakanishi, Y., Bastow, K. F., Cragg, G., & Lee, K. H. (2006). Cytotoxic *Alangium* alkaloids from *Alangium longiflorum*. *Phytochemistry*, *67*(9), 894-897.

Sanna, M. G., Liao, J., Jo, E., Alfonso, C., Ahn, M. Y., Peterson, M. S., Webb, B., Lefebvre, S., Chun, J., Gray, N., & Rosen, H. (2004). Sphingosine 1-phosphate (S1P) receptor subtypes $S1P_1$ and $S1P_3$, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem, 279*(14), 13839-13848.

Sankala, H. M., Hait, N. C., Paugh, S. W., Shida, D., Lépine, S., Elmore, L. W., Dent, P., Milstien, S., & Spiegel, S. (2007). Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. *Cancer Res, 67*(21), 10466-10474.

Sarkar, S., Maceyka, M., Hait, N. C., Paugh, S. W., Sankala, H., Milstien, S., & Spiegel, S. (2005). Sphingosine kinase 1 is required for migration, proliferation and survival of MCF-7 human breast cancer cells. *FEBS Lett, 579*(24), 5313-5317.

Scarlatti, F., Sala, G., Somenzi, G., Signorelli, P., Sacchi, N., & Ghidoni, R. (2003). Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells via *de novo* ceramide signaling. *FASEB J*, *17*(15), 2339-2341.

Schwartz, G. K., Haimovitz-Friedman, A., Dhupar, S. K., Ehleiter, D., Maslak, P., Lai, L., Loganzo, F., Jr., Kelsen, D. P., Fuks, Z., & Albino, A. P. (1995). Potentiation of apoptosis by treatment with the protein kinase c-specific inhibitor safingol in mitomycin c-treated gastric cancer cells. *J Natl Cancer Inst, 87*(18), 1394-1399.

Schwartz, G. K., Ward, D., Saltz, L., Casper, E. S., Spiess, T., Mullen, E., Woodworth, J., Venuti, R., Zervos, P., Storniolo, A. M., & Kelsen, D. P. (1997). A pilot clinical/pharmacological study of the protein kinase C-specific inhibitor safingol alone and in combination with doxorubicin. *Clin Cancer Res, 3*(4), 537-543.

Shapiro, G. I., & Harper, J. W. (1999). Anticancer drug targets: cell cycle and checkpoint control. *J Clin Invest, 104*(12), 1645-1653.

Shimizu, H., Takahashi, M., Kaneko, T., Murakami, T., Hakamata, Y., Kudou, S., Kishi, T., Fukuchi, K., Iwanami, S., Kuriyama, K., Yasue, T., Enosawa, S., Matsumoto, K., Takeyoshi, I., Morishita, Y., & Kobayashi, E. (2005). KRP-203, a novel synthetic immunosuppressant, prolongs graft survival and attenuates chronic rejection in rat skin and heart allografts. *Circulation, 111*(2), 222-229.

Shu, X., Wu, W., Mosteller, R. D., & Broek, D. (2002). Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. *Mol Cell Biol, 22*(22), 7758-7768.

Siddik, Z. H. (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene, 22*(47), 7265-7279.

Smith, C. (2003). Drug target validation: Hitting the target. *Nature, 422*(6929), 341, 343, 345 passim.

Snyder, S. A., Gollner, A., & Chiriac, M. I. (2011). Regioselective reactions for programmable resveratrol oligomer synthesis. *Nature, 474*(7352), 461-466.

Sotheeswaran, S., & Pasupathy, V. (1993). Distribution of resveratrol oligomers in plants. *Phytochemistry*, *32*(5), 1083-1092.

Sotheeswaran, S., Sultanbawa, M. U. S., Surendrakumar, S., & Bladon, P. (1983). Polyphenols from dipterocarp species–copalliferol A and stemonoporol. *Journal of the Chemical Society-Perkin Transactions 1*, 699-702.

Stahelin, R. V., Hwang, J. H., Kim, J. H., Park, Z. Y., Johnson, K. R., Obeid, L. M., & Cho, W. (2005). The mechanism of membrane targeting of human sphingosine kinase 1. *J Biol Chem, 280*(52), 43030-43038.

Strub, G. M., Paillard, M., Liang, J., Gomez, L., Allegood, J. C., Hait, N. C., Maceyka, M., Price, M. M., Chen, Q., Simpson, D. C., Kordula, T., Milstien, S., Lesnefsky, E. J., & Spiegel, S. (2011). Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J, 25*(2), 600-612.

Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., & Dannenberg, A. J. (1998). Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J Biol Chem*, *273*(34), 21875-21882.

