University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Sciences

# Sphingosine kinases: evaluation of therapeutic potential using prostate cancer cell models

by

Francesca R. Tonelli

A thesis presented in fulfillment of the requirements for the degree of

Doctor of Philosophy

2012

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:

**ACKNOWLEDGEMENTS**

I would like to sincerely thank the University of Strathclyde for granting me a Research Scholarship, thereby giving me the chance to reach a professional and personal maturity I could have not gained elsewhere.

I am truly grateful to my project supervisors, Prof. Nigel Pyne and Prof. Susan Pyne, for being excellent mentors and for always being willing to share their time and deep knowledge with me. The invaluable guidance they have provided was instrumental for the completion of this study.

Finally, my greatest appreciation goes to all those that with unconditional love and support have been by my side through the darkest as well as the brightest moments of this long journey. The strength they have given me, made everything possible.

**TABLE OF CONTENTS**

**LIST OF FIGURES**

**CHAPTER 1**



**CHAPTER 3**

**CHAPTER 4**



**CHAPTER 5**

**CHAPTER 6**



**APPENDIX**

**LIST OF TABLES**

## CHAPTER 2



## APPENDIX

## ABBREVIATIONS

ABC294640, 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide; AR, androgen receptor; ATP, adenosine triphosphate; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; Cer, ceramide; CerS, ceramide synthase; CHX, cycloheximide; CIB1, calcium and integrin-binding protein 1; DAG, diacylglycerol; DAPI, 4,6-diamidino-2-phenylindole; DHC, dihydroceramide; DHS, dihydrosphingosine; dihydroS1P, dihydrosphingosine 1-phosphate; DMSO, dimethyl sulphoxide; eEF1A, eukaryotic elongation factor 1A; EFCS, European foetal calf serum; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FB1, fumonisin B1; FITC, fluorescein isothiocyanate; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPCR, G protein coupled receptor; GSH, reduced glutathione; GSSG, oxidised glutathione; HDAC, histone deacetylase; HEK, human embryonic kidney; HER2, human epidermal growth factor receptor-2; HIF, hypoxia-inducible factor; hPASMC, human pulmonary aortic smooth muscle cells; $IC_{50}$, half maximal inhibitory concentration; $IP_3$, inositol trisphosphate; JNK, c-Jun N-terminal kinase; KAT, lysine acetyltransferase; KDAC, lysine deacetylase; $K_{ic}$, competitive inhibition constant; $K_{iu}$, uncompetitive inhibition constant; $K_M$, Michaelis constant; LC-MS, liquid chromatography-mass spectrometry; LPP, lipid phosphate phosphatase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MG132, Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; MS, mass spectrometry; NAC, N-acetyl L-cysteine; PARP, polyADP ribose polymerase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PDGF, platelet derived growth factor; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonyl fluoride; PP2A, protein phosphatase 2A; (*R*)-FTY720 methyl ether, (*R*)-FTY720-OMe, (*R*)-OMe (2R)-2-amino-3-(O-methyl)-(2-(4′-n-octylphenyl)ethyl)propanol; qPCR, quantitative PCR; RIP1, receptor interacting protein 1; RTK, receptor tyrosine kinase; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; SK, sphingosine kinase; SKi, 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole; Sph, sphingosine; Stat3, Signal transducer and activator of transcription 3; (*S*)-vinylPn, (*S*)-FTY720 vinylphosphonate; S1P, sphingosine 1-phosphate; S1PP, S1P phosphatase; S1PR, S1P receptor; TRAF2, tumor necrosis factor receptor-associated factor 2; TRITC, tetramethyl rhodamine isothiocyanate; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

**PUBLICATIONS**

**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, 1536-1542

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, 38841-38852

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, 18633-18640

Pyne, N.J., **Tonelli, F.**, Lim, K.G., Long, J., Edwards, J., Pyne, S. (2011) Targeting sphingosine kinase 1 in cancer. *Adv. Enzyme Regul.* 52, 31-38

Pyne, N.J., **Tonelli, F.**, Lim, K.G., Long, J.S., Edwards, J., Pyne, S. (2012) Sphingosine 1-phosphate signalling in cancer. *Biochem. Soc. Trans.* 40, 94-100

Lim, K.G., **Tonelli, F.**, Berdyshev, E., Gorshkova, I., Leclercq, T., Pitson, S.M., Bittman, R., Pyne, S., Pyne, N.J. (2012) Inhibition kinetics and regulation of sphingosine kinase 1 expression in prostate cancer cells: Functional differences between sphingosine kinase 1a and 1b. *Int. J. Biochem. Cell Biol.* 44, 1457-1464

**ABSTRACT**

Sphingosine kinase 1 and 2 (SK1 and SK2) catalyse the formation of the bioactive lipid sphingosine 1-phosphate. Alterations in SK1 function have been implicated in human prostate cancer, being involved in the acquisition of therapy resistance and progression to androgen independence, two major issues in the clinical management of this disease.

This study investigated the effect of down-regulating SK1 in androgen-dependent (LNCaP) and androgen-independent (LNCaP-AI) prostate cancer cells. The SK1 inhibitor, 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi) activates the proteasome by acutely inhibiting SK1 activity. Consequently, SKi induces the proteasomal degradation of SK1 isoforms, SK1a and SK1b, in LNCaP cells, which is associated with the accumulation of the pro-apoptotic lipid C22:0 ceramide and the induction of apoptosis. In contrast, SK1b is resistant to SKi-induced proteasomal degradation in LNCaP-AI cells, and this is associated with the failure to elevate ceramide levels and to induce apoptosis. However, a different SK1 inhibitor, (*S*)-FTY720 vinylphosphonate overcomes this resistance to induce the proteasomal degradation of both SK1a and SK1b in LNCaP-AI cells, resulting in C16:0 ceramide accumulation and activation of apoptosis. The analysis of the effects of a selective inhibitor of SK2 revealed that SK1 and SK2 might regulate distinct functional pools of sphingolipids in prostate cancer cells. Additionally, SK1 inhibitors markedly reduce androgen receptor (AR) expression in prostate cancer cells. In particular, SKi down-regulates AR via a reactive oxygen species-dependent mechanism. Indeed, SKi treatment induces a pronounced oxidative stress response in LNCaP and LNCaP-AI cells.

Thus, this study highlights a significant role of SK1 in promoting androgen receptor-dependent signalling and maintaining the survival of prostate cancer cells. This study also provides the first documented evidence of increased stability of SK1b compared with SK1a, which is associated with resistance to apoptosis. Taken together, these findings provide useful information regarding SK1-targeted therapeutic intervention for the treatment of (prostate) cancer.

# CHAPTER 1:

# GENERAL INTRODUCTION

# 1. GENERAL INTRODUCTION

## 1.1 Sphingolipid metabolism

First discovered as structural components of cellular membranes, it is now well established that sphingolipids are signalling molecules that play key roles in the regulation of many fundamental cellular processes, including cell proliferation, survival, senescence, migration and differentiation, under both physiological and pathological conditions (Hannun and Obeid, 2008).
Sphingolipids are structurally characterised by the presence of a sphingoid backbone, a long-chain (mainly C18 in mammalian cells) amino alcohol (Figure 1.1).

Sphingolipid metabolism (Figure 1.1) is a complex network of reactions catalysed by enzymes that are localized to different sub-cellular compartments, leading to the generation of a multitude of lipids with signalling functions (Gault et al., 2010). Ceramide is the key hub of sphingolipid metabolism (Hannun and Obeid, 2008). Ceramide can be formed by two distinct metabolic pathways, termed *de novo* and salvage pathway. The *de novo* synthesis of ceramide occurs at the cytoplasmic surface of endoplasmic reticulum (ER) and is initiated by the condensation of serine and fatty acyl CoA to form 3-ketosphinganine catalysed by serine palmitoyltransferase (Mandon et al., 1992). 3-ketosphinganine is then reduced by 3-ketosphinganine reductase to form dihydrosphingosine, which is then acylated at the C2-amino group to dihydroceramide in a reaction catalysed by (dihydro)ceramide synthase. Six distinct (dihydro)ceramide synthases exist in mammals that use various fatty acyl CoA molecules to generate distinct dihydroceramide/ceramide species that differ for the acyl chain they are composed of (varying in length from C14 to C28) (Gault et al., 2010).
In the last step of the *de novo* pathway, dihydroceramide desaturase converts dihydroceramide into ceramide (Michel et al., 1997). Ceramide formed at the ER is transported to the Golgi apparatus either through vesicular trafficking or through a transport protein (CERT) (Hanada et al., 2003).

In the salvage pathway, ceramide is formed by the hydrolysis of plasma membrane sphingomyelin which is catalysed by sphingomyelinase (Okazaki et al., 1989; Liu et al., 1998). This enables the synthesis of ceramide via a less energetically exhaustive route compared with the *de novo* pathway.

Besides being a signalling molecule itself, ceramide is also the precursor of other bioactive sphingolipids. Ceramide can be deacylated by ceramidase to generate sphingosine (el Bawab et al., 2002) which can be further metabolised to sphingosine 1-phosphate (S1P) by the action of sphingosine kinase (see below). Alternatively, ceramide can be glycosylated by glucosylceramide synthase to form glucosylceramides, that are employed for the synthesis of complex glycosphingolipids (Ichikawa et al., 1996). Ceramide can also be converted back to sphingomyelin by the action of sphingomyelin synthase (Tafesse et al., 2006). Complex glycosphingolipids and sphingomyelin generated from ceramide at the Golgi can then be transferred to the plasma membrane by vesicular transport. Finally, ceramide can be phosphorylated by ceramide kinase to generate ceramide 1-phosphate (Kolesnick and Hemer, 1990).

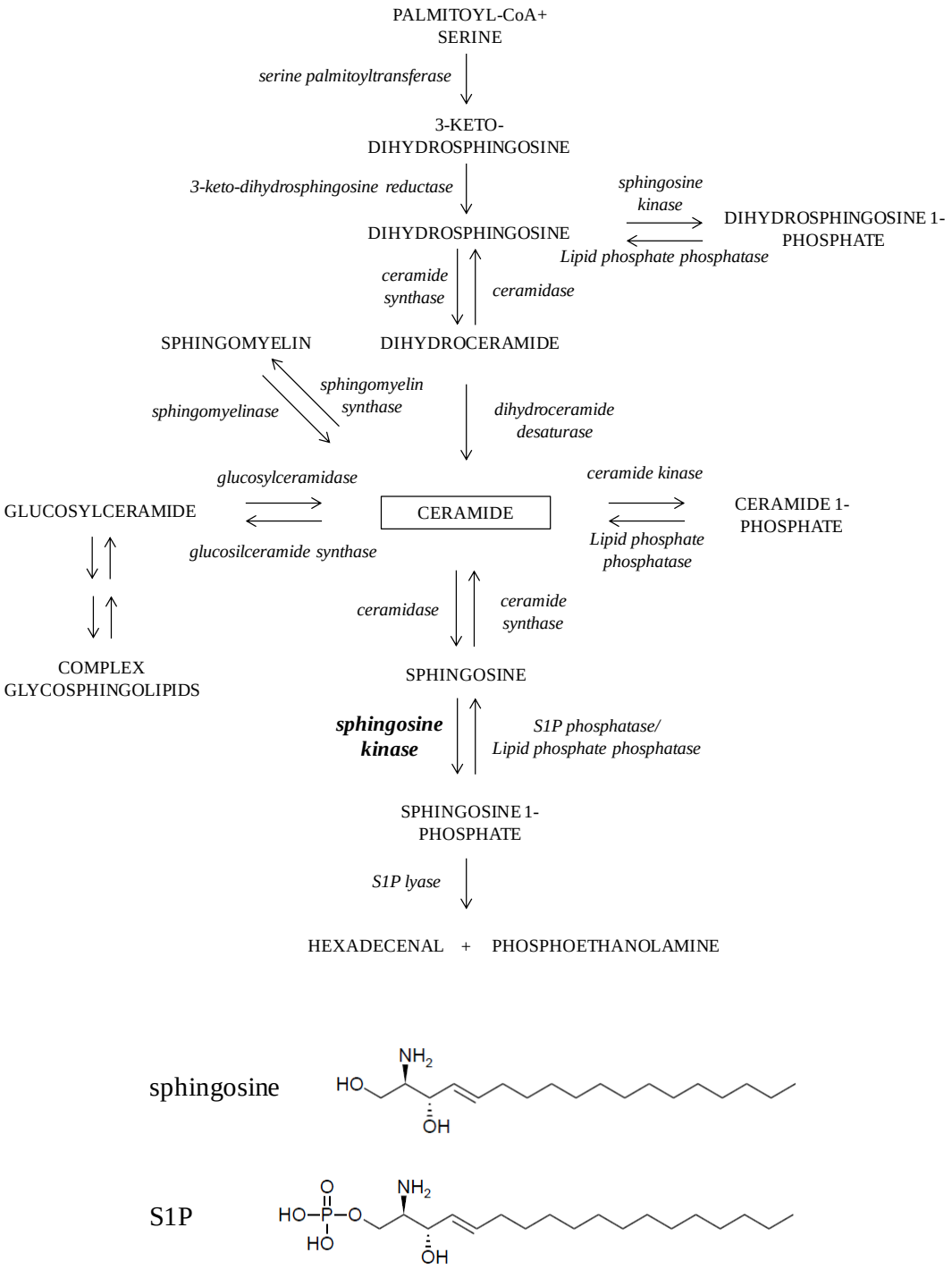



**Figure 1.1** ***Sphingolipid metabolism: synthetic and catabolic pathways (adapted from Pitson, 2011).*** *The enzymes that catalyse each reaction are reported in italics. Also shown are the chemical structures of sphingosine (the sphingoid backbone) and sphingosine 1-phosphate (S1P).*

Most of the reactions described above are reversible, so that sphingolipid metabolism is a highly dynamic process. In addition, since virtually all of the molecules that are formed by these reactions are biologically active (i.e. with signalling functions), their levels are tightly regulated by the balance between their synthesis and degradation. This is accomplished by a fine modulation of the activity of the enzymes involved in sphingolipid metabolism.

Among the bioactive sphingolipids, ceramide, sphingosine and S1P are the most well-characterised. Interestingly, while S1P promotes cell proliferation and suppresses apoptosis, its precursors ceramide and sphingosine have been widely associated with the inhibition of cell growth and activation of apoptosis (Ogretmen and Hannun, 2004). Since these opposing acting lipids are interconvertible, a "sphingolipid rheostat" model has been proposed whereby the intracellular levels of ceramide/sphingosine and S1P are in a dynamic balance and alterations in this balance determine whether a cell survives and proliferates or undergoes apoptosis, depending on the relative amounts of these three lipids (Cuvillier et al., 1996) (Figure 1.3). Indeed, published reports demonstrate that the pro-survival function of S1P is in part due to its ability to antagonize ceramide action, i.e. S1P protects cells against ceramide-induced apoptosis (Cuvillier et al., 1996; Cuvillier et al., 1998; Xia et al., 1999). The function of the sphingolipid rheostat in the regulation of cell survival will be discussed in section 1.2.3.

Although several enzymes are potentially involved in the regulation of the sphingolipid rheostat (Figure 1.3), SK has a crucial role (Spiegel and Milstien, 2003), being able to reduce the intracellular levels of pro-apoptotic ceramide/sphingosine while simultaneously generating pro-survival S1P (section 1.3.3). In particular, a number of studies have demonstrated that SK1 activity inversely correlates with the intracellular content of ceramide in a variety of cell systems (Olivera et al., 1999; Xia et al., 2000; Nava et al., 2002; French et al., 2003; Pitson et al., 2003; Taha et al., 2004; Maceyka et al., 2005), providing evidence that SK1 is a key regulator of cell fate.

## 1.2 Sphingosine 1-phosphate (S1P) metabolism and signalling

### 1.2.1 S1P biosynthesis and degradation

Sphingosine formed from ceramide (synthesized either *de novo* or via the salvage pathway) can be phosphorylated on its primary hydroxyl group to generate sphingosine 1-phosphate (S1P) in an ATP-dependent reaction catalysed by the enzyme sphingosine kinase (SK) (Figure 1.1). Two isoforms of SK1 have been identified so far: SK1 (Olivera et al., 1998; Kohama et al., 1998; Melendez et al., 2000; Nava et al., 2000a) and SK2 (Liu et al., 2000a), that will be described in details in section 1.3. The chemical structure of S1P is reported in Figure 1.1.

The removal of S1P may occur via different routes: reversible dephosphorylation or irreversible cleavage (Figure 1.1). S1P can be reversibly dephosphorylated to sphingosine by the action of lipid phosphate phosphatases (LPP1, LPP2 and LPP3) or S1P-specific phosphatases (SPP1 and SPP2) localized to the ER (Pyne et al., 2005). Sphingosine localized at the ER can then be converted to ceramide by the action of ceramide synthase and re-utilized for the synthesis of complex sphingolipids, which allows cells to minimize the energetically exhaustive *de novo* synthesis of ceramide. In this regard, a number of studies provided evidence that sphingosine kinase and S1P phosphatase act in concert to regulate sphingosine recycling into the sphingolipid metabolic pathway (Maceyka et al., 2005; Le Stunff et al., 2007; Siow et al., 2010). Location of LPPs at the plasma membrane has also been reported (Pyne et al., 2005). The catalytic site of these enzymes is predicted to face the extracellular side of the plasma membrane (Pyne et al., 2005). Thus, LPPs have the potential to regulate not only the intracellular levels of S1P, but also S1P extracellular pool via their ecto-activity, thereby modulating the availability of S1P at its cognate membrane receptors (see below).

Alternatively, S1P can be cleaved at the C2-C3 bond by ER-localized S1P lyase to generate *trans*-2-hexadecenal and ethanolamine phosphate (Van Veldhoven and Mannaerts, 1993). The irreversible cleavage of the sphingosine backbone by S1P lyase is the only “exit point” from the sphingolipid metabolic pathway.

Interestingly, the degradative fate of S1P does not depend on the subcellular localization where S1P is produced. In particular, S1P generated at the plasma membrane is equally accessible to ER-localized degradative enzymes as S1P generated at the ER (Siow et al., 2010). This suggests that S1P can move from the site where it is produced to other intracellular compartments (Siow et al., 2010), although the mechanisms involved in S1P intracellular transport are unknown.