Sugimoto, N., Takuwa, N., Okamoto, H., Sakurada, S., & Takuwa, Y. (2003). Inhibitory and stimulatory regulation of Rac and cell motility by the $G_{12/13}$-Rho and G_i pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol Cell Biol, 23*(5), 1534-1545.

Sukocheva, O., Wadham, C., Holmes, A., Albanese, N., Verrier, E., Feng, F., Bernal, A., Derian, C. K., Ullrich, A., Vadas, M. A., & Xia, P. (2006). Estrogen transactivates EGFR via the sphingosine 1-phosphate receptor EDG-3: the role of sphingosine kinase-1. *J Cell Biol, 173*(2), 301-310.

Sukocheva, O., Wang, L., Verrier, E., Vadas, M. A., & Xia, P. (2009). Restoring endocrine response in breast cancer cells by inhibition of the sphingosine kinase-1 signaling pathway. *Endocrinology, 150*(10), 4484-4492.

Sukocheva, O. A., Wang, L., Albanese, N., Pitson, S. M., Vadas, M. A., & Xia, P. (2003). Sphingosine kinase transmits estrogen signaling in human breast cancer cells. *Mol Endocrinol, 17*(10), 2002-2012.

Sun, J., Yan, G., Ren, A., You, B., & Liao, J. K. (2006). FHL2/SLIM3 decreases cardiomyocyte survival by inhibitory interaction with sphingosine kinase-1. *Circ Res, 99*(5), 468-476.

Taha, T. A., Hannun, Y. A., & Obeid, L. M. (2006a). Sphingosine kinase: biochemical and cellular regulation and role in disease. *J Biochem Mol Biol, 39*(2), 113-131.

Taha, T. A., Kitatani, K., El-Alwani, M., Bielawski, J., Hannun, Y. A., & Obeid, L. M. (2006b). Loss of sphingosine kinase-1 activates the intrinsic pathway of programmed cell death: modulation of sphingolipid levels and the induction of apoptosis. *FASEB J, 20*(3), 482-484.

Takabe, K., Kim, R. H., Allegood, J. C., Mitra, P., Ramachandran, S., Nagahashi, M., Harikumar, K. B., Hait, N. C., Milstien, S., & Spiegel, S. (2010). Estradiol induces export of sphingosine 1-phosphate from breast cancer cells via ABCC1 and ABCG2. *J Biol Chem, 285*(14), 10477-10486.

Takabe, K., Paugh, S. W., Milstien, S., & Spiegel, S. (2008). "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. *Pharmacol Rev, 60*(2), 181-195.

Takasugi, N., Sasaki, T., Suzuki, K., Osawa, S., Isshiki, H., Hori, Y., Shimada, N., Higo, T., Yokoshima, S., Fukuyama, T., Lee, V. M., Trojanowski, J. Q., Tomita, T., & Iwatsubo, T. (2011). BACE1 activity is modulated by cell-associated sphingosine-1-phosphate. *J Neurosci, 31*(18), 6850-6857.

Tanaka, T., Ito, T., Ido, Y., Son, T., Nakaya, K., Iinuma, M., Ohyama, M., & Chelladurai, V. (2000). Stilbenoids in the stem bark of *Hopea parviflora*. *Phytochemistry*, *53*(8), 1015-1019.

Tonelli, F., Lim, K. G., Loveridge, C., Long, J., Pitson, S. M., Tigyi, G., Bittman, R., Pyne, S., & Pyne, N. J. (2010). FTY720 and (*S*)-FTY720 vinylphosphonate inhibit sphingosine kinase 1 and promote its proteasomal degradation in human pulmonary artery smooth muscle, breast cancer and androgen-independent prostate cancer cells. *Cell Signal, 22*(10), 1536-1542.

Tsai, C. J., Ma, B., & Nussinov, R. (1999). Folding and binding cascades: Shifts in energy landscapes. *Proc Natl Acad Sci U S A, 96*(18), 9970-9972.

Vadas, M., Xia, P., McCaughan, G., & Gamble, J. (2008). The role of sphingosine kinase 1 in cancer: oncogene or non-oncogene addiction? *Biochim Biophys Acta, 1781*(9), 442-447.

Valentine, W. J., Kiss, G. N., Liu, J., E, S., Gotoh, M., Murakami-Murofushi, K., Pham, T. C., Baker, D. L., Parrill, A. L., Lu, X., Sun, C., Bittman, R., Pyne, N. J., & Tigyi, G. (2010). (*S*)-FTY720-vinylphosphonate, an analogue of the immunosuppressive agent FTY720, is a pan-antagonist of sphingosine 1-phosphate GPCR signaling and inhibits autotaxin activity. *Cell Signal, 22*(10), 1543-1553.

Van Brocklyn, J. R., Gräler, M. H., Bernhardt, G., Hobson, J. P., Lipp, M., & Spiegel, S. (2000). Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. *Blood, 95*(8), 2624-2629.

Van Brocklyn, J. R., Jackson, C. A., Pearl, D. K., Kotur, M. S., Snyder, P. J., & Prior, T. W. (2005). Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. *J Neuropathol Exp Neurol, 64*(8), 695-705.

Van Brocklyn, J. R., Tu, Z., Edsall, L. C., Schmidt, R. R., & Spiegel, S. (1999). Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J Biol Chem, 274*(8), 4626-4632.

Venkataraman, K., Thangada, S., Michaud, J., Oo, M. L., Ai, Y., Lee, Y. M., Wu, M., Parikh, N. S., Khan, F., Proia, R. L., & Hla, T. (2006). Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. *Biochem J, 397*(3), 461-471.

Vessey, D. A., Kelley, M., Zhang, J., Li, L., Tao, R., & Karliner, J. S. (2007). Dimethylsphingosine and FTY720 inhibit the SK1 form but activate the SK2 form of sphingosine kinase from rat heart. *J Biochem Mol Toxicol, 21*(5), 273-279.

Visentin, B., Vekich, J. A., Sibbald, B. J., Cavalli, A. L., Moreno, K. M., Matteo, R. G., Garland, W. A., Lu, Y., Yu, S., Hall, H. S., Kundra, V., Mills, G. B., & Sabbadini, R. A. (2006). Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages. *Cancer Cell, 9*(3), 225-238.

Walker, I., & Newell, H. (2008). Do molecularly targeted agents in oncology have reduced attrition rates? *Nat Rev Drug Discov*, *8*(1):15-16.

Walzer, T., Chiossone, L., Chaix, J., Calver, A., Carozzo, C., Garrigue-Antar, L., Jacques, Y., Baratin, M., Tomasello, E., & Vivier, E. (2007). Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor. *Nat Immunol, 8*(12), 1337-1344.

Wang, F., Van Brocklyn, J. R., Hobson, J. P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., & Spiegel, S. (1999a). Sphingosine 1-phosphate stimulates cell migration through a G_i-coupled cell surface receptor. Potential involvement in angiogenesis. *J Biol Chem*, *274*(50), 35343-35350.

Wang, F., Van Brocklyn, J. R., Edsall, L., Nava, V. E., & Spiegel, S. (1999b). Sphingosine-1-phosphate inhibits motility of human breast cancer cells independently of cell surface receptors. *Cancer Res, 59*(24), 6185-6191.

Wang, W., Graeler, M. H., & Goetzl, E. J. (2005). Type 4 sphingosine 1-phosphate G protein-coupled receptor ($S1P_4$) transduces S1P effects on T cell proliferation and cytokine secretion without signaling migration. *FASEB J, 19*(12), 1731-1733.

Waters, C. M., Long, J., Gorshkova, I., Fujiwara, Y., Connell, M., Belmonte, K. E., Tigyi, G., Natarajan, V., Pyne, S., & Pyne, N. J. (2006). Cell migration activated by platelet-derived growth factor receptor is blocked by an inverse agonist of the sphingosine 1-phosphate receptor-1. *FASEB J, 20*(3), 509-511.

Watson, C. M., Long, J. S., Orange, C., Tannahill, C. L., Mallon, E., McGlynn, L. M., Pyne, S., Pyne, N. J., & Edwards, J. (2010). High expression of sphingosine 1-phosphate receptors, $S1P_1$ and $S1P_3$, sphingosine kinase 1, and extracellular signal-regulated kinase-1/2 is associated with development of tamoxifen resistance in estrogen receptor-positive breast cancer patients. *Am J Pathol, 177*(5), 2205-2215.

Wattenberg, B. W., Pitson, S. M., & Raben, D. M. (2006). The sphingosine and diacylglycerol kinase superfamily of signaling kinases: localization as a key to signaling function. *J Lipid Res, 47*(6), 1128-1139.

Weber, J. F. F., Wahab, I. A., Marzuki, A., Thomas, N. F., Kadir, A. A., Hadi, A. H. A., Awang, K., Latiff, A. A., Richomme, P., & Delaunay, J. (2001). Heimiol a, a new dimeric stilbenoid from *Neobalanocarpus heimii*. *Tetrahedron Lett*, *42*(29), 4895-4897.