It is important to appreciate that *trans*-2-hexadecenal is not simply an end-product of the sphingolipid pathway. In fact, it has been recently shown that *trans*-2-hexadecenal is a biologically active molecule, being able to induce cytoskeletal reorganization thereby leading to cell detachment and cell death by apoptosis via a c-Jun N-terminal kinase (JNK)-dependent signalling pathway in various mammalian cell lines (Kumar et al., 2011). Thus, according to this study, S1P lyase may function not only to attenuate S1P-dependent signalling but also to generate a bioactive molecule.

As for other signalling molecules, the cellular level of S1P is tightly regulated (by the balance between its formation and degradation) and is low under basal conditions (Spiegel et al., 1998), although S1P levels can rapidly increase upon activation of SK by a variety of extracellular stimuli (section 1.3.2). Significantly, alterations in the balance between S1P synthesis and removal result in various pathological conditions, including cancer (section 1.4).

### 1.2.2 Biological activities of S1P

S1P is a pleiotropic mediator that regulates a wide range of biological activities including cell proliferation and survival, cytoskeletal rearrangement and cell motility, cell adhesion and $Ca^{2+}$ homeostasis (Taha et al., 2006a). By regulating these cellular processes, S1P contributes to a variety of physiological functions, such as embryonic development, regulation of the immune system and homeostasis of the cardiovascular system, whereas aberrant S1P signalling is implicated in the

pathogenesis and progression of various diseases, including cancer and autoimmune disorders (Taha et al., 2006a; Takabe et al., 2008).

The first reports on S1P showed that various mitogens stimulate SK activity and S1P synthesis to induce cell proliferation, which raised the concept that S1P was an intracellular second messenger (Zhang et al., 1991; Olivera and Spiegel, 1993). It was only afterwards that cell surface receptors with high affinity for S1P were identified, revealing the complexity of S1P signalling and function. It is now well established that S1P has a dual modality of action, being able to act both as an intracellular second messenger and an extracellular agonist to activate specific G protein-coupled receptors (GPCRs), as described in the following sections.

The process by which intracellularly generated S1P acts on cell surface receptors is defined as "inside-out" signalling (Takabe et al., 2008) (Figure 1.3). To bind to its receptors on the outer leaflet of plasma membrane, S1P generated inside cells must be released in the extracellular space. Given the hydrophilic nature of S1P, its release from cells cannot occur via spontaneous diffusion through the bilayer but has to be mediated by a transporter. In this regard, several transporter proteins have been implicated in S1P export from mammalian cells, based on the evidence that manipulating the expression of these proteins affects S1P release. These include members of the ATP-Binding Cassette family (Mitra et al., 2006; Sato et al., 2007; Takabe et al., 2010) and the recently identified SPNS2 (spinster homologue 2) (Hisano et al., 2012).
S1P released from cells can act on the receptors present both on the same cells and on the neighbouring cells, i.e. via an autocrine and paracrine manner (Alvarez et al., 2007).

Interestingly, S1P can be also produced directly in the extracellular environment. Indeed, it has been reported that a variety of cell types release enzymatically active SK1 into the extracellular space, either constitutively (Ancellin et al., 2002; Waters et al., 2003; Venkataraman et al., 2006) or under stimulation (Hammad et al., 2006; Soldi et al., 2007). SK1 lacks a conventional secretory signal sequence and the

mechanism that mediates SK1 secretion is currently unknown (Rigogliuso et al., 2010). Significantly, SK1 constitutively secreted by vascular endothelial cells is a major contributor of S1P present in plasma (Venkataraman et al., 2006).

The release of a catalytically active truncated form of SK2 into the extracellular milieu has been reported to occur in apoptotic cells, which is dependent on caspase-1-mediated cleavage of SK2 (Weigert et al., 2010).

Extracellular SKs may utilize sphingosine present at the outer leaflet of the plasma membrane (derived from sphingomyelin degradation) (Tani et al., 2007) and ATP released by cells (Elliott et al., 2009) to generate S1P.

A recent study reported the presence of active SK1 and sphingosine in vesicles shed by tumour cells, providing evidence of an additional mechanism for extracellular production of S1P by cancer cells (Rigogliuso et al., 2010).

It may be speculated that S1P generated directly in the extracellular space might allow its privileged binding to cell surface receptors. Additionally, Natarajan and colleagues reported that extracellular S1P can be used as a source for generating intracellular S1P (Zhao et al., 2007). In particular, these authors demonstrated that extracellular S1P can be dephosphorylated to sphingosine by the ecto-activity of plasma membrane LPP1; sphingosine is then able to cross the plasma membrane and is converted back to S1P by intracellular SK1 (Zhao et al., 2007).

#### *1.2.2.1 S1P as a receptor agonist*

The majority of the known cellular effects elicited by S1P are due to the ability of this lipid to bind to and activate specific GPCRs. Five related membrane receptors for S1P (termed $S1P_{1-5}$) have been identified so far, for which S1P exhibits low nanomolar affinity (Chun et al., 2002). These receptors share approximately 50% sequence similarity (Chun et al., 2002). $S1P_1$, $S1P_2$ and $S1P_3$ are expressed ubiquitously, while $S1P_4$ and $S1P_5$ show a more restricted expression pattern, localizing predominantly in lymphoid tissues and central nervous system,

respectively (Chun et al., 2002). As S1P receptors are widely expressed in human tissues, virtually every cell is able to respond to S1P.

GPCRs are seven-transmembrane domain receptors that mediate signal transduction from extracellular ligands via their coupling with heterotrimeric G proteins (guanine nucleotide-binding proteins) which consist of three subunits named Gα, Gβ and Gγ (Berg et al., 2002). G proteins exist in an inactive state (bound to guanosine diphosphate, GDP) and in an active state (bound to guanosine triphosphate, GTP). Upon binding by its extracellular agonist, GPCR activates the Gα subunit by promoting the switch from GDP to GTP via its guanine nucleotide exchange factor activity. This results in the dissociation of the G proteins from the receptor and of the GTP-bound Gα subunit from the Gβγ complex. Both Gα-GTP and Gβγ can then act on their specific downstream effectors, resulting in the modulation of intracellular signalling pathways (Berg et al., 2002). Different types of G proteins exist (termed $G_s$, $G_i$, $G_q$ and $G_{12/13}$) which act on distinct downstream effectors to control different signalling cascades. Each GPCR can couple to and activate more than one type of G protein (Berg et al., 2002).

S1P receptor subtypes regulate different cellular processes via their coupling with distinct G proteins, as diagrammatically described in Figure 1.2. The ability of S1P receptors to modulate different intracellular cascades accounts for the variety of biological effects elicited by S1P, as detailed below. Interestingly, through the modulation of distinct signalling pathways S1P receptors may exert opposing effects on cells. This is exemplified by the differential effects of $S1P_1$, $S1P_2$ and $S1P_3$ on cell motility (see below). These characteristics provide a molecular explanation as to why cellular responses to S1P are cell-type specific, depending on the receptor expression pattern. Indeed, S1P receptor subtypes are differentially expressed in distinct cell types.

Interestingly, S1P receptors are also involved in "cross-talk" phenomena with other membrane receptors, as will be detailed in section 1.2.4.

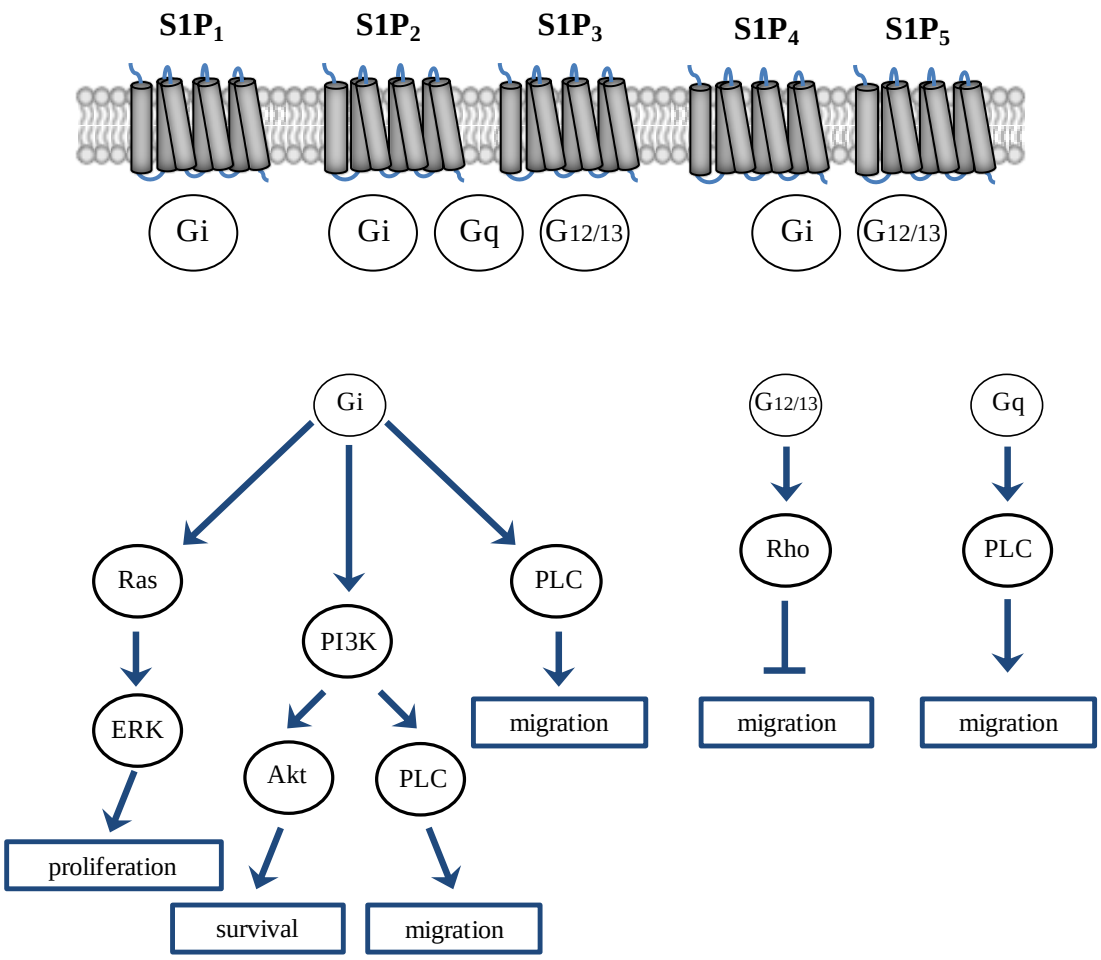



**Figure 1.2** ***S1P receptor subtypes: G protein coupling and down-stream effectors (adapted from Brinkmann, 2007; Pyne and Pyne, 2010).*** *Other signalling pathways initiated by S1P receptors have been omitted for clarity. PLC, phospholipase C.*

A few examples of actions of S1P that are mediated by its binding to membrane receptors are described below. With relevance to the current study, the functions of S1P that are relevant to its role in cancer development and progression will be discussed herein.

*Proliferation*

S1P exerts mitogenic effects in a wide variety of cell types, including normal and cancer cells (Spiegel et al., 1998). Indeed, numerous mitogens, including foetal calf serum, promote cell proliferation through the activation of SK and production of S1P (Olivera and Spiegel, 1993).

A number of studies have shown that $S1P_1$ positively regulates proliferation in many different cell types via the activation of the extracellular signal-regulated kinase (ERK) pathway, the principal pathway involved in promoting cell proliferation (Kluk and Hla, 2002). Thus, over-expression of $S1P_1$ results in enhanced S1P-stimulated, $G_i$-dependent activation of ERK-1/2 in various cell types (Lee et al., 1996; Okamoto et al., 1998).

Additional receptor subtypes and intracellular pathways are involved in mediating the mitogenic effect of S1P, at least in some cell types. For instance, inhibition of $G_i$-mediated signalling only partially inhibits S1P-stimulated activation of ERK-1/2 and cell proliferation in fibroblasts and astrocytes (Wu et al., 1995; Pébay et al., 2001). Indeed, stimulation of ERK-1/2 activity can also occur via the S1P-induced activation of $G_q$-coupled receptors (Radeff-Huang et al., 2004). Furthermore, over-expression of $S1P_2$ and $S1P_3$ results in enhanced cell proliferation via $G_i$- and Rho-dependent pathways in Jurkat T cells (An et al., 2000). Indeed, evidence exists that $G_{12/13}$-dependent activation of Rho may contribute to the nuclear translocation and/or sustained activation of ERK-1/2 to promote cell proliferation (Radeff-Huang et al., 2004).

Phosphatidylinositol 3-kinase (PI3K) might also mediate the pro-proliferative effect of S1P, as suggested by the finding that pharmacological inhibition of PI3K suppresses S1P-stimulated activation of ERK-1/2 and proliferation in astrocytes (Pébay et al., 2001). Similarly, a PI3K-dependent activation of ERK-1/2 has been reported in smooth muscle cells (Rakhit et al., 1999).

It is important to appreciate that the mitogenic effect of S1P also involves intracellular actions, e.g. via the activation of the transcription factor NF-κB (Alvarez et al., 2010) (section 1.2.2.2).

Intriguingly, a recent study demonstrated that $S1P_5$ co-localises with SK1/SK2 in the centrosome of mammalian cells, suggesting a role for this signalling system in mitotic spindle formation and cell division (Gillies et al., 2009). Thus, this study provides evidence for a novel mechanism by which S1P may regulate mitosis which involves an intracellular role for $S1P_5$.

*Migration*

S1P regulates the migration of many cell types through the activation of its cell-surface receptors (Kluk and Hla, 2002). Interestingly, $S1P_1$, $S1P_2$ and $S1P_3$ have differential effects on the regulation of cell motility due to opposing actions on the reorganization of cytoskeleton. This, in turn, depends on the activation of different intracellular pathways by these receptors, which results in the modulation of the activity of distinct members of the Rho family of GTPase (guanosine triphosphatases), proteins that regulate cytoskeleton rearrangements and cell motility. Thus, $S1P_1$ and $S1P_3$ promote cell migration by activating the Rac pathway which stimulates the rearrangement of cytoskeleton to induce lamellipodia protrusion and extension (Kon et al., 1999; Okamoto et al., 2000; Hobson et al., 2001). In contrast, $S1P_2$ inhibits cell motility by stimulating Rho and Rho-associated kinase, leading to the formation of stress fibers that anchor the cell to the substrate (Goparaju et al., 2005; Lepley et al., 2005).

Thus, S1P has a dual effect on cell migration, depending on the receptor that it activates. Hence, the migratory response of a cell to S1P might depend on the relative expression levels of the three different receptor subtypes. Indeed, this is confirmed by studies demonstrating that manipulation of S1P receptor levels to alter the balance between the distinct subtypes affects the migratory response to S1P in various cell systems (Okamoto et al., 2000; Yamaguchi et al., 2003; Yamashita et al., 2006).

Interestingly, a study by Spiegel and colleagues describes a mechanism that coordinates S1P/$S1P_1$-mediated directed cell movement (Maceyka et al., 2008). In particular, these authors demonstrated that SK1 and $S1P_1$ (but not $S1P_2$) are recruited to nascent lamellipodia in response to chemoattractants. This results in the localized production of S1P and its privileged interaction with migration-promoting $S1P_1$ (rather than migration-inhibiting $S1P_2$) and subsequent cell movement directed along a chemotactic gradient (Maceyka et al., 2008) (section 1.2.2.2).

The physiological relevance of $S1P_1$ in mediating S1P-induced migration is exemplified by the involvement of the S1P/$S1P_1$ axis in the trafficking of immune cells. In particular, $S1P_1$ mediates the egress of lymphocytes from lymphoid organs (Matloubian et al., 2004). An S1P gradient exists between blood/lymph (high nanomolar/ low micromolar concentration) and lymphoid tissues (low nanomolar concentration) which is generated and maintained by the activity of sphingosine kinase associated with red blood cells and vascular endothelial cells (Pappu et al., 2007; Venkataraman et al., 2008), coupled with a high S1P lyase activity associated with the lymphoid tissues to reduce the release of S1P from lymphoid tissue cells into the extracellular space (Schwab et al., 2005). As S1P stimulates the migration of lymphocytes acting via $S1P_1$, the modulation of $S1P_1$ expression in these cells controls the ability of lymphocytes to respond to the S1P gradient to exit from the lymphoid organs into the circulation (Matloubian et al., 2004).
This represents the molecular basis of the action of the immunosuppressant drug FTY720, which has been recently approved by the U.S. Food and Drug Administration and the European Medicines Agency for treating relapsing multiple sclerosis (Chun and Brinkmann, 2011). FTY720 is a synthetic analogue of sphingosine that is taken up by cells, where it is effectively phosphorylated by SK2 to generate FTY720-phosphate. The latter can be released and bind to four of the five S1P receptors, $S1P_2$ being the exception (Brinkmann et al., 2002). Interestingly, FTY720-phosphate acts as a "functional antagonist" of $S1P_1$ (Matloubian et al., 2004; Brinkmann et al., 2004). Indeed, FTY720-phosphate binding to $S1P_1$ induces the internalization and proteolytic degradation of this receptor, which results in a sustained down-regulation of its expression on lymphocytes. Consequently, FTY720

blocks the S1P-directed, $S1P_1$-mediated migration of lymphocytes out of lymphatic tissues thereby inducing lymphopenia and exerting a potent immunosuppressive effect (Matloubian et al., 2004; Brinkmann et al., 2004).

Interestingly, S1P mediates cell migration in response to various chemoattractants. Thus, pharmacological inhibition of SK suppresses chemotaxis in many cell types (Spiegel and Milstien, 2003). For instance, the S1P/$S1P_1$ axis is required for cell migration toward platelet-derived growth factor (PDGF), as demonstrated by the finding that ablating SK1 or $S1P_1$ blocks PDGF-induced cell motility (Hobson et al., 2001). This involves the functional interaction ("cross-talk") between PDGF and S1P signalling pathways (section 1.2.4). In contrast with $S1P_1$, $S1P_2$ inhibits PDGF-induced cell migration (Goparaju et al., 2005), in keeping with the opposing effects of the two receptor subtypes in regulating cell motility.

*Angiogenesis*

S1P signalling through its receptors is critical for the development of the vascular system during embryogenesis as well as for angiogenesis in the adult (Hla et al., 2001; Mizugishi et al., 2005). S1P acts predominantly via $S1P_1$ to promote angiogenesis. Indeed, genetic knock-down of this receptor inhibits angiogenesis *in vivo* (Lee et al., 1999). Significantly, deletion of $S1P_1$ in mice is embryonically lethal due to severe defects in vascular development resulting in massive hemorrhages (Liu et al., 2000b; Allende et al., 2003), a phenotype similar to that observed in mice lacking both *Sk1* and *Sk2* (Mizugishi et al., 2005).

The S1P/$S1P_1$ signalling contributes to blood vessel formation by stimulating proliferation, survival and migration of vascular endothelial cells (Lee et al., 1999; LaMontagne et al., 2006). The expression of $S1P_1$ on endothelial cells is also required for vascular maturation as it promotes the migration of perycites and smooth muscle cells to envelop the newly-formed endothelial tubes, as well as the formation of cell-cell junctions (Allende et al., 2003; Paik et al., 2004).

Interestingly, cross-talk between S1P and other pro-angiogenic molecules, including PDGF, epidermal growth factor (EGF), and vascular endothelial growth factor

(VEGF), has been described that may be relevant to angiogenesis as S1P signalling mediates the actions of these molecules on cells (section 1.2.4). Thus, inhibition of $S1P_1$ expression/function may also reduce the effects of PDGF, EGF and VEGF on endothelial cells.

*$Ca^{2+}$ signalling*

S1P is also involved in the regulation of $Ca^{2+}$ signalling (which in turn modulates numerous cellular functions, including cell proliferation and survival: Berridge et al., 2000) acting via diverse mechanisms. S1P can increase $Ca^{2+}$ intracellular concentration through a "classical" pathway, involving the S1P receptor/protein G-mediated activation of phospholipase C (PLC) leading to formation of inositol trisphosphate ($IP_3$) that acts on ER-localized receptors to induce $Ca^{2+}$ release (An et al., 1999). However, S1P can also elicit $Ca^{2+}$ mobilization independently of $IP_3$ receptors (Mattie et al., 1994), providing evidence for the existence of additional mechanisms. Indeed, S1P-induced release of $Ca^{2+}$ from ER was not suppressed by pharmacological inhibition of $IP_3$ activity in fibroblasts (Mattie et al., 1994).
Interestingly, the photolysis-induced intracellular release of caged S1P induces $Ca^{2+}$ mobilization in various cell types, while extracellular S1P fails to do so (Meyer zu Heringdorf et al., 2003), suggesting an intracellular action of the lipid mediator.

Significantly, many external stimuli, including growth factors, induce $Ca^{2+}$ mobilization acting via SK/S1P (Meyer zu Heringdorf, 2004). Indeed, interfering with SK1 function using pharmacological inhibitors or antisense oligonucleotides attenuates the increase in intracellular $Ca^{2+}$ concentration induced by these stimuli.

More recently it has been shown that S1P modulates $Ca^{2+}$ homeostasis by an additional mechanism, involving $S1P_2$ and PLC activation to facilitate $Ca^{2+}$ entry rather than mobilization from intracellular stores (Gratschev et al., 2009).

### *1.2.2.2 S1P as an intracellular messenger*

A number of studies in mammalian cells have provided compelling evidence that S1P has receptor-independent functions. These include the regulation of $Ca^{2+}$

homeostasis, as mentioned above (Meyer zu Heringdorf et al., 2003). Additionally, SK1 over-expression (which results in increased intracellular S1P) promotes growth and survival in various cell lines genetically engineered to be devoid of S1P receptors (Olivera et al., 2003; Kohno et al., 2006). Furthermore, not all of the effects of S1P are recapitulated by dihydroS1P, although these two molecules bind to and activate S1P receptors with similar potencies. For instance, S1P, but not dihydroS1P, exerts cytoprotective effects in various cell lines (Van Brocklyn et al., 1998).

Despite the evidence of receptor-independent actions, the intracellular targets of S1P have remained elusive until recently. Examples of intracellular actions of S1P are described below. It should be noted that some of the actions of S1P that are reported in the literature are not ascribable to the activation of known receptors/intracellular targets of this lipid, suggesting that additional S1P targets are yet to be identified.

S1P produced in the nucleus by SK2 regulates gene transcription by modulating histone acetylation (Hait et al., 2009). In particular, SK2-generated S1P binds to and inhibits the enzymatic activity of histone deacetylases (HDAC), resulting in enhanced histone acetylation and transcription of target genes. This is contingent on the association of SK2 with HDAC, which results in S1P production in proximity to its nuclear targets (Hait et al., 2009). SK2 has been shown to selectively associate with specific promoter regions, which results in the modulation of the expression of specific target genes, including the cyclin-dependent kinase inhibitor *p21* and the transcriptional regulator *c-fos* (Hait et al., 2009).

Another intracellular target of S1P is the tumor necrosis factor receptor-associated factor 2 (TRAF2) (Alvarez et al., 2010). In particular, SK1-generated S1P is a cofactor necessary to activate the ubiquitin ligase activity of TRAF2. This results in the regulatory (Lysine 63-linked) polyubiquitination of receptor interacting protein 1 (RIP1), which activates the signalling pathway leading to NF-κB activation (Alvarez et al., 2010). Interestingly, this study provides an explanation as to why S1P, but not dihydro-S1P exerts anti-apoptotic functions although the two molecules exhibit the same potency for the five S1P receptors. In fact, S1P, but not dihydroS1P stimulates

TRAF2 activity and promotes RIP1 polyubiquitination in *in vitro* assays (Alvarez et al., 2010).

S1P has also been shown to interact with prohibitin 2 (PHB2), a protein that controls the assembly and the function of mitochondria (Strub et al., 2011). Mitochondrial S1P is mainly produced by SK2 that localizes to the inner membrane of mitochondria (Strub et al., 2011). Significantly, SK2 knock-down results in impaired mitochondrial respiration due to defective assembly of the respiratory chain complex, suggesting that the interaction between PHB and S1P produced by SK2 has a key role in the regulation of mitochondrial function (Strub et al., 2011).

The p21-activated kinase 1 (PAK1), an important regulator of actin reorganization and cell migration, may represent an additional intracellular target of S1P (Maceyka et al., 2008). In particular, it has been shown that S1P directly activates PAK1, and this is required for PAK1-induced phosphorylation of the actin binding protein filamin A and subsequent filamin A-mediated rearrangement of actin into membrane ruffles to promote cell migration (Maceyka et al., 2008). These authors propose a model in which SK1 and PAK1 interact with filamin A and this drives their translocation to nascent lamellipodia; here, SK1-generated S1P stimulates PAK1 activity; S1P can also be secreted and act on $S1P_1$, which is also recruited at membrane ruffles via filamin A; this results in Rac activation and subsequent PAK1 stimulation (Maceyka et al., 2008). Thus, this study suggests that S1P acts in a dual manner to promote cell migration: intracellularly to directly activate PAK1 and extracellularly to activate $S1P_1$ and its downstream effector Rac, which in turn stimulates PAK1 (Maceyka et al., 2008). Importantly, the filamin A-dependent co-localization of SK1 and $S1P_1$ at lamellipodia favours the interaction of released S1P with this receptor subtype rather than with $S1P_2$, thereby resulting in directed cell movement.

### 1.2.3 SK1, S1P and cell fate control

As mentioned above, a "sphingolipid rheostat" exists in cells whereby the balance between the cellular levels of ceramide and S1P is a critical determinant of cell fate (Figure 1.3).
The regulation of cell survival by SK1 and S1P is complex and involves several different mechanisms, discussed herein.

Exposure of cells to stress stimuli results in the accumulation of ceramide due to stimulation of *de novo* biosynthesis or sphingomyelin hydrolysis (Oskouian and Saba, 2010). Ceramide then acts on intracellular targets (e.g. PP2A and cathepsin D) to activate intrinsic and extrinsic apoptotic pathways (Ogretmen and Hannun, 2004). In particular, ceramide promotes apoptosis by modulating the activity of members of the Bcl-2 protein family. A number of intracellular targets of ceramide have been identified that mediate the pro-apoptotic function of this lipid. One of these is the protein phosphatase PP2A which, upon ceramide-induced activation, catalyses the dephosphorylation of pro-apoptotic Bax and anti-apoptotic Bcl-2. This, in turn, results in Bax activation (Xin and Deng, 2006) and Bcl-2 proteasomal degradation (Lin et al., 2006), and subsequent activation of caspases and onset of apoptosis (Pastorino et al., 1999). Ceramide has also been shown to activate the protease cathepsin D to cleave the Bcl-2 family member Bid. This, in turn, results in the translocation of Bid to the mitochondrial outer membrane and ensuing initiation of the apoptotic pathway (Heinrich et al., 2004). Significantly, tumour necrosis factor-induced activation of apoptosis, which is mediated by cathepsin D-induced cleavage of Bid, is dependent on the expression of sphingomyelinase, the enzyme that catalyses the hydrolysis of sphingomyelin to generate ceramide (Heinrich et al., 2004).

In addition to its effect on the regulation of the intrinsic and extrinsic apoptotic pathways, ceramide controls cell fate by modulating intracellular signalling cascades involved in the regulation of cell survival, e.g. ERK-1/2, Akt and JNK pathways, as detailed below.

Over-expression of SK1 protects cells from apoptosis induced by stress stimuli or by ceramide addition (Olivera et al., 1999). Indeed, by reducing ceramide levels (section 1.3.3), SK1 has the potential to shift the sphingolipid rheostat toward a pro-survival programme thereby protecting cells from stress-induced apoptosis.

In addition to the effect of SK1 on ceramide levels, S1P itself can counteract the pro-apoptotic action of ceramide. This is evident from a study by Cuvillier et al. demonstrating that exogenously added S1P prevents ceramide-mediated apoptosis in response to stress stimuli (Cuvillier et al., 1996). It is now well established that the antagonistic actions of S1P and ceramide are in part due to their opposing effects in the regulation of key signalling pathways that control cell survival and apoptosis: ERK, Akt and JNK. In this regard, it has been shown that both ERK and Akt promote survival by regulating the function of Bcl-2 family members to inhibit the release of cytocrome *c* from mytochondria and caspase activation (Franklin and McCubrey, 2000). Additionally, Akt activates NF-κB thereby promoting the expression of pro-survival genes (Franklin and McCubrey, 2000). In contrast, JNK stimulates apoptosis by modulating the activity of mitochondrial pro- and anti-apoptotic proteins through various mechanisms (Dhanasekaran and Reddy, 2008). Additionally, JNK promotes the expression of pro-apoptotic genes (Dhanasekaran and Reddy, 2008).
Ceramide activates the JNK cascade and negatively regulates the ERK and Akt pathways to promote apoptosis (Westwick et al., 1995; Verheij et al., 1996; Oskouian and Saba, 2010). S1P opposes the pro-apoptotic action of ceramide by suppressing JNK activation induced by ceramide while activating ERK and Akt (Wu et al., 1995; Cuvillier et al., 1996; Limaye et al., 2005).

It is important to appreciate that evidence exists that S1P negatively regulates ceramide biosynthesis (section 1.3.3). Thus, this effect might also contribute to the ability of S1P to suppress ceramide-mediated apoptosis.

Interestingly, a recent study demonstrated that S1P inhibits the protein phosphatase 2A (PP2A) (Salas et al., 2011) (detailed in section 1.4.3), which is one of the mediators of ceramide pro-apoptotic action (Ogretmen and Hannun, 2004).
Thus, it is possible that inhibition of PP2A might be another means by which S1P counteracts ceramide-induced apoptosis.

Another mechanism by which S1P promotes cell survival involves its ability to modulate the expression and activity of various members of the Bcl-2 family. In particular, S1P up-regulates the expression of anti-apoptotic proteins (e.g. Bcl-2) while down-regulating pro-apoptotic proteins (e.g. Bad and Bax) (Limaye et al., 2005; Sauer et al., 2005; Avery et al., 2008; Li et al., 2008b). Similarly, over-expression of SK1 increases the expression of Bcl-2 while reducing cellular content of the pro-apoptotic Bim (Limaye et al., 2005). Additionally, both over-expression of SK1 and exogenous S1P inhibit the release of mitochondrial cytocrome *c* and caspase activation by modulating the function of Bcl-2 family members (Betito and Cuvillier, 2006; Bonhoure et al., 2008).
As described above, ceramide has an opposite function on the Bcl-2 family members to induce caspase activation and apoptosis (Ogretmen and Hannun, 2004).
Finally, SK1-generated S1P mediates a pro-survival signal by activating NF-κB (Alvarez et al., 2010) (section 1.2.2.2).

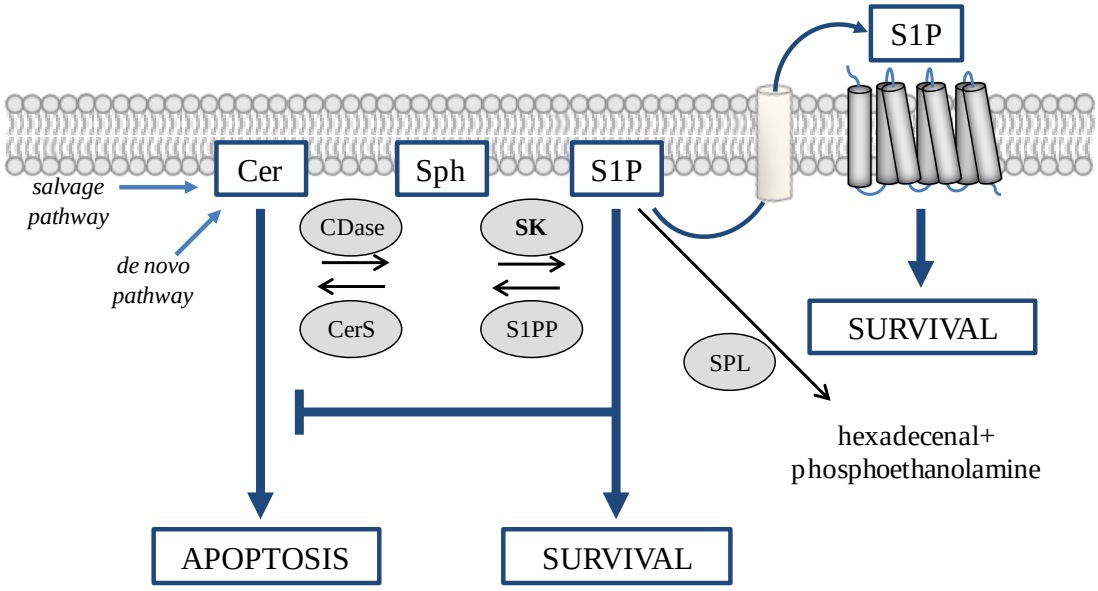



**Figure 1.3** ***Sphingolipid rheostat and S1P "inside-out" signalling in the regulation of cell survival (adapted from Pyne and Pyne, 2010).*** *CDase, ceramidase; CerS, ceramide synthase: S1PP, S1P phosphatases; SPL, S1P lyase.*

### 1.2.4 Cross-talk between S1P and other signalling pathways

An important aspect in the biology of S1P concerns the existence of functional interactions, or cross-talk, between S1P and other signalling pathways. Distinct mechanisms of cross-talk have been described and are discussed in this section.

A well characterized mechanism of cross-talk involves the stimulation of SK1 activity by growth factors acting via their cognate receptor tyrosine kinases (RTKs) (section 1.3.2). Growth factor-induced activation of SK1 results in the generation of S1P that can then act either intracellularly or via its cell surface receptors to regulate various cellular processes (e.g. proliferation, migration). Thus, S1P acts as a mediator of the effects of these growth factors. In addition, S1P can induce the transactivation of various RTKs, resulting in signal amplification. As mentioned above, examples of this mechanism of cross-talk include the interactions of S1P with EGF, PDGF and VEGF pathways.

EGF stimulates S1P production by enhancing the activity and expression of SK1 to promote cellular responses in various cell types, including cell proliferation and migration (Döll et al., 2005; Sarkar et al., 2005). In addition, S1P binding to its receptors and stimulation of downstream pathways results in EGF receptor (EGFR) transactivation, via a mechanism that involves the activation of matrix metalloproteases which act on EGF-heparin binding proteins to release active EGF that binds to and activates EGFR (Shida et al., 2004).

PDGF also activates SK1 (Olivera and Spiegel, 1993). This results in S1P production, release and interaction with $S1P_1$, which is required for PDGF-stimulated proliferation and migration (Olivera and Spiegel, 1993; Hobson et al., 2001). In addition, S1P can transactivate PDGF receptor (PDGFR) via the $S1P_3$-mediated stimulation of tyrosine phosphorylation of PDGFR, which is necessary for S1P-induced activation of Akt in various cell lines (Baudhuin et al., 2004).

Similar to EGF and PDGF, cross-talk between S1P and VEGF pathways involves the VEGF-induced stimulation of SK1 activity and production of S1P (Shu et al., 2002). By inhibiting Ras GTPase activity, S1P promotes the VEGF-stimulated activation of the Ras-ERK signalling pathway (Shu et al., 2002; Wu et al., 2003). Indeed, inhibiting SK1 prevents ERK activation in response to VEGF in various cancer cell lines (Shu et al., 2002; Wu et al., 2003). This effect does not appear to involve S1P receptors. Indeed, S1P inhibits Ras GTPase activity *in vitro* (Wu et al., 2003). However, it has been reported that an anti-S1P antibody inhibits endothelial cell migration and angiogenesis induced by VEGF in various *in vivo* cancer models (Visentin et al., 2006), providing evidence that S1P is released from cells to mediate VEGF-stimulated tumour-associated angiogenesis.

S1P has also been shown to transactivate VEGF receptors and this is required for S1P-induced migration of endothelial cells (Endo et al., 2002).

A distinct mechanism of cross-talk, termed “integrative signalling”, involves the formation of functional complexes between a receptor tyrosine kinase and an S1P receptor to regulate a common signalling pathway. The close proximity of the receptors enables them to share signalling components. For instance, the association between PDGFR and $S1P_1$ has been reported in smooth muscle cells and mouse

embryonic fibroblasts, where constitutively active $S1P_1$ enhances PDGF-induced stimulation of ERK-1/2 to promote cell migration (Waters et al., 2003; Long et al., 2006; Waters et al., 2006). Indeed, inhibition of $S1P_1$ resulted in reduced ERK-1/2 activation and cell migration in response to PDGF (Waters et al., 2003; Long et al., 2006; Waters et al., 2006). In contrast, SK1 activity inhibition did not reduce the effect of PDGF, indicating that the formation of S1P is not involved (Waters et al., 2003; Long et al., 2006).
It is important to appreciate that multiple mechanisms of cross-talk can exist for the same agonist, as exemplified by PDGF, that might be functional in distinct cell systems.

Another mechanism of cross-talk between signalling pathways is the so-called "criss-cross" transactivation, which involves three different ligand-receptor systems (Sukocheva et al., 2006). This is exemplified by the oestrogen-induced transactivation of EGFR via SK1/$S1P_3$ in breast cancer cells (Sukocheva et al., 2006). Acting on its specific membrane receptors, oestrogen induces the ERK-1/2-mediated activation of SK1, followed by S1P release and binding to $S1P_3$; this, in turn, leads to the metalloprotease-dependent release of EGF which activates EGFR to further stimulate the ERK pathway (Sukocheva et al., 2006). This mechanism may underlie the role of SK1 in mediating breast cancer cell growth in response to oestrogen stimulation *in vitro* and *in vivo* (Nava et al., 2002; Sukocheva et al., 2003).

Finally, an example of cross-talk involving SK2 has also been described. In particular, Spiegel and colleagues demonstrated that EGF stimulates the ERK-dependent phosphorylation and consequent activation of SK2, and this is required for EGF-induced migration of breast cancer cells (Hait et al., 2007). Indeed, both SK1 and SK2 are necessary for the migration of these cells in response to EGF (Hait et al., 2005).

## 1.3 Human sphingosine kinases (SK)

Sphingosine kinases catalyse the ATP-dependent phosphorylation of the two sphingoid bases that are present in mammalian cells, sphingosine and dihydrosphingosine, to generate S1P and dihydrosphingosine 1-phosphate (dihydroS1P), respectively (Olivera et al., 1998; Kohama et al., 1998; Liu et al., 2000a; Nava et al., 2000; Pitson et al., 2000a).

Two isoforms of sphingosine kinase have been identified in mammalian cells, named SK1 and SK2 (Olivera et al., 1998; Kohama et al., 1998; Nava et al., 2000a; Liu et al., 2000a), which differ for their structure, kinetic properties, subcellular localization, tissue distribution and expression pattern during embryogenesis (Taha et al., 2006b; Wattenberg et al., 2006), suggesting that these enzymes may have distinct cellular functions.

Despite catalysing the same reaction, SK1 and SK2 have differential effects. In particular, while SK1 promotes cell proliferation and protects cells against apoptotic stimuli (Olivera et al., 1999; Olivera et al., 2003; Xia et al., 2000; Hofmann et al., 2008), SK2 is generally associated with cell growth suppression and activation of apoptosis independently of S1P receptors (Liu et al., 2003; Igarashi et al., 2003; Okada et al., 2005; Maceyka et al., 2005). In agreement with these studies, genetic knock-down of SK2 blocks apoptosis induced by various stimuli (Okada et al., 2005; Hofmann et al., 2008).

The pro-apoptotic effect of SK2 was initially ascribed to a Bcl-2 homology domain 3 (BH3)-like motif present in its sequence (Liu et al., 2003). Indeed, similar to BH3-only proteins (pro-apoptotic members of the Bcl-2 family), SK2 interacts with and sequesters the anti-apoptotic protein Bcl-$X_L$ to promote apoptosis, which is preceded by cytocrome *c* release from mitochondria and caspase-3 activation (Liu et al., 2003). However, mutations in the SK2 putative BH3 domain to disrupt its interaction with Bcl-$X_L$ do not completely abrogate SK2-induced apoptosis (Liu et al., 2003), indicating that additional mechanisms are involved in the pro-apoptotic effect of

SK2. There is now strong evidence that the opposing functions of SK1 and SK2 on cell survival are partly mediated by their differential effects on ceramide levels which, in turn, are dependent on the different localization of the two enzymes (Maceyka et al., 2005). Indeed, growing evidence supports the notion that the intracellular localization of SK is critical in regulating the cellular function of this enzyme, as will be discussed in section 1.3.2.

Despite a number of studies supporting a role for SK2 in promoting apoptosis, evidence exists that this enzyme can exert a pro-survival function in some cell types. Indeed, SK2 down-regulation inhibits cell proliferation and sensitizes cells to apoptotic stimuli in various cell systems both *in vitro* and *in vivo* (Van Brocklyn et al., 2005; Sankala et al., 2007; Weigert et al., 2009; Gao and Smith, 2011). Thus, in contrast with SK1, that almost universally promotes cell proliferation and survival, SK2 function is likely to be complex and possibly cell type-specific. This might reflect the fact that SK2 localization appears to be dependent on cell type (Maceyka et al., 2005).

Additionally, it is important to appreciate that, although a number of studies provide evidence that SK1 and SK2 have opposing cellular functions, these enzymes might have redundant roles *in vivo*, i.e. they might be able to compensate for a deficiency in each other activity. This is evident during embryonic development. Indeed, *Sk1*/*Sk2* double knockout is embryonic lethal in mice due to severe abnormalities in neural and vascular development, while single knockout (*Sk1* or *Sk2*) mice are viable, fertile and without phenotypic abnormalities (Allende et al., 2004; Mizugishi et al., 2005).

Interestingly, non-catalytic functions of SK1 and SK2 have also been described, that do not depend on the formation of S1P. Evidence for a non-kinase function of SK1 is provided by the finding that siRNA knock-down of SK1 inhibits EGF-induced activation of ERK-1/2 in breast cancer cells while pharmacological inhibition of this enzyme fails to do so (Pyne et al., 2009). As mentioned above, SK2-induced activation of apoptosis is in part mediated by a non-catalytic action via its BH3

putative domain (Liu et al., 2003), although SK2 pro-apoptotic function also involves its enzymatic activity (Maceyka et al., 2005) (section 1.3.2).

### 1.3.1 SK isoforms: sequence and structural features

The SK isoforms that have been identified in human cells, SK1 (Melendez et al., 2000; Nava et al., 2000a; Pitson et al., 2000a) and SK2 (Liu et al., 2000a), are generated from the expression of two separate genes. For both human SKs, a number of N-terminal variants have been reported that arise from alternative splicing, all with similar enzymatic properties and slightly different subcellular localization (Billich et al., 2003; Alemany et al., 2007).

SK1 and SK2 show a high degree of amino acid sequence similarity (80%), although SK2 is larger than SK1 (383 amino acids for human SK1 *versus* 618 for human SK2) as it possesses two additional regions that are not present in SK1: one at the N-terminus and the other in the central part of its sequence (Liu et al., 2000a).

The sequences of SKs are evolutionary well conserved among eukaryotic organisms. In particular, five highly conserved regions (C1-C5) have been identified in the polypeptide sequence of all the known eukaryotic SKs, which contain the catalytic site (domain C1-C3), the ATP-binding site (domain C2) and the sphingosine-binding site (domain C4) (Kohama et al., 1998; Liu et al., 2000a; Pitson al., 2002; Yokota et al., 2004). Interestingly, SK1 and SK2 differ in the sequence, and thus likely in the structure, of the sphingosine-binding site (Liu et al., 2000a). This might at least in part account for the different kinetic properties of the two enzymes (Pitson, 2011). For instance, human SK2 shows a broader substrate specificity compared with human SK1 (Liu et al., 2000a; Pitson et al., 2000a). Thus, SK2 phosphorylates the sphingosine analogue FTY720 more efficiently than SK1 (Billich et al., 2003; Paugh et al., 2003; Sanchez et al., 2003).

The SKs share sequence similarity with the catalytic domain of the diacylglycerol (DAG) kinases (DGKs) (Sugiura et al., 2002). However, the sequences of SKs do not contain any other known protein domain.

The crystal structures of SK1 and SK2 have not been reported yet. A recent study, however, provided some insights into SK1 structure (Lim et al., 2011b). In particular, the authors propose that SK1 is an oligomeric protein, minimally a dimer, based on the finding that differentially-tagged recombinant SK1 co-immunoprecipitate when over-expressed in cells (Lim et al., 2011b). This possibility is further supported by structural data available for prokaryotic DGKs (Bakali et al., 2007; Nichols et al., 2007; Miller et al., 2008). Indeed, these lipid kinases, that have significant amino acid sequence homology with eukaryotic SK, are arranged as dimers (Bakali et al., 2007; Nichols et al., 2007; Miller et al., 2008).
Additionally, data obtained from kinetic analysis studies provide evidence that SK1 contains an allosteric site, and that the allosteric site exhibits auto-inhibitory activity on the catalytic site (Lim et al., 2011b).

The lack of information on SK structure has hampered the understanding of the molecular basis of the regulation of this enzyme. Nonetheless, a number of mechanisms that modulate SK1/SK2 function have been reported, and are described in the following section.

### 1.3.2 SK regulation

SK1 is catalytically active under basal conditions (Pitson et al., 2000a; Pitson et al., 2000b; Pitson et al., 2003). Stimulation of cells by a variety of growth factors, cytokines and other extracellular agonists results in further activation of this enzyme and S1P production (Pitson et al., 2000b; Taha et al., 2006b). Interestingly, S1P itself stimulates SK1 activity by binding to its cognate membrane receptors (Meyer zu Heringdorf et al., 2001) thereby creating a positive feedback amplification loop that may enhance S1P signalling. In addition to this rapid and transient activation of SK1 (after minutes of stimulation), a number of stimuli also induce a delayed increase in

SK1 activity (after hours of stimulation) due to transcriptional up-regulation of *SK1* expression to chronically increase S1P production (Shida et al., 2008).

The rapid increase in SK1 activity that is observed in response to extracellular stimuli is mediated by the ERK-1/2-dependent phosphorylation of SK1 at serine 225 (Ser225) (Pitson et al., 2003). Phosphorylation-dependent activation of SK1 is transient, as this enzyme is deactivated by dephosphorylation of phospho-Ser225 catalysed by the protein phosphatase PP2A (Barr et al., 2008). In particular, the B'α regulatory subunit of PP2A interacts with the C-terminus of SK1 to reduce SK1 phosphorylation (Pitman et al., 2011).

The extracellular agonists that promote SK1 activation also induce the rapid translocation of this enzyme from the cytoplasm to the plasma membrane (Pitson et al., 2003). This is mediated by the interaction of SK1 with the calmodulin-related protein CIB1 (calcium and integrin-binding protein 1), a $Ca^{2+}$-myristoyl switch protein (Jarman et al., 2010). SK1 activation results in increased $Ca^{2+}$ intracellular concentration (Spiegel and Milstien, 2003). Upon binding to $Ca^{2+}$, CIB1 undergoes a conformational change that has a dual effect: it enables CIB1 interaction with SK1 and it causes the exposure of CIB1 myristoylation site, which drives the translocation of the CIB1-SK1 complex to the plasma membrane (Jarman et al., 2010).

Besides increasing SK1 catalytic activity, phosphorylation at Ser225 is also involved in regulating the agonist-induced relocalization of SK1 (Pitson et al., 2003; Stahelin et al., 2005; Jarman et al., 2010; Pitman et al., 2011). In particular, CIB1 interacts with both phosphorylated and non-phosphorylated SK1 (Jarman et al., 2010). However, only phosphorylated SK1 appears to localize to the plasma membrane (Jarman et al., 2010). Indeed, phosphorylation of SK1 at Ser225 is necessary for prolonged retention of this enzyme at the plasma membrane as it induces conformational changes in SK1 that enhance its interaction with the phospholipid phosphatidylserine, which is localized on the inner leaflet of the plasma membrane (Stahelin et al., 2005). Interaction of SK1 with another membrane phospholipid, phosphatidic acid, has also been implicated in the localization of this enzyme to the

plasma membrane, although it has not been reported whether phosphorylation of SK1 increases its affinity for this lipid (Delon et al., 2004).
In keeping with a role for phosphorylation in promoting SK1 association with the plasma membrane, over-expression of B'α-PP2A blocks agonist-induced translocation of SK1 to the plasma membrane (Pitman et al., 2011).
Thus, ERK-1/2 and PP2A modulate SK1 activity and subcellular localization (and therefore SK1 function) by regulating its phosphorylation status.

The mechanisms that regulate SK2 are still largely unknown. Similar to SK1, SK2 regulation mediated by agonist-induced phosphorylation has been reported. In particular, EGF activates SK2 by inducing its ERK-1-dependent phosphorylation at Ser351 and Thr578 (Hait et al., 2005; Hait et al., 2007). In contrast with SK1, however, SK2 phosphorylation does not promote the translocation of this enzyme to the plasma membrane (Hait et al., 2005; Hait et al., 2007). EGF-induced activation of SK2 is physiologically relevant, as interfering with SK2 phosphorylation or down-regulating SK2 expression blocks cell migration in response to EGF (Hait et al., 2005; Hait et al., 2007).

Another mechanism of regulation of SK2 involves its phosphorylation by protein kinase D, which results in the export of SK2 from the nucleus to the cytoplasm (Ding et al., 2007).

Interestingly, proteomic analysis of cultured cells and tissue extracts revealed the presence of other phosphorylatable sites in endogenous SK2 in addition to those that have been characterised so far (Pitson, 2011), suggesting the existence of other mechanisms of phosphorylation-dependent regulation of SK2.

An additional mechanism of regulation of SK1/SK2 function involves their interaction with other proteins. Several proteins have been described that modulate SK activity via protein-protein interactions. One of these is the eukaryotic elongation factor 1A (eEF1A), that enhances the catalytic activity of both SK1 and SK2 in *in vitro* assays and in cells (Leclercq et al., 2008; Leclercq et al., 2011). Interestingly,

eEF1A had been previously shown to be involved in regulating cell growth and promoting tumorigenesis (Thornton et al., 2003). Significantly, a truncated, constitutively active isoform of eEF1A, termed prostate tumour inducer-1, induces neoplastic transformation via the activation of SK1 (Leclercq et al., 2011).

Conversely, other proteins interact with SK1 and/or SK2 to inhibit their activity. For instance, SK1-interacting protein (SKIP) inhibits the catalytic activity of SK1 *in vitro* and attenuates the pro-proliferative and anti-apoptotic effects of SK1 in cells (Lacana et al., 2002).

Protein-protein interactions also regulate SK1 function by controlling its intracellular localization. In addition to CIB1, another example is filamin A, that recruits SK1 at nascent lamellipodia to promote cell migration (Maceyka et al., 2008).

#### *1.3.2.1 Spatial regulation of SK*

A growing body of evidence indicates that the intracellular localization of SK is a key determinant of the function of SK, raising the concept of "spatial regulation" of SK.

SK1 resides predominantly in the cytosol in unstimulated cells, while it rapidly translocates to the plasma membrane upon agonist-induced activation (Pitson et al., 2003), as detailed above. In contrast, SK2 is found both in the cytosol and the nucleus, as well as in association with the plasma membrane and internal membranes, with its subcellular distribution that appears to differ depending on cell type (Igarashi et al., 2003; Maceyka et al., 2005; Sankala et al., 2007).

SK localization affects the function of SKs in several ways. First, the localization of SK to specific subcellular sites may result in the synthesis of its product, S1P in close proximity of its downstream effectors (either cell-surface receptors or intracellular targets). Second, the intracellular localization of SK may affect the access of this enzyme to its substrate. In this regard, it is important to appreciate that SK substrates (sphingosine and dihydrosphingosine) are hydrophobic and are therefore localized mainly within the membranes. In addition to this, it has been shown that sphingosine and dihydrosphingosine are localized to different membrane compartments, and SK

localization may therefore determine which substrate is utilized and therefore which product is synthesized. Third, the differential subcellular localization of SK may result in the regulation of distinct pools of sphingolipids, possibly via the functional interaction of SK1/SK2 with different S1P metabolising enzymes.
Examples of how SK localization affects the signalling of these enzymes are discussed below.

Several studies demonstrated that the localization of SK1 at the plasma membrane, rather than the phosphorylation-induced increase in its catalytic activity, is critical for the mitogenic and anti-apoptotic functions of this enzyme and therefore for the oncogenic effect of SK1 (Pitson et al., 2005; Hengst et al., 2009; Jarman et al., 2010). Indeed, a non-phosphorylatable mutant SK1 exhibits pro-survival and pro-mitogenic properties when artificially targeted to the plasma membrane (Pitson et al., 2005). In contrast, preventing SK1 translocation to the plasma membrane blocks SK1-induced cell growth (Pitson et al., 2005; Hengst et al., 2009; Jarman et al., 2010).
Significantly, plasma membrane targeting of SK1 results in enhanced S1P generation and release from cells (Pitson et al., 2005). In this regard, it is important to appreciate that SK1 substrate, sphingosine, mainly resides in the plasma membrane (Slife et al., 1989). In addition, the production of S1P at the plasma membrane might favour its release from cells due to privileged interaction with membrane transporters. It is therefore logical to propose that production of S1P at the plasma membrane would also result in the privileged binding of S1P to its cognate cell-surface receptors and thus in enhanced S1P inside-out signalling.

Localization of SK2 to the nucleus has been shown to be necessary for cell cycle arrest and cell growth suppression induced by this enzyme (Igarashi et al., 2003; Okada et al., 2005). A more recent study provided an explanation for these findings demonstrating that SK2-generated nuclear S1P inhibits the activity of HDAC thereby promoting the expression of the cyclin-dependent kinase inhibitor p21 (Hait et al., 2009). Indeed, depletion of SK2 from the nucleus results in decreased levels of nuclear S1P and histone acetylation (Hait et al., 2009). Significantly, artificial

targeting of SK1 to the nucleus results in the inhibition of DNA synthesis (Igarashi et al., 2003).

A study by Wattenberg and colleagues provides evidence that "substrate compartmentalization" is another means by which SK1 localization may affect the function of this enzyme (Siow et al., 2010). In particular, cytosolic and ER-localized SK1 have access to dihydrosphingosine to produce dihydroS1P, while dihydrosphingosine is not available for SK1 localized at the plasma membrane (Siow et al., 2010). Thus, SK1 subcellular localization determines the bioactive product that is generated by this enzyme, depending on substrate availability. Significantly, this affects the downstream signalling of SK1. Indeed, it has been shown that S1P and dihydroS1P may have opposite effects in cells. For instance, formation of dihydroS1P is associated with the inhibition of cell migration and $Ca^{2+}$ signalling in response to S1P (Berdyshev et al., 2006).

Significantly, the differential effect that SK1 and SK2 have on cell fate (pro-survival *versus* pro-apoptotic, respectively) is in part related to their distinct subcellular localization which, in turn, accounts for the opposing functions of the two enzymes in regulating ceramide levels (Maceyka et al., 2005). Indeed, SK1 reduces, while SK2 increases intracellular ceramide content when over-expressed in cells (Maceyka et al., 2005). Spiegel and colleagues demonstrated that the localization of SK2 to the ER is functional for its ability to increase ceramide levels and consequently for the pro-apoptotic effect of this enzyme (Maceyka et al., 2005). Consistent with this possibility, SK1 increases ceramide and promotes apoptosis to a similar extent as SK2 when artificially targeted to the ER (Maceyka et al., 2005). Spiegel and colleagues proposed that ER-localized SK2 might act in concert with S1P phosphatases to generate sphingosine which is then converted back to ceramide; this sphingosine "recycling" pathway would enable cells to minimize the energetically exhaustive *de novo* synthesis of ceramide (Maceyka et al., 2005). Consequently, SK2 over-expression results in enhanced recycling of sphingosine and generation of pro-apoptotic ceramide. The model proposed by Spiegel is further supported by other studies providing evidence for the existence of a functional interaction between SK

and S1P phosphatases which is active in sphingosine recycling (Le Stunff et al., 2007; Siow et al., 2010).

### 1.3.3 SK1: housekeeping *versus* signalling function

Based on the finding that SK1 has a high basal activity (Pitson et al., 2000a), Wattenberg and colleagues proposed that SK1 might have a "housekeeping function" (which consists of controlling the intracellular levels of ceramide and sphingolipid metabolism) that is distinct from its "signalling function" (dependent on agonist-induced activation and translocation to the plasma membrane of SK1 and generation of S1P) (Wattenberg et al., 2006).
Indeed, as mentioned above, there is strong evidence showing that SK1 has a unique role in modulating ceramide levels and sphingolipid metabolism. SK1 controls the sphingolipid biosynthetic pathway at several points. First, SK1 can reduce ceramide levels by utilizing sphingosine as a substrate to produce S1P. It is also important to appreciate that, since the cleavage of S1P catalysed by S1P lyase is the only way that cells have to eliminate the sphingosine backbone, SK1 has a critical role in controlling the cellular levels of sphingolipids via the generation of S1P.

Second, SK1 can utilize dihydrosphingosine as a substrate to generate dihydroS1P. Indeed, SK1 (but not SK2) over-expression increases the intracellular levels of dihydroS1P in various cell systems (Maceyka et al., 2005; Berdyshev et al., 2006; Siow et al., 2010). By doing so, SK1 prevents dihydrosphingosine from entering the ceramide *de novo* pathway (Figure 1.1), thereby reducing the amount of *de novo*-synthesized ceramide.

Finally, evidence exists that SK1-generated S1P might negatively regulate ceramide *de novo* biosynthesis. Indeed, it has been shown that phosphorylated analogues of sphingosine inhibit the activity of serine palmitoyltransferase (van Echten-Deckert et al., 1997), that catalyses the first step of this pathway (Figure 1.1). Additionally, SK1-generated S1P might inhibit (dihydro)ceramide synthase activity. Indeed, over-expression of SK1 (but not SK2) results in the accumulation of the

(dihydro)ceramide synthase substrate, dihydrosphingosine, while reducing ceramide levels (Maceyka et al., 2005; Berdyshev et al., 2006; Siow et al., 2010). The ability of S1P to inhibit the activity of these enzymes was confirmed by *in vitro* assays (Laviad et al., 2008).

It is important to appreciate that, given that virtually all of the sphingolipids have signalling functions, SK1 has a crucial role in cell physiology by modulating sphingolipid metabolism, which might be as important as the generation of a bioactive molecule, S1P.

## 1.4 SK/S1P signalling and cancer

There is now substantial evidence in support of a major role of the SK/S1P axis in human cancers (Pyne and Pyne, 2010). As discussed in the previous sections, S1P signalling regulates cell proliferation, survival and migration. Thus, it is not surprising that aberrant activation of this signalling pathway contributes to tumour development and progression being able to promote the major hallmarks of cancer, as defined by Hanahan and Weinberg (2000), including enhanced proliferation and evasion of growth suppression, reduced apoptosis, angiogenesis and invasiveness/metastasis.

Although the vast majority of studies on S1P and cancer focused on investigating the role of SK1, it is now evident that all of the components of the S1P pathway, including S1P receptors and S1P-metabolising enzymes (that together with SKs modulate S1P levels) participate to aberrant cellular processes that are integral to cancer pathogenesis (Pyne and Pyne, 2010). Interestingly, despite a number of studies linking SK2 to growth inhibitory and pro-apoptotic effects, recent reports provided evidence for a critical role of this enzyme in cancer cells.

The current knowledge of how dysregulated S1P signalling contributes to cancer development and progression is discussed in the following section. With relevance to

the topic of the current study, attention will be focused on the role of SKs (Figure 1.4). The potential of these enzymes as therapeutic targets for cancer treatment will also be discussed. Significantly, SKs have also a crucial role in the acquisition of resistance to therapeutic agents by cancer cells, as detailed in section 1.4.3. Additionally, the involvement of SK1 and S1P signalling in various aspects of prostate cancer biology, including the progression to androgen independence and the resistance to therapeutic agents, will be discussed in section 1.5.

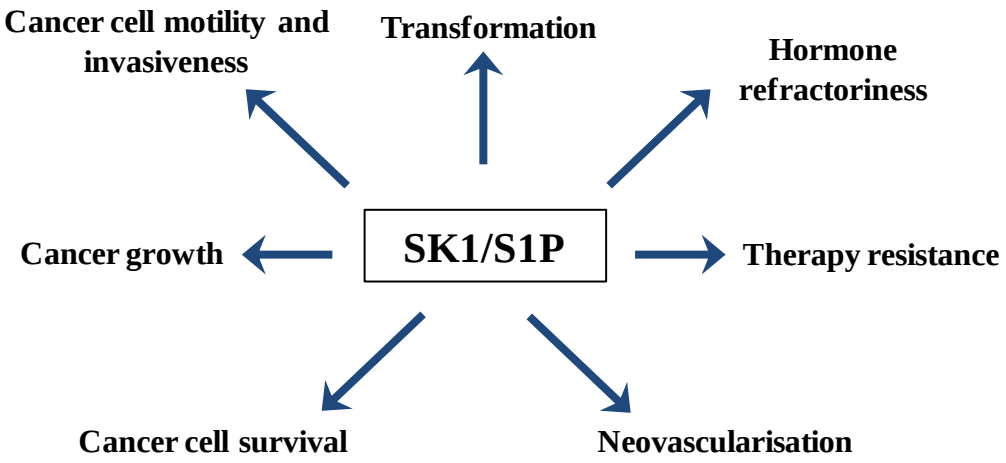



**Figure 1.4** ***Role of SK1/S1P signalling in cancer: an overview (adapted from Pyne and Pyne, 2010).***

### 1.4.1 S1P signalling in cancer development and progression

The first demonstration that S1P signalling was involved in oncogenesis came from a study showing that the over-expression of catalytically active SK1 in fibroblasts was sufficient to induce their transformation and the acquisition of tumorigenicity by these cells, i.e. the ability to form tumours when injected in mice (Xia et al., 2000). Significantly, cells over-expressing SK1 show a reduced dependence on serum for proliferation and escape contact inhibition (as demonstrated by increased clonogenicity) (Xia et al., 2000), two major characteristics of cancer cells. The oncogenic action of SK1 was found to be dependent on the translocation of this

enzyme to the plasma membrane (Xia et al., 2000; Pitson et al., 2005). Based on the ability of SK1 to induce cell transformation (Xia et al., 2000), it was assumed that *Sk1* was an oncogene, although no tumour-inducing mutations of this gene have been reported yet.

Analysis of clinical samples support the relevance of SK1 in human cancers demonstrating that SK1 mRNA and protein levels are markedly up-regulated in numerous types of cancer (including both solid tumours and haematological malignancies) compared with their normal counterparts (French et al., 2003; Johnson et al., 2005; Van Brocklyn et al., 2005; Kawamori et al., 2006). Significantly, clinical studies also demonstrate that high SK1 expression correlates with adverse clinical features such as increased tumour grade, development of resistance to anticancer agents and reduced patient survival (Van Brocklyn et al., 2005; Li et al., 2008b; Ruckäberle et al., 2008; Li et al., 2009; Long et al., 2010a; Watson et al., 2010; Ohotski et al., 2012), providing compelling evidence for a functional role of SK1 in the progression of human cancers. Conversely, SK2 was not found to be over-expressed in human cancers (including solid tumours and haematological malignancies) (Van Brocklyn et al., 2005; Sobue et al., 2006; Bayerl et al., 2008; Ruckhäberle et al., 2008), although a limited number of clinical studies as yet have investigated the relation between SK2 and cancer.

Altered expression of S1P receptors, as well as S1P lyase and phosphatases, has also been reported in human cancers, although in most cases the relevance of these deregulations remains to be determined (Pyne and Pyne, 2010). However, a clear correlation between high expression of $S1P_1$/$S1P_3$ and reduced patient survival was demonstrated in oestrogen-positive breast cancer patients (Watson et al., 2010).

It is well established that SK1 stimulates cell proliferation and induces protection against apoptotic stimuli in a wide variety of cell systems (section 1.2.2; section 1.2.3). Significantly, SK1 over-expression promotes cell proliferation in the absence of growth factors (Olivera et al., 1999; Sukocheva et al., 2003), which characterises cancer cells.

SK1 is a critical regulator of cancer cell growth and survival. Indeed, SK1 over-expression promotes proliferation and protects cells from apoptosis in a variety of cancer cell lines (Nava et al., 2002; Bektas et al., 2005; Sarkar et al., 2005; Van Brocklyn et al., 2005; Bonhoure et al., 2008; Sukocheva et al., 2009). Accordingly, siRNA knock-down of SK1 expression or pharmacological inhibition of SK1 increases the ceramide/S1P ratio to inhibit cell-cycle progression and cell proliferation while inducing apoptosis in multiple cancer cell lines (Taha et al., 2004; Sarkar et al., 2005; Van Brocklyn et al., 2005; Bonhoure et al., 2006; Taha et al., 2006a; Baran et al., 2007; Guillermet-Guibert et al., 2009; Huwiler et al., 2011; Antoon et al., 2012). Thus, the sphingolipid rheostat is functional in cancer cells.
Similar results were obtained from studies in animal models of cancer. Thus, cancer cells over-expressing SK1 form larger tumours when injected in nude mice compared to cells that express endogenous SK1 only (Nava et al., 2002), while genetic deletion of SK1 results in tumour regression (Kohno et al., 2006).

Taken together, these findings support the notion that SK1 over-expression might confer a growth and survival advantage to tumour cells that results in their positive selection and clonal expansion during cancer progression.

A role for SK2 in cancer was supported by a study showing that the growth of tumours formed by SK2-deficient breast cancer cells injected in nude mice was substantially reduced compared with control cells (Weigert et al., 2009). Genetic ablation of SK2 was then found to inhibit proliferation in multiple cancer cell lines (section 1.4.2). Similar results were obtained using a pharmacological inhibitor of this enzyme that reduced cancer cell growth *in vitro* and *in vivo* (French et al., 2010; Antoon et al., 2010), as detailed in section 1.4.4.

Another means by which S1P signalling may contribute to cancer progression is by stimulating cancer cell migration to promote their invasiveness and metastatic potential. Indeed, S1P induces a migratory phenotype in a receptor-dependent manner in a variety of cancer cell lines (Van Brocklyn et al., 2003; Park et al., 2007; Bergelin et al., 2009; Long et al., 2010a; Kim et al., 2011). As discussed in section

1.2.2.1, however, S1P has a dual action on cell motility, as it exerts a pro-migratory effect through $S1P_1$/$S1P_3$ and an anti-migratory effect through $S1P_2$. Thus, the migratory response of tumour cells to S1P is determined by the relative abundance of these receptor subtypes. This is exemplified by the finding that S1P stimulates cell migration in gastric cancer cells whereas it inhibits the migration of B16 melanoma cells which exclusively express $S1P_3$ or $S1P_2$, respectively (Yamashita et al., 2006; Yamaguchi et al., 2003). Consistent with the notion that S1P/$S1P_1$ signalling stimulates cell motility, over-expression of this receptor subtype in cancer cells results in enhanced metastatic spread in mice (Yamaguchi et al., 2003).
Importantly, siRNA knock-down of SK1 expression inhibits breast cancer cell migration in response to various chemoattractants (Sarkar et al., 2005; Döll et al., 2005).

SK2 is also involved in promoting cell migration in some cancer cell types. For instance, SK2 is required for the migration of MDA-MB-453 breast cancer cells in response to EGF (Hait et al., 2005).

Interestingly, an additional mechanism has been recently described by which S1P may promote tumour cell invasiveness and metastasis. In fact, S1P binding to its receptors on cancer cells enhances the synthesis or stimulates the activity of matrix metalloproteases that degrade the extracellular matrix to enable cancer cells to penetrate into the circulation (Young et al., 2009; Kim et al., 2011).

A fundamental contributor to the progression of solid cancers is the neovascularisation of the tumour mass to ensure oxygen and nutrient supply that sustain cancer cell growth. Additionally, tumour vascularisation is also crucial for the spread of cancer cells to distant sites via the bloodstream, i.e. for the formation of metastases.
S1P signalling is critical for tumour-associated angiogenesis. Indeed, a recent *in vitro* study demonstrated that SK1-derived S1P secreted from cancer cells acts in a paracrine manner on co-cultured endothelial cells to induce angiogenesis (Anelli et al., 2010). More importantly, cancer cells over-expressing SK1 form tumours with

increased vascularisation when injected in nude mice (Nava et al., 2002). *In vivo* studies also demonstrated that $S1P_1$ expression is markedly up-regulated in tumour vessels and siRNA knock-down of this receptor strongly inhibits angiogenesis and therefore tumour growth (Chae et al., 2004). Additionally, an antibody against S1P suppressed blood vessel formation induced by VEGF, basic fibroblast growth factor and multiple cytokines in murine models of various types of cancer (Visentin et al., 2006), providing evidence that S1P is required for the pro-angiogenic effect of these molecules. Importantly, S1P also induces the release of pro-angiogenic cytokines and growth factors from cancer cells (Visentin et al., 2006).
In keeping with the correlation between tumour vascularisation and metastatic spread of cancer cells, the anti-S1P antibody also reduced tumour metastasis *in vivo* (Visentin et al., 2006).

Hypoxia, a condition defined by subnormal tissue oxygen levels, is a common characteristic of solid tumours (Vaupel and Mayer, 2007). Interestingly, hypoxia promotes the transcriptional up-regulation of SK1 expression via the hypoxia-inducible factors HIF1α and HIF2α in both normal and cancer cells (Ahmad et al., 2006; Anelli et al., 2008). This, in turn, results in increased production and release of S1P from hypoxic cells that may promote the formation of blood vessels to re-supply oxygen and nutrients to cancer cells.

Actively growing solid tumours may also experience nutrient insufficiency. Interestingly, nutrient starvation has been shown to activate SK1 in breast cancer cells and this, in turn, activates autophagy to sustain cell growth in the absence of extracellular nutrients (Lavieu et al., 2006). Thus, SK1-induced autophagy may represent a transient adaptive response that enables cancers cells to survive and proliferate in the presence of low nutrient levels until neovascularisation of the tumour mass takes place.
Interestingly, SK1 activity is stimulated under hypoxic conditions in multiple cancer cell lines (Ader et al., 2008). Increased SK1 activity results in the accumulation of HIF1α due to inhibition of its proteasomal degradation (Ader et al., 2008). Conversely, pharmacological inhibition of SK1 or down-regulation of SK1

expression reduces HIF1α levels and activity in these cells (Ader et al., 2008). Thus, a positive feedback loop might exist that amplifies SK1 and HIF1α signalling and this might be crucial for cancer progression. Indeed, HIF activates the expression of proteins involved in tumour growth, angiogenesis, metastasis and drug resistance thereby contributing to the development of an aggressive phenotype (Ader et al., 2008).

SK2 expression and activity are also increased by hypoxia (Schnitzer et al., 2009). This results in S1P release and activation of $S1P_1$/$S1P_3$ that protects lung cancer cells from etoposide-induced apoptosis (Schnitzer et al., 2009). Thus, S1P signalling mediates hypoxia-induced chemoresistance.

From the studies reported above, it is clear that in addition to the oncogenic activity that the SK/S1P pathway exerts on its own, S1P signalling also contributes to cancer progression by mediating the effects of other molecules implicated in cancer (e.g. EGF, VEGF, oestrogen, HIF). Furthermore, functional interactions exist between oncogenes and components of S1P signalling, that mediate oncogene actions or facilitate oncogene-dependent signal transmission. For instance, oncogenic Ras stimulates SK1 activity in fibroblast and this is required for Ras-induced transformation of these cells (Xia et al., 2000). Another example is the functional interaction between $S1P_4$ and HER2 (human EGF receptor 2), an oncogene involved in breast cancer progression (Borg et al., 1990). In particular, S1P binding to $S1P_4$ results in HER2 transactivation and enhanced stimulation of the downstream ERK-1/2 pathway in breast cancer cells (Long et al., 2010b), which has a well-established role in breast cancer metastasis. Also, high SK1 expression is associated with reduced survival in breast cancer patients whose tumours are HER2 positive (Ohotski et al., 2012).

Finally, a functional interaction has been recently described between SK1 and the tumour suppressor p53 (Heffernan-Stroud et al., 2012). In particular, the authors of this study demonstrated that p53 negatively regulates SK1 expression. Genotoxic stress was found to induce the proteolytic degradation of SK1 via the p53-dependent activation of caspase-2; this, in turn, shifts the ceramide/S1P balance in favour of

ceramide to promote apoptosis. Conversely, p53 knock-out resulted in increased levels of SK1 and reduced ceramide/S1P ratio to promote carcinogenesis. Importantly, deletion of SK1 in p53 null mice protected from tumour development. Thus, this study demonstrates that the tumour suppressive function of p53 is at least in part mediated by the effect of p53 on SK1 expression and ensuing deregulation of sphingolipid levels. Moreover, this study provides evidence that p53 inactivating mutations (that are common in diverse types of human tumours: Hollstein et al., 1991) might promote carcinogenesis because of increased SK1 signalling. Significantly, these findings suggest that inhibition of SK1 is a promising therapeutic option for the treatment of p53-mutated cancers.

### 1.4.2 SK1 *versus* SK2 in cancer cells

Unexpectedly, Gao and Smith have recently demonstrated that in various cancer cell lines (including kidney and breast cancer cells) ablation of SK2 has stronger anticancer effects than ablation of SK1 (Gao and Smith, 2011), suggesting that some cancer cell types might rely on SK2 more than on SK1 function. In particular, genetic knock-down of SK2 was more effective in inhibiting cell proliferation and migration than genetic knock-down of SK1. Moreover, combined treatment with both SK1 siRNA and SK2 siRNA did not further reduce cell proliferation nor migration compared with SK2 siRNA alone.
Similar results were obtained in glioblastoma cells, where down-regulation of SK2 expression suppressed cell proliferation to a higher extent than knock-down of SK1 (Van Brocklyn et al., 2005).

Gao and Smith also demonstrated that SK1- and SK2-selective ablation exerts differential effects on various signalling pathways (p53, p21 and ERK-1/2, involved in cell-cycle control and proliferation; FAK and VCAM, involved in cell migration) which are consistent with their effects on cell-cycle and cell motility.
Interestingly, genetic knock-down of SK2 resulted in increased SK1 expression and activity. However, the ensuing elevation in cellular S1P levels could not compensate the effect of SK2 loss on cell proliferation (Gao and Smith, 2011). This finding

suggests that SK2 might regulate a pool of S1P that is functionally distinct from S1P formed by SK1 in the cancer cell systems examined. An alternative explanation is that SK2 might regulate cell proliferation of these cells via yet unidentified non-catalytic functions.

Taken together, the results of the study by Gao and Smith support the notion that the functions of SK1 and SK2 are not completely redundant in the cancer cell types studied, i.e. SK1 and SK2 cannot substitute for each other.
Another study demonstrated that both SK1 and SK2 are required for EGF-induced migration in MDA-MB-453 breast cancer cells (Hait et al., 2005). In fact, down-regulating the expression of SK1 or SK2 with specific siRNA suppresses cell migration in response to EGF while cells are still responsive to exogenous S1P.
Understanding whether SK1 and SK2 have redundant, complementary or opposing functions in cancer cells requires further investigation.

### 1.4.3 SK and chemotherapy resistance

The development of resistance to radio- and chemo-therapy by tumour cells is obviously a major problem in terms of successful treatment of cancer and contributes significantly to mortality associated with this disease. SK1 has a critical role in regulating the response of cancer cells to therapeutic treatments, i.e. sensitivity *versus* resistance to apoptosis induced by these treatments.
This is exemplified by numerous studies in cellular and animal systems showing that enforced SK1 over-expression results in enhanced resistance to chemotherapeutic drugs; conversely, pharmacological inhibition or siRNA knock-down of SK1 sensitise cancer cells to chemotherapeutic drugs (Bektas et al., 2005; Bonhoure et al., 2006; Baran et al., 2007; Sobue et al., 2008; Guillermet-Guibert et al., 2009; Marfe et al., 2011). Significantly, these effects are associated with changes in the ceramide/S1P balance, indicating that the sphingolipid rheostat is functional in regulating the cellular response to chemotherapeutic agents.
*In vitro* studies also showed that cancer cell lines that are resistant to chemotherapeutic drugs have higher expression levels of SK1 compared with their

chemotherapeutic-sensitive counterparts and that only the latter produce ceramide upon drug treatment (Bonhoure et al., 2006; Baran et al., 2007; Sobue et al., 2008). In this regard, it is important to appreciate that a number of stress stimuli, including chemotherapeutic agents and ionizing radiations, induce the generation of ceramide in cancer cells (either via the *de novo* or the sphingomyelinase pathway) and this mediates apoptosis in response to these agents (Oskouian and Saba, 2010).
Taken together, these findings are consistent with the possibility that SK1 might at least in part contribute to chemoresistance of cancer cells by reducing cellular levels of ceramide (section 1.3.3).

A number of mechanisms have been described by which SK1 induces resistance to chemotherapeutic drugs, some of which are discussed herein. The role of SK1 in mediating the resistance of prostate cancer cells to therapeutic agents is well established and will be discussed in more detail in section 1.5.
Huwiler and colleagues demonstrated that genetic down-regulation or pharmacological inhibition of SK1 increases the intracellular levels of reactive oxygen species in multiple cancer cell types (Huwiler et al., 2011). This, in turn, results in DNA damage which might prime cancer cells for additional damage by the genotoxic agent doxorubicin. Indeed, SK1-deficient cancer cells are more sensitive to DNA damage and ensuing apoptosis induced by doxorubicin than control cells (Huwiler et al., 2011).
A recent study by Ogretmen and colleagues elucidated the molecular mechanism by which SK1 induces the acquisition of resistance to the anticancer agent imatinib in chronic myeloid leukaemia (CML) (Salas et al., 2011). CML patients express the oncoprotein Bcr/Abl, a constitutively active tyrosine kinase that activates multiple signalling pathways to promote cancer progression. Imatinib, a Bcr/Abl inhibitor, is the mainstay therapeutic option for treatment of CML. SK1 contributes to the resistance of CML cells to this anticancer agent as demonstrated by the finding that siRNA knock-down of SK1 expression sensitises resistant CML cells to imatinib whereas enforced expression of SK1 prevents apoptosis in response to this drug in sensitive CML cells (Baran et al., 2007).

Salas and colleagues demonstrated that S1P/$S1P_2$ signalling regulates Bcr/Abl stability in CML cells by inhibiting the activity of the protein phosphatase PP2A. This prevents the dephosphorylation of Bcr/Abl and therefore its proteasomal degradation, resulting in the accumulation of the oncoprotein. Thus, inhibition of the SK1/S1P/$S1P_2$ pathway sensitises CML cells to imatinib by restoring the PP2A-dependent dephosphorylation and subsequent degradation of Bcr/Abl (Salas et al., 2011).
Interestingly, FTY720 was previously reported to induce apoptosis in CML cells via direct activation of PP2A (Neviani et al., 2007). In light of the studies by Salas et al. and Tonelli et al. (2010) reporting that FTY720 is a novel SK1 inhibitor, it is conceivable that FTY720-induced apoptosis in CML cells might be due not only to its direct action on PP2A but also to the inhibition of SK1 activity and consequent disruption of the S1P/$S1P_2$ signalling.

Interestingly, evidence also exists that the SK1/S1P/S1P receptors pathway enhances the expression and activity of drug efflux pumps in cancer cells, which are major contributors to the development of multidrug resistance in cancer (Pilorget et al., 2007). This might represent an additional mechanism by which SK1 might contribute to chemotherapy resistance.

SK2 is also involved in resistance to chemotherapy. This is exemplified by the finding that siRNA knock-down of SK2 expression enhances apoptosis induced by the anti-cancer agent doxorubicin in breast and colon cancer cells, an effect that was associated with a reduction in the expression of the cell-cycle regulator protein p21 (Sankala et al. 2007). Indeed, SK2-generated S1P promotes p21 expression by modulating the activity of histone deacetylases (Hait et al., 2009).
Similarly, pharmacological inhibition of SK2 sensitised hepatocellular carcinoma cells to the anti-cancer drug sorafenib and, more importantly, potentiated its anti-tumour activity *in vivo* (Beljanski et al., 2011).

Taken together, these studies indicate that combination therapies of SK1/SK2 inhibitors with conventional anticancer agents might be a valuable option for the

clinical management of therapeutic-resistant cancers. Significantly, it is expected that this approach would also enable the administration of lower doses of chemotherapeutic drugs, thereby reducing the risk of side-effects.

### 1.4.4 SK as a therapeutic target

The data from the studies presented in the previous sections provide compelling evidence that targeting S1P signalling is an attractive therapeutic approach for cancer treatment. Indeed, inhibiting S1P pathway in cancer has the potential to impact a number of processes that are instrumental in cancer progression. Significantly, SK/S1P signalling appears to be almost universally involved in human tumours, so that therapies targeted to this pathway may be widely applied for cancer treatment.

Among the potential strategies for inhibiting S1P signalling, inhibition of SK1 offers several major advantages, which are: 1) shift of the sphingolipid rheostat due to reduction of S1P and concurrent increase of cellular levels of pro-apoptotic ceramide; 2) inhibition of both extracellular and intracellular actions of S1P; 3) inhibition of growth factor- and hypoxia-dependent responses; 4) sensitisation of cancer cells to therapeutic agents.

The results of pre-clinical studies using SK inhibitors in a variety of cancer models confirmed that SK-targeted therapy may be a valuable option for clinical management of cancer, as detailed below.
Thus, the development of SK1 inhibitors for clinical use as anti-cancer agents has attracted considerable attention over the last few years. Despite a high interest in these compounds, however, there are only very few established SK inhibitors to date and the development of new SK inhibitors with improved potency and selectivity and effective *in vivo* remains a problem to be addressed.

Until recently, the SK inhibitors most commonly used in pharmacological studies were sphingosine analogues, namely N,N-dimethylsphingosine and D,L-*threo*-dihydrosphingosine. These compounds inhibit cell growth and induce apoptosis in

multiple cancer cell types and overcome resistance to therapeutic agents (Endo et al., 1991; Sweeney et al., 1996; Nava et al., 2002). However, although showing low-micromolar potency against SK1 and SK2 activity *in vitro* (Olivera et al., 1998; Liu et al., 2000a), sphingosine analogues lack of selectivity as they also inhibit protein kinase C and affect other signalling pathways (Pyne and Pyne, 2010). Consequently, despite being effective in reducing tumour growth and metastasis, these compounds cause severe side effects when used *in vivo* (Endo et al., 1991; Shirahama et al., 1997).

Novel, non-lipid inhibitors of SK were identified by French et al. by screening a library of structurally diverse synthetic compounds, that were found to potently inhibit SK activity without affecting other lipid kinases or protein kinases (French et al., 2003). Among these, 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole was the most potent and selective in inhibiting SK activity in *in vitro* assays (French et al., 2003). Significantly, this compound was found to inhibit cell proliferation and induce apoptosis in a variety of human cancer cell lines and to effectively suppress tumour growth *in vivo* without causing side effects (French et al., 2003; French et al., 2006).

More recently, amidine-based sphingosine analogues have been synthesised and characterized which exhibit nanomolar potency in inhibiting SK1 and/or SK2 activity *in vitro* and in cancer cells (Mathews et al., 2010; Kennedy et al., 2011).

FTY720 has anticancer effects in multiple models of cancer, being able to induce growth arrest and apoptosis in cancer cell lines and to effectively reduce tumour growth, vascularisation and metastasis in mice, as will be detailed in Chapter 4. Interestingly, FTY720 was recently found to inhibit SK1 activity *in vitro* (Tonelli et al., 2010). Thus, the reported anticancer actions of FTY720 are likely to be dependent on both functional antagonism of $S1P_1$ and inhibition of SK1.
An FTY720 analogue (OSU-2S) has been recently synthesised that lacks $S1P_1$ receptor activity and therefore does not induce lymphopenia, thereby reducing the risk of potential side effects (Omar et al., 2011). OSU-2S is more potent than

FTY720 in inhibiting SK1 activity *in vitro* and suppresses tumour growth *in vivo* without inducing adverse effects (Omar et al., 2011).

The first validation of SK2 as a therapeutic target for cancer treatment was provided by a recent study from French et al. (2010). In fact, this study reported that an SK2-selective inhibitor, ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide] inhibited cell viability and migration when tested in a panel of cancer cell types (French et al., 2010). ABC294640 also exhibits anticancer activity *in vivo* being able to inhibit tumour growth associated with reduction in S1P levels (French et al., 2010). Interestingly, the reduction of cancer cell viability by ABC294640 was associated with the activation of autophagy-mediated death (Beljanski et al., 2010). Additionally, it has been demonstrated that ABC294640 binds to ER acting as an antagonist of this receptor, thereby inhibiting ER signalling (Antoon et al., 2010). This provides an additional means by which ABC294640 might suppress breast cancer cell growth (French et al., 2010; Antoon et al., 2010). Importantly, ABC294640 shows favourable chemico-physical and pharmacological properties and low toxicity in mice (French et al., 2010).

## 1.5 SK1 and prostate cancer

SK1 and S1P signalling exert multiple effects on prostate cancer cells that are conducive to cancer progression, which are discussed in the following sections. This will be preceded by a general introduction on prostate cancer focused on the mechanisms that underlie the development of androgen-independent prostate cancer.

### 1.5.1 Prostate cancer: an overview

The latest statistics available indicate that prostate cancer is the most common cancer in men in the UK, with 40,841 new cases diagnosed in 2009 (25% of all new cases of cancer in males), and the second most common cancer in men worldwide (14% of all new cases of cancer in males) (http://www.statistics.gov.uk, *last accessed 01 June*

*2012*). Although improvement in early detection techniques resulted in the reduction in prostate cancer death rate over the last few years (Siegel et al., 2012), prostate-cancer related mortality is still high and this pathology is the second leading cause of cancer-related deaths in the male population of Western countries, with 10,721 deaths in 2010 in UK (http://www.statistics.gov.uk, *last accessed 01 June 2012*). This is due to the fact that although localized prostate cancers are curable through surgery (prostatectomy) or radiation therapy, no curative therapies are currently available for locally advanced or metastatic tumours, for which the prognosis is almost invariably fatal (see below).

The molecular mechanisms underlying prostatic carcinogenesis are still poorly understood, although it has been shown that androgen signalling through the androgen receptor (AR) is necessary for this process. Indeed, AR knock-out mice do not develop prostate cancer (Yeh et al., 2002).

AR is a ligand-activated nuclear transcription factor belonging to the superfamily of steroid hormone receptors (MacLean et al., 1997). Its sequence consists of an amino-terminal transcriptional activation domain, a central DNA-binding domain, a hinge region (containing a nuclear localization signal) and a carboxy-terminal ligand-binding domain (Grossmann et al., 2001). Ligand-free AR is localized in the cytoplasm and bound to inhibitory chaperones such as heat-shock proteins that prevent its nuclear translocation but also stabilize AR in a conformation that favours androgen binding (Yeh et al., 1999b). Upon binding with its ligand (testosterone and its intracellular derivative dihydrotestosterone: Lindzey et al., 1994), AR undergoes conformational changes that result in its dissociation from inhibitory proteins. This is followed by receptor homodimerization and phosphorylation and subsequent translocation from the cytoplasm to the nucleus. Here, AR binds to specific genomic regions in the promoter and enhancer regions of target genes, which results in the recruitment of cofactors and the formation of an active transcription apparatus (Shang et al., 2002) to activate the expression of genes involved in pro-growth and pro-survival signalling (Mangelsdorf et al., 1995). Noteworthy, in addition to this "genotropic signalling", a different mechanism of action of AR has been described

which involves the modulation of intracellular cascades by AR via direct protein-protein interactions (Unni et al., 2004). The term “non-genotropic” has been used to describe this signalling mechanism which does not involve AR nuclear translocation nor direct interaction with DNA (Unni et al., 2004).
The transcriptional program activated by AR is essential for the differentiation, growth and function of normal prostate (Dehm and Tindall, 2006).

The growth and survival of prostate cancer cells is initially dependent on androgen stimulation (Denmeade et al., 1996) and this reliance is exploited in the clinic. Indeed, the main therapeutic approach for the treatment of locally advanced or metastatic disease is based on preventing the expression of AR-regulated genes. This is achieved by suppressing androgen production via surgical and/or chemical castration (androgen ablation therapy) and/or by blocking AR activation via administration of AR antagonists (antiandrogen therapy) (Denis and Griffiths, 2000). However, despite the initial effectiveness in inducing tumour regression, these AR-directed therapies ultimately fail in the large majority of the patients due to the development of an androgen-independent (also termed hormone-refractory or castration-resistant) prostate cancer which occurs within a period of approximately 18 months from treatment (Lee et al., 2008). The mechanisms underlying the acquisition of androgen independence are still not fully understood, which hampered the development of effective therapies. Indeed, castration-resistant prostate cancer is currently incurable and has an inevitably fatal outcome with a median survival of approximately 18 months from its onset (Lee et al., 2008).
Clinical studies have shown that AR is expressed in nearly all prostate cancers, both androgen-dependent and androgen-independent and regardless of tumour stage (Sadi et al., 1991; van der Kwast et al., 1991; Ruizeveld de Winter et al., 1994; Tilley et al., 1994; Hobish et al., 1995; van der Kwast et al., 1996). Significantly, gene expression analysis of human prostate cancer samples demonstrated that AR-regulated genes (including prostate-specific antigen, PSA) that are suppressed upon androgen deprivation therapy are re-expressed in tumours that have progressed to hormone independence (Holzbeierlein et al., 2004). This indicates that AR function has been inappropriately restored in androgen-independent prostate cancers, i.e. AR

signalling is active despite the (virtual) absence of androgen stimulation. Thus, the reactivation of AR signalling is a key determinant of "androgen escape" and therefore therapeutic failure.

A number of mechanisms have been described by which AR activation may occur in the presence of castration levels of androgen. These mechanisms, that are not mutually exclusive and may operate together, include: 1) AR amplification/over-expression, 2) AR mutations, 3) alterations of AR co-factors, 4) AR transactivation via cross-talk with other signalling pathways, and 5) generation of constitutively active AR variants. These aberrant mechanisms of AR activation are discussed below.

*1) AR amplification/over-expression*-- AR over-expression is common in hormone-refractory human prostate cancers (Chen et al., 2008). *AR* gene amplification is reported in 25-30% of patients with castration-resistant prostate cancer while it is present only in 1-2% of primary tumours (Visakorpi et al., 1995; Koivisto et al., 1997; Bubendorf et al., 1999; Linja et al., 2001; Edwards et al., 2003; Ford et al., 2003) and is associated with increased AR protein levels (Edwards et al., 2003; Ford et al., 2003). AR over-expression in androgen-independent prostate cancers has also been reported in the absence of *AR* gene amplification, due to post-transcriptional mechanisms (e.g. increased AR mRNA and/or protein stability) (Edwards et al., 2003). Remarkably, it has been shown that enforced expression of AR is sufficient to confer androgen-independent growth in animal models of prostate cancer (Chen et al., 2004). Indeed, over-expressed AR is activated by extremely low concentration of androgens; additionally, other endogenous steroids and even antiandrogens act as an agonist of AR when this receptor is over-expressed (Chen et al., 2004).

*2) AR mutations*-- Clinical studies have shown that the progression of prostate cancer to a hormone-refractory state is associated with a significant increase in the frequency of AR mutations (Taplin et al., 1995; Tilley et al., 1996; Marcelli et al., 2000). These mutations broaden the ligand specificity of AR, enabling its activation by other steroid hormones and even by antiandrogens. In addition, a nonsense

mutation in the *AR* gene has also been described that results in the expression of a truncated AR receptor with constitutive, ligand-independent activity (Ceraline et al., 2004).

*3) Alterations of AR co-factors*-- Once bound to specific DNA sequences, AR recruits a number of "coactivators", proteins that facilitate the recruitment of other transcription factors and chromatin remodelling to promote transcription (Shang et al., 2002). Altered expression of AR co-activators in prostate cancer cell lines has been linked to ligand-independent AR activation, increased AR transcriptional activity in the presence of very low androgen concentrations and altered ligand specificity, which allows AR activation by nonandrogenic steroids or by antiandrogens (Edwards and Bartlett, 2005).

*4) AR transactivation via cross-talk with other signalling pathways*-- *In vitro* studies reported that AR can be activated in the absence of its ligand or in the presence of very low levels of androgens through the interaction with other signalling pathways. For instance, a number of growth factors (including insulin-like growth factor-I, keratinocyte growth factor and EGF) can bind to their cognate tyrosine kinase receptors to stimulate signalling cascades that result in the ligand-independent activation of AR and ensuing androgen-independent growth (Culig et al., 1994). Significantly, the synthesis of these growth factors is up-regulated in prostate cancer (Feldman and Feldman, 2001). Similarly, enforced over-expression of HER2 receptor tyrosine kinase in androgen-dependent prostate cancer cells results in increased AR transcriptional activity and subsequent expression of AR target genes in the absence of androgen stimulation (Craft et al., 1999; Yeh et al., 1999a; Wen et al., 2000; Mellinghoff et al., 2004). Significantly, HER2 expression is increased with progression to androgen independence, as observed in cell lines, animal models and patients who have developed castration resistant-tumours (Craft et al., 1999; Signoretti et al., 2000; Shi et al., 2004; Mukherjee et al., 2011). It is possible that similar to HER2, other receptor tyrosine kinases become over-expressed or constitutively active in prostate cancer cells, resulting in aberrant AR

phosphorylation and transcriptional activation and androgen-independent growth of prostate cancer cells.

The downstream effectors of the receptor tyrosine kinase involved in AR transactivation include the ERK-1/2 and Akt pathways. Indeed, both ERK-1/2 and Akt phosphorylate AR to enhance its transcriptional activity in prostate cancer cell lines (Yeh et al., 1999a; Wen et al., 2000; Lin et al., 2001; Lin et al., 2003). Thus, HER2-induced transactivation of AR can be blocked by inhibiting ERK-1/2 or Akt (Yeh et al., 1999a; Wen et al., 2000). Remarkably, Akt activation and AR phosphorylation are strongly correlated in samples from hormone-refractory prostate cancer patients (McCall et al., 2008), supporting the clinical relevance of Akt-induced transactivation of AR.

*5) Generation of constitutively active AR variants*-- A number of truncated AR variants have been described whose expression is reported to be increased in androgen-independent *versus* androgen-dependent prostate cancer cell lines, animal models and clinical samples. Preclinical studies demonstrated that these receptor isoforms are constitutively active and induce the expression of AR-regulated genes in a ligand-independent manner to promote cell growth in the absence of androgen stimulation (Ceraline et al., 2004; Libertini et al., 2007; Dehm et al., 2008; Guo et al., 2009; Sun et al., 2010; Watson et al., 2010; Chan et al., 2012). Various mechanisms have been described by which these constitutively active truncated AR variants can be generated, that include aberrant splicing of AR mRNA (Dehm et al., 2008; Guo et al., 2009; Sun et al., 2010; Watson et al., 2010), protease-mediated cleavage of AR (Libertini et al., 2007; Yang et al., 2008) and nonsense mutations (Ceraline et al., 2004).

Another mechanism that might account for the failure of androgen ablation therapy involves the intracrine production of androgens by prostate cancer cells. Indeed, clinical studies reported an increase in intratumoral androgen levels associated with the progression of prostate cancer to androgen independence following androgen ablation therapy, despite lower levels of serum androgens (Holzbeierlein et al., 2004;

Stanbrough et al., 2006; Locke et al., 2008; Montgomery et al., 2008). These studies also reported that the expression of various enzymes involved in the synthesis of androgens is up-regulated in castration-resistant tumours, which might account for the increased intra-prostatic androgen levels (Holzbeierlein et al., 2004; Stanbrough et al., 2006; Locke et al., 2008; Montgomery et al., 2008). Thus, the intracrine synthesis of androgens by tumour cells may allow prostate cancer growth in the presence of very low serum androgen levels (Holzbeierlein et al., 2004; Stanbrough et al., 2006; Locke et al., 2008; Montgomery et al., 2008).

Interestingly, Li and colleagues demonstrated that androgen removal activates autophagy in androgen-dependent prostate cancer cells and this results in increased cell viability under androgen deprivation conditions (Li et al., 2008a). Thus, this study suggests that the activation of the autophagic pathway might sustain prostate cancer cell survival in the absence of androgen stimulation during the transition to androgen independence.

It is not known whether hormone-refractory prostate cancer develops from a minority of pre-existent androgen-independent cells that are positively selected for under androgen deprivation conditions, or whether androgen ablation itself promotes the acquisition of an androgen-independent phenotype. In this regard, it has been recently reported that androgen withdrawal induces the acquisition of a senescence-associated secretory phenotype in prostate cancer cells both *in vitro* and in human prostate tumours (Pernicová et al., 2011). This finding is particularly interesting, as it has been shown that senescent cells are able to secrete various factors that act in a paracrine manner to promote cancer development and progression, both *in vitro* and *in vivo* (Coppé et al., 2008). Thus, the study by Pernicová et al. provides a mechanism by which castration may contribute to the development of hormone-refractory prostate cancer.

Remarkably, castration-resistant prostate cancers are still dependent on AR signalling for growth and survival. Indeed, down-regulation of AR results in growth suppression and apoptotic death in *in vitro* and *in vivo* models of hormone-refractory

prostate cancer (Haag et al., 2005; Liao et al., 2005; Yuan et al., 2006; Snoek et al., 2009).

Given that AR has a key role in both hormone-sensitive and castration-resistant prostate cancer, the AR pathway represent an attractive therapeutic target for the treatment of this disease, and the development of more effective AR-directed strategies is an active area of research in this field (Chen et al., 2008).

### 1.5.2 Functions of the SK1/S1P pathway in prostate cancer models

A number of studies demonstrate that endogenous SK1 has a critical role in promoting proliferation and maintaining survival of prostate cancer cells. Enforced expression of SK1 markedly stimulates prostate cancer cell proliferation (Brizuela et al., 2010). Conversely, siRNA knock-down of SK1 expression increases the ceramide/S1P ratio and this is associated with inhibition of cell proliferation and activation of apoptosis in prostate cancer cells (Akao et al., 2006; Pchejetski et al., 2005). Similarly, pharmacological inhibitors of SK1 reduced cell proliferation and induced apoptosis in prostate cancer cells by shifting the ceramide/S1P balance toward ceramide (Nava et al., 2000b; Akao et al., 2006; Pchejetski et al., 2005; Pchejetski et al., 2008; Brizuela et al., 2010; Pchejetski et al., 2010). The effect of SK1 inhibitors on cell survival could be reversed by over-expression of SK1. Indeed, prostate cancer cells that over-express SK1 have a significantly lower ceramide/S1P ratio than control cells. In these cells, treatment with SK1 inhibitors fails to shift the ceramide/S1P balance toward ceramide and this is associated with the failure to activate apoptosis (Pchejetski et al., 2005; Pchejetski et al., 2008; Brizuela et al., 2010; Pchejetski et al., 2010).

Interestingly, treatment with pertussis toxin to inhibit $G_i$-coupled S1P receptors markedly inhibits prostate cancer cell growth (Akao et al., 2006), indicating that S1P/S1PR signalling participates to cell proliferation. Indeed, exogenous S1P stimulates prostate cancer cell proliferation associated with the activation of ERK-1/2 (Gibbs et al., 2009).

Conversely, genetic knock-down of S1P receptors ($S1P_1$-$S1P_5$), either individually or altogether, does not significantly reduce prostate cancer cell viability (Pchejetski et al., 2010), suggesting that S1P inside-out signalling might not be involved in promoting the survival of these cells.

*In vivo* studies support a role for SK1 in prostate cancer growth. Indeed, SK1 over-expressing prostate cancer cells develop significantly larger tumours when implanted in nude mice compared with cells that express endogenous SK1 only (Pchejetski et al., 2005; Brizuela et al., 2010). These tumours have a reduced ceramide/S1P ratio compared with control tumours (Pchejetski et al., 2005; Brizuela et al., 2010). Conversely, SK1 inhibition induce tumour regression associated with increased ceramide/S1P ratio (Pchejetski et al., 2008; Brizuela et al., 2010).

Taken together, these findings demonstrate that the sphingolipid rheostat is critically regulated by SK1 in prostate cancer cells.

In addition to its effect on tumour growth, SK1 enhances prostate cancer cell invasiveness and ability to metastasize. Indeed, SK1 inhibition is associated with a significant reduction of metastatic dissemination in prostate cancer animal models (Pchejetski et al., 2005; Brizuela et al., 2010). This is consistent with the ability of exogenous S1P to stimulate prostate cancer cell migration and invasion, which involves the $S1P_2$/$S1P_3$-dependent activation of the transcription factor Stat3 (signal transducer and activator of transcription 3) via ERK-1/2 (Sekine et al., 2011). It is important to appreciate that the ability of S1P to activate Stat3 may be relevant to prostate cancer progression. In fact, Stat3 promotes the expression of genes involved in cell growth and survival and a high expression/activity of Stat3 is associated with an aggressive phenotype in human prostate cancer (Horinaga et al., 2005). Significantly, a positive feedback loop has been demonstrated in cancer cells whereby Stat3 promotes the expression of $S1P_1$ and this, in turn, is required for persistent activation of Stat3 in tumour cells (Lee et al., 2010).

Another mechanism by which S1P promotes prostate cancer invasiveness has been recently elucidated (Beckham et al., 2012). In this study it was shown that S1P activates signalling pathways that culminate in the transcriptional up-regulation of the expression of cathepsin B, a protease involved in the degradation the extracellular matrix (Beckham et al., 2012).

Finally, an additional mechanism by which SK1 might contribute to the progression of prostatic carcinoma is through its ability to mediate the response of prostate cancer cells to hypoxia. Indeed, exposure of prostate cancer cells to hypoxic conditions results in SK1 activation and subsequent increase in the expression and transcriptional activity of hypoxia inducible factor HIF1α (Ader et al., 2008).
Significantly, the exposure of prostate cancer cells to hypoxic conditions and ensuing elevation in HIF levels has been shown to promote the acquisition of an aggressive phenotype and resistance to apoptotic stimuli including chemotherapeutic drugs (Ghafar et al., 2003). SK1, that functions both upstream and down-stream of HIF (section 1.4.1), might contribute to this.

The relevance of SK1 in human prostate cancer development and progression is confirmed by a clinical study showing that SK1 expression/activity in patient tumour samples is significantly increased compared with healthy counterparts and that a high expression of SK1 is predictive of a poor clinical outcome (Malavaud et al., 2010).

The role of SK2 in prostate cancer is poorly characterised. The only published study in regard reports that pharmacological inhibition of SK2 by ABC294640 reduces prostate cancer cell proliferation (French et al., 2010), although it has not been investigated whether this is due to SK2 inhibition or an off-target effect of this compound. As mentioned above, ABC294640 is an ER antagonist (Antoon et al., 2010), which raises the possibility that this inhibitor might interfere with AR signalling to inhibit prostate cancer cell proliferation.

Interestingly, cross-talk exists between SK1 and AR signalling in prostate cancer cells. In fact, SK1 mediates the mitogenic effect of androgen in these cells.

Treatment of androgen-dependent prostate cancer cells with dihydrotestosterone (DHT) induces a rapid and transient stimulation of SK1 activity via a PI3K/Akt-dependent mechanism (Dayon et al., 2009). Indeed, it has been shown that activation of the PI3K/Akt pathway via direct interaction between AR and PI3K (i.e. via a non-genotropic mechanism) mediates the rapid effects of androgen stimulation (Sun et al., 2003; Baron et al., 2004). Significantly, pharmacological inhibition of SK1 suppressed DHT-induced proliferation of androgen-dependent prostate cancer cells (Dayon et al., 2009), supporting a crucial role of SK1.

SK1 also mediates the response of hormone-sensitive prostate cancer cells to androgen depletion. Acute androgen deprivation induces a rapid and transient inhibition of SK1 activity in androgen-dependent, but not in androgen-independent prostate cancer cells which is associated with cell growth inhibition (Dayon et al., 2009). Significantly, over-expression of SK1 prevents the suppression of cell proliferation induced by androgen withdrawal (Dayon et al., 2009).
Similar results were obtained *in vivo*, where castration induced a substantial shrinkage of androgen-dependent tumours which was associated with a significant reduction in SK1 activity (Dayon et al., 2009).

Of note, chronic androgen deprivation has an opposite effect on SK1, inducing the up-regulation of SK1 expression (Dayon et al., 2009), as detailed in the next subsection.

#### *1.5.2.1 SK1 and acquisition of androgen independence*

As mentioned in the previous section, androgen-dependent prostate cancer cells that are enforced to over-express SK1 do not undergo cell growth arrest in response to acute androgen removal (Dayon et al., 2009). This finding indicates that SK1 over-expression is sufficient to sustain the proliferation of prostate cancer cells in the absence of hormonal stimulation, which is the hallmark of androgen-independence.

Remarkably, a study by Cuvillier and colleagues demonstrates that SK1 is necessary for androgen escape (Dayon et al., 2009). Androgen-dependent prostate cancer cells

subjected to long-term androgen deprivation progress to an androgen-independent state (Murillo et al., 2001; Shi et al., 2004; Dayon et al., 2009), recapitulating the development of hormone-refractory prostate cancer in patients treated with androgen ablation therapy. Interestingly, Cuvillier and colleagues demonstrated that the transition to androgen independence *in vitro* is associated with a substantial increase in SK1 expression and activity (Dayon et al., 2009). Significantly, pharmacological inhibition of SK1 prevented the transition of androgen-dependent prostate cancer cells to androgen independence (Dayon et al., 2009). Thus, this study provides evidence that inhibiting SK1 might represent a successful therapeutic approach to prevent the transition to androgen independence.

The elevation of SK1 levels observed in prostate cancer cells under chronic androgen deprivation conditions was dependent on PI3K/Akt signalling, as pharmacological inhibition of this pathway suppressed SK1 accumulation (Dayon et al., 2009). Of note, aberrant activation of Akt has been described as a central mechanism involved in androgen escape (Murillo et al., 2001; Pfeil et al., 2004; Lu et al., 2006) and clinical studies have demonstrated that Akt activation is associated with the development of hormone-refractory prostate cancer and poor clinical outcome (Edwards et al., 2003; Kreisberg et al., 2004; McCall et al., 2008). In light of the findings from the study by Dayon et al., it is tempting to speculate that the activation of SK1 might mediate some of the effects of Akt.

The mechanisms by which increased SK1 promotes the progression of prostate cancer cells to androgen independence have not been reported. Several mechanisms might be involved. For instance, SK1 over-expression confers a growth advantage to prostate cancer cells (Pchejetski et al., 2005; Dayon et al., 2009) which may enable these cells to bypass the need for androgen stimulation.
Another mechanism might involve the SK1-induced up-regulation of Bcl-2 (Limaye et al., 2005). Indeed, a number of studies reported that Bcl-2 levels are up-regulated in prostate cancer cell lines and human tumours that have progressed to androgen independence (McDonnell et al., 1992; Colombel et al., 1993; Shi et al., 2004). Significantly, *in vitro* and *in vivo* studies demonstrated that enforced over-expression

of Bcl-2 protects androgen-dependent prostate cancer cells from androgen ablation-induced apoptosis, while genetic knock-down of Bcl-2 inhibits androgen escape following castration in an animal model of prostate cancer (Raffo et al., 1995; Gleave et al., 1999).

Furthermore, S1P activates intracellular cascades and interacts with signalling pathways that have been implicated in androgen-independent activation of AR (e.g. ERK-1/2, Akt, HER2, EGF) (paragraph 1.5.1). The involvement of SK1/S1P signalling in AR transactivation and in AR bypass in prostate cancer requires formal investigation.

Interestingly, SK1 is also involved in the acquisition of oestrogen independence in breast cancer cells (Sukocheva et al., 2009). Indeed, over-expression of SK1 confers resistance to tamoxifen (an ER antagonist), which is due to constitutive activation of ER. Conversely, inhibition of SK1 activity/expression restores responsiveness to this drug in breast cancer cells (Sukocheva et al., 2009). This was confirmed by clinical studies demonstrating that high SK1 expression correlates with induction of resistance to tamoxifen in oestrogen receptor-positive breast cancer patients, where time for disease recurrence in patients receiving tamoxifen was significantly shortened in patients with high levels of SK1 (Long et al., 2010a; Watson et al., 2010).

### 1.5.3 SK1 and resistance to therapy

*In vitro* and *in vivo* studies demonstrated a correlation between SK1 activity and resistance to therapeutic treatments in prostate cancer. In particular, the inhibition of SK1 and ensuing alteration in the ceramide/S1P balance is a key element in chemotherapy- and radiotherapy-induced apoptosis in prostate cancer cell lines and animal models.

Chemotherapeutic agents (e.g. docetaxel, camptothecin) substantially reduce SK1 activity and elevate the ceramide/S1P ratio in prostate cancer cells that are sensitive to these drugs, and this is associated with the activation of apoptosis (Pchejetski et

al., 2005; Pchejetski et al., 2008; Sauer et al., 2009; Pchejetski et al., 2010). Indeed, SK1 is required for prostate cancer cell survival, as demonstrated by the finding that genetic deletion of this enzyme substantially reduces cell viability (Pchejetski et al., 2005) (1.5.2).

Over-expression of SK1 impairs the efficacy of chemotherapeutic agents. Indeed, prostate cancer cells over-expressing SK1 have a lower ceramide/S1P ratio which is not shifted in response to chemotherapy, and this is associated with resistance to chemotherapy-induced apoptosis (Pchejetski et al., 2005).

Accordingly, the failure to inhibit SK1 and elevate ceramide levels is associated with chemoresistance of prostate cancer cells. Indeed, treatment with chemotherapeutics does not induce inhibition of SK1 activity and does not alter the ceramide/S1P ratio in chemotherapy resistant prostate cancer cells (Pchejetski et al., 2005).

The relevance of SK1 and the ceramide/S1P rheostat in mediating the response to chemotherapeutics is supported by studies in *in vivo* models of prostate cancer, where chemotherapy-induced reduction in tumour mass correlates with SK1 inhibition and elevation in the ceramide/S1P ratio (Pchejetski et al., 2005). Consistent with the *in vitro* studies, prostate cancer cells over-expressing SK1 develop tumours with increased resistance to chemotherapeutic treatment, associated with the failure of the anticancer agents to shift the ceramide/S1P balance toward pro-apoptotic ceramide (Pchejetski et al., 2005).

Significantly, the efficacy (i.e. the ability to reduce cell viability) of the chemotherapeutic drugs *in vitro* and *in vivo* is proportional to the extent to which SK1 activity is inhibited, reflected by the shift of the ceramide/S1P balance, demonstrating that SK1 is a “chemotherapy sensor” in prostate cancer (Pchejetski et al., 2005).

In line with these findings, SK1 down-regulation increases the efficacy of chemotherapy. In fact, pharmacological or siRNA-mediated inhibition of SK1 strongly sensitises prostate cancer cells to chemotherapy-induced apoptosis and overcomes chemotherapy resistance by shifting the ceramide/S1P balance, both *in vitro* and *in vivo* (Pchejetski et al., 2008; Sauer et al., 2009).

Similarly, a correlation between SK1 and resistance to radiotherapy has also been shown. Ionizing radiation strongly inhibits SK1 activity and elevates ceramide levels in radiation-sensitive prostate cancer cells whereas SK1 activity is not affected in radiation-resistant prostate cancer cells (Nava et al., 2000b).
Pharmacological inhibitors of SK1 and SK1 siRNA sensitise prostate cancer cells to γ-irradiation-induced apoptosis (Nava et al., 2000b; Pchejetski et al., 2010), an effect that is abrogated by SK1 over-expression and partially reversed by exogenous S1P (Pchejetski et al., 2010). Addition of cell-permeable ceramide mimicked the effect of SK1 inhibition in sensitising prostate cancer cells to γ-irradiation (Pchejetski et al., 2010).

The mechanism(s) by which chemotherapeutic drugs and ionizing radiations inhibit SK1 in sensitive prostate cancer cells have not been reported. In this context, it has been shown that docetaxel and camptothecin fail to inhibit SK1 activity in *in vitro* assays (Pchejetski et al., 2005). In the case of docetaxel, it has been shown that SK1 inhibition in response to this drug involves an initial reduction in SK1 catalytic activity followed by the down-regulation of *Sk1* gene expression (Sauer et al., 2009). Interestingly, SK1 expression has been shown to be reduced by γ-irradiation in breast cancer cells due to cathepsin- and caspase-mediated proteolytic degradation (Taha et al., 2004). Whether this mechanism mediates the loss of SK1 activity in irradiated prostate cancer cells is not known.
Likewise, the mechanisms underlying the failure of chemotherapeutic agents and γ-irradiation to reduce SK1 activity in resistant cancer cells have not been reported.
Surprisingly, some chemotherapy agents can increase SK1 activity and expression in prostate cancer cells and this is associated with the failure to activate apoptosis (Akao et al., 2006; Pchejetski et al., 2008). Although the molecular basis of SK1 up-regulation is not known, this event is crucial for the resistance of prostate cancer cells to the anticancer drugs. Indeed, cotreatment with anticancer agents and SK1 inhibitors to prevent the activation of SK1 and the reduction of the ceramide/S1P ratio, overcomes the resistance of these cells to apoptosis (Pchejetski et al., 2008).

Collectively, these studies clearly indicate that increased SK1 activity confers resistance to chemotherapy and radiotherapy in prostate cancer via the modulation of the sphingolipid rheostat. It is important to appreciate that chemotherapeutic drugs induce apoptosis in prostate cancer cells via both SK1-dependent and SK1-independent mechanisms (Sauer et al., 2009). Nonetheless, the studies reported in this section provide strong evidence that inhibition of SK1 can substantially improve the response to therapeutic treatments.

In contrast, SK2 does not appear to be involved in the acquisition of chemotherapy resistance by prostate cancer cells (Pchejetski et al., 2010).

Of note, the recently uncovered functional interaction between S1P and NF-κB might be relevant to the acquisition of resistance to docetaxel by prostate cancer cells. Indeed, NF-κB is a prognostic marker for resistance to docetaxel in prostate cancer and inhibition of NF-κB sensitises prostate cancer cells to this drug *in vitro* (Domingo-Domenech et al., 2006). As discussed in section 1.2.2.2, S1P binds to and stimulates TRAF2 to activate NF-κB (Alvarez et al., 2010). Thus, S1P-induced activation of NF-κB might contribute to decrease the response of prostate cancer cells to docetaxel.

## 1.6 Project aim

Compelling evidence implicates SK1 in human prostate cancer. SK1 expression is up-regulated in clinical tumour specimens and correlates with adverse clinical features, and SK1 is instrumental for prostate cancer growth, progression to androgen independence and resistance to therapeutic agents both in prostate cancer cell lines and animal models. Pre-clinical studies provide strong evidence that targeting SK1 might be a successful approach for the clinical management of prostate cancer. This is remarkable, as the development of effective therapeutic treatments for advanced and hormone-resistant prostate cancer is an unmet clinical need.

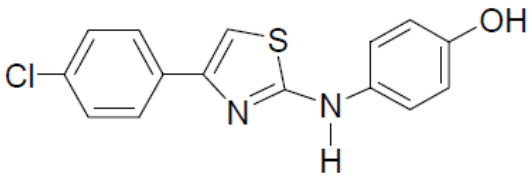
In light of these issues, the overall aim of the present study was to further investigate the functions of SK1 in prostate cancer to evaluate its potential as a therapeutic target for the treatment of this disease. For this purpose, the effects of pharmacological inhibition of SK1 were investigated in *in vitro* models of prostate cancer, including an androgen-dependent cell line and an androgen-independent subline derived from it. Comparing these two cell lines enabled the evaluation of any alteration in SK1 functions occurring in conjunction with the progression of prostate cancer cells to androgen independence.

In particular, the study focused on investigating the mechanisms by which SK1 regulates cell survival and androgen receptor-dependent signalling. The contribution of two SK1 isoforms (SK1a and SK1b) was assessed and comparison with SK2 was made.

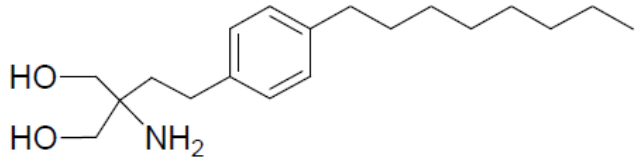
In the course of these investigations, an entirely novel mechanism of action of SK1 inhibitors was identified, i.e. the down-regulation of SK1 expression by these compounds. Therefore, this study focused on elucidating the mechanisms by which SK1 inhibitors regulate SK1 expression, as well as on characterising the molecular basis of the different sensitivities of SK1a and SK1b to degradation.

The chemical structures of the SK inhibitors that were used in the current study are reported in Figure 1.5.

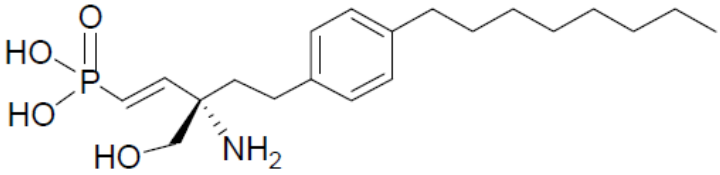
**SKi**




**FTY720**




**(*S*)-vinylPn**




**(*R*)-FTY720-OMe**

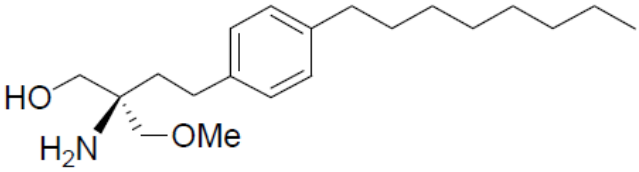



***Figure 1.5 Chemical structures of SK inhibitors:*** *2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole [SKi], 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol [FTY720], (S)-FTY720 vinylphosphonate [(S)-vinylPn] and (R)-FTY720 methyl ether [(R)-FTY720-OMe].*

# CHAPTER 2:

# MATERIALS AND METHODS

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 General reagents

All biochemical reagents were purchased from Sigma-Aldrich (UK) unless otherwise stated.

Bio-Rad (UK)
BIO-RAD protein assay reagent

GE Healthcare (UK)
Hybond$^{TM}$ ECL$^{TM}$ Nitrocellulose membrane; Kodak X-ray Films (X-Omat LS)

Merck Chemicals (UK)
β-glycerophosphate disodium salt

Promega UK
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit

Thermo Scientific
Pierce BCA Protein Assay kit

### 2.1.2 Cell culture

RPMI 1640, OptiMEM®, European foetal calf serum (EFCS), penicillin-streptomycin (10,000 units/ml penicillin and 10,000 μg/ml streptomycin), L-glutamine, trypsin/EDTA solution and Lipofectamine$^{TM}$2000 were from Invitrogen (Paisley, UK). Charcoal filtered foetal bovine serum was from Lonza (Switzerland). LNCaP and LNCaP-AI (Androgen-Independent) cells were gifted from Professor

Hing Leung (Beatson Institute, Glasgow, UK). LNCaP-AI cells were derived by culturing LNCaP cells in androgen-deprivation conditions (Halkidou et al., 2003).

### 2.1.3 Antibodies

BD Transduction Laboratories (Oxford, UK)
Monoclonal anti-ERK-2 antibody (#610104)

New England Biolabs Ltd. (Hitchin, UK)
Polyclonal anti-PARP antibody (#9542S); polyclonal anti-cleaved caspase-3 (Asp175) (5A1E) antibody (#96445)

Santa Cruz Biotechnology (California, USA)
Monoclonal anti-phosphorylated ERK-1/2 (E-4) antibody (#sc-7383); polyclonal anti-androgen receptor (C-19) antibody (#sc-815); polyclonal anti-cyclin D1 (M-20) antibody (#sc-718); monoclonal anti-c-Myc (9E10) antibody (#sc-40); polyclonal anti-c-Myc (A-14) antibody (#sc-789)

Sigma-Aldrich (Poole, UK)
Polyclonal anti-actin antibody (#A2066); monoclonal anti-p53 (DO-7) antibody (#P8999); tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG; fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG; Protein G-Sepharose 4B; reporter horseradish peroxidase-anti-mouse IgG; reporter horseradish peroxidase-anti-rabbit IgG

Stratagene (La Jolla, USA)
Monoclonal anti-FLAG M2 antibody (#200472)

Polyclonal anti-SK1 antibodies (Ab-64 and Ab-94) were a kind gift from Dr Andrea Huwiler (University of Bern, Switzerland) (Huwiler et al., 2006). It should be noted that, due to the limited availability of these antibodies, for some of the experiments

reported in the current study it was not possible to assess the expression of both SK1a and SK1b.

### 2.1.4 Agonists and inhibitors

Avanti Polar Lipids (Alabaster, USA)
Sphingosine; sphingosine 1-phosphate

Cayman Chemicals (Tallinn, Estonia)
FTY720

Enzo Life Sciences (Exeter, UK)
MG132; PD98059; PD150606; myriocin; Ac-DEVD-CHO; Fumonisin B1; C2-dihydroceramide; C2-ceramide; dihydrosphingosine

Merck Biosciences (Nottingham, UK)
2-(p-Hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi); cycloheximide; CA074Me; caspase-2 inhibitor ICH-1; Protease Inhibitor Cocktail Set I

Sigma-Aldrich (Poole, UK)
Phenylmethylsulphonyl fluoride; N-acetyl L-cysteine

FTY720 analogues (*S*)-FTY720 vinylphosphonate and (*R*)-FTY720 methyl ether were gifted from Professor Robert Bittman (Queens College of the City University of New York, New York, USA).

### 2.1.5 Molecular Biology

Bioline (London, UK)
Bioscript™ reverse transcriptase enzyme; HyperLadder™ II

Biomers.net

SK1 PCR primers; SK2 PCR primers; $S1P_2$ PCR primers; $S1P_3$ PCR primers

Dharmacon (Cromlington, UK)

DharmaFECT$^{TM}$2 reagent; ON-TARGETplus SMARTpool® SK1 siRNA; Solaris qPCR Gene Expression Assay kits

Eurofins MWG Operon (Ebersberg, Germany)

Oligo$(dT)_{12-18}$; $S1P_1$ PCR primers

GE Healthcare (UK)

dNTP mix

Invitrogen (Paisley, UK)

GAPDH PCR primers; LPP1 PCR primers; LPP2 PCR primers; LPP3 PCR primers; UltraPure Agarose; pcDNA3.1 and pcDNA4.0 plasmid constructs

NBS Biologicals

SafeView Nucleic Acid Stain

QIAGEN (Crawley, UK)

Scrambled siRNA

Sigma-Aldrich (Poole, UK)

Amplification Grade DNAse I

Thermo Scientific (Abgene/Thermo Fisher Scientific, Epsom, UK)

NucleoSpin® RNA II kit; *Taq* DNA polymerase

Sphingosine kinase (SK) plasmid constructs (pcDNA3.1-SK1a-Myc, pcDNA3.1-SK1b-FLAG, pcDNA4.0-SK2-Myc) were gifted from Professor Stuart Pitson (Centre of Cancer Biology, Adelaide, Australia).

### 2.1.6 Radioisotopes

PerkinElmer (UK)

Adenosine 5'-triphosphate [$\gamma$-$^{32}$P] (specific activity: 10 Ci/mMol); L-[$^{35}$S]-Methionine (specific activity: >1000 Ci/mMol)

## 2.2 Cell culture

### 2.2.1 Maintenance of LNCaP and LNCaP-AI cells

Human prostate cancer LNCaP (androgen-dependent) and LNCaP-AI (androgen-independent) cell lines were maintained in culture in RPMI 1640 medium supplemented with 10% (v/v) EFCS or 10% (v/v) delipidated serum, respectively, 50 U/ml penicillin/50 μg/ml streptomycin and 1% (v/v) L-glutamine. Cells were cultivated in T-75 cell culture flasks at a temperature of 37°C in a humidified atmosphere with 5% $CO_2$.

Confluent cells were detached from the flask surface by incubating them in a trypsin/EDTA solution for 2-3 minutes at 37°C, after removing medium and washing once with serum-free RPMI 1640 medium. Complete medium was then added to the cell/trypsin mixture and the cell suspension was transferred to a new flask after diluting appropriately in complete medium (1:8-1:10).

LNCaP and LNCaP-AI cells between passage 20 and 30 were used for the experiments reported in the current study.

### 2.2.2 Treatment protocol

For experimentation, cells were plated and grown to approximately 60-70% confluence, unless otherwise specified, before being treated as described in each figure legend in the Results sections. Cells were treated with inhibitors or vehicle for 8-48 h, as indicated for each experiment. When cells were treated for 48 hours, the inhibitors or vehicle were replaced after 24 hours.

SKi, FTY720, (*S*)-FTY720 vinylphosphonate, (*R*)-FTY720 methyl ether, C2-ceramide, C2-dihydroceramide, dihydrosphingosine, S1P, MG132, PD98059, Ac-DEVD-CHO, CA074Me, fumonisin B1, myriocin, PD150606, and ICH-1 were reconstituted in DMSO before being added to the culture medium for cell stimulation, at a final concentration of DMSO <0.5%. Phenylmethylsulphonyl fluoride (PMSF) and cycloheximide (CHX) were dissolved in methanol (MeOH) before being used for treating cells, at a final concentration of MeOH <0.4%. N-acetyl L-cysteine was reconstituted in sterile distilled $H_2O$.

### 2.3 Cell transfections

#### 2.3.1 SK1 siRNA transfection

siRNA transfection was performed using SK1 siRNA, which specifically targets SK1 mRNA, or scrambled siRNA, as a negative control, at a final concentration of 200 nM. DharmaFECT® 2 transfection reagent was used for delivering siRNA into cells, following the protocol provided by the manufacturer. Transfection was performed in 12 well-plates on approximately 40% confluent cells. For each well, a mix of 50 µl of 4 µM siRNA, 1.6 µl DharmaFECT® 2 transfection reagent and 150 µl of serum-free, antibiotic-free RPMI 1640, was prepared, and incubated for 20 minutes at room temperature to allow for the formation of siRNA/delivery reagent complexes. After that, 800 µl of antibiotic-free RPMI 1640 medium supplemented with 10% (v/v) serum (EFCS or delipidated serum for LNCaP cells or LNCaP-AI cells, respectively) and 1% L-glutamine were added to the transfection mix, which was then added to the well after removing the culture medium. Cells were maintained at 37°C in 5% $CO_2$ for 48/72 hours before being treated and/or assayed for protein expression by western blotting analysis. Adherent cells and any floating cells (present in different proportions depending on the treatment) were harvested in their culture medium, transferred to microcentrifuge tubes and pelletted by centrifugation at 1,000 rpm for 5 minutes, before being resuspended in boiling sample buffer [125 mM Tris-Base, pH 6.7, 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 0.5% w/v SDS, 1.25% v/v glycerol,

0.06% w/v bromophenol blue and 50 mM dithiothreitol (DTT)]. Cell lysates were homogenized by repeated passages (10x) through a 0.24 mm gauge needle and syringe and kept at -20°C if not used immediately for SDS-PAGE and western blotting analyis.

### 2.3.2 Transient transfection with Myc-tagged SK