Wei, S. H., Rosen, H., Matheu, M. P., Sanna, M. G., Wang, S. K., Jo, E., Wong, C. H., Parker, I., & Cahalan, M. D. (2005). Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nat Immunol, 6*(12), 1228-1235.

Weinstein, I. B., & Joe, A. K. (2006). Mechanisms of disease: oncogene addiction—a rationale for molecular targeting in cancer therapy. *Nat Clin Pract Oncol, 3*(8), 448-457.

Windh, R. T., Lee, M. J., Hla, T., An, S., Barr, A. J., & Manning, D. R. (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G_i, G_q, and G_{12} families of heterotrimeric G proteins. *J Biol Chem, 274*(39), 27351-27358.

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. *Biochem J, 322 (Pt 3)*, 681-692.

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M., & Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature 430*(7000), 686-689.

Worrall, D., Liang, Y. K., Alvarez, S., Holroyd, G. H., Spiegel, S., Panagopulos, M., Gray, J. E., & Hetherington, A. M. (2008). Involvement of sphingosine kinase in plant cell signalling. *Plant J, 56*(1), 64-72.

Xia, P., Gamble, J. R., Wang, L., Pitson, S. M., Moretti, P. A., Wattenberg, B. W., D'Andrea, R. J., & Vadas, M. A. (2000). An oncogenic role of sphingosine kinase. *Curr Biol, 10*(23), 1527-1530.

Xia, P., Wang, L., Gamble, J. R., & Vadas, M. A. (1999). Activation of sphingosine kinase by tumor necrosis factor-α inhibits apoptosis in human endothelial cells. *J Biol Chem, 274*(48), 34499-34505.

Xia, P., Wang, L., Moretti, P. A., Albanese, N., Chai, F., Pitson, S. M., D'Andrea, R. J., Gamble, J. R., & Vadas, M. A. (2002). Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-α signaling. *J Biol Chem, 277*(10), 7996-8003.

Yamada, K., Sakane, F., Matsushima, N., & Kanoh, H. (1997). EF-hand motifs of α, β and γ isoforms of diacylglycerol kinase bind calcium with different affinities and conformational changes. *Biochem J, 321* (Pt 1), 59-64.

Yan, K. X., Terashima, K., Takaya, Y., & Niwa, M. (2001). A novel oligostilbene named (+)-viniferol A from the stem of *Vitis vinifera* 'kyohou'. *Tetrahedron*, *57*(14), 2711-2715.

Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., & Igarashi, Y. (1996). N,N-dimethylsphingosine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. *Biochemistry, 35*(2), 626-633.

Yokota, S., Taniguchi, Y., Kihara, A., Mitsutake, S., & Igarashi, Y. (2004). Asp177 in C4 domain of mouse sphingosine kinase 1a is important for the sphingosine recognition. *FEBS Lett, 578*(1-2), 106-110.

Young, K. W., Willets, J. M., Parkinson, M. J., Bartlett, P., Spiegel, S., Nahorski, S. R., & Challiss, R. A. (2003). Ca^{2+}/calmodulin-dependent translocation of sphingosine kinase: role in plasma membrane relocation but not activation. *Cell Calcium, 33*(2), 119-128.

Young, N., & Van Brocklyn, J. R. (2007). Roles of sphingosine-1-phosphate (S1P) receptors in malignant behavior of glioma cells. Differential effects of $S1P_2$ on cell migration and invasiveness. *Exp Cell Res, 313*(8), 1615-1627.

Zebol, J. R., Hewitt, N. M., Moretti, P. A., Lynn, H. E., Lake, J. A., Li, P., Vadas, M. A., Wattenberg, B. W., & Pitson, S. M. (2009). The CCT/TRiC chaperonin is required for maturation of sphingosine kinase 1. *Int J Biochem Cell Biol, 41*(4), 822-827.

Zhang, Y. J., Nagao, T., Tanaka, T., Yang, C. R., Okabe, H., & Kouno, I. (2004). Antiproliferative activity of the main constituents from *Phyllanthus emblica*. *Biol Pharm Bull, 27*(2), 251-255.

Zhao, M., Yang, B., Wang, J., Liu, Y., Yu, L., & Jiang, Y. (2007). Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn.) pericarp. *Int Immunopharmacol, 7*(2), 162-166.

Zhu, R., Snyder, A. H., Kharel, Y., Schaffter, L., Sun, Q., Kennedy, P. C., Lynch, K. R., & Macdonald, T. L. (2007). Asymmetric synthesis of conformationally constrained fingolimod analogues—discovery of an orally active sphingosine 1-phosphate receptor type-1 agonist and receptor type-3 antagonist. *J Med Chem, 50*(25), 6428-6435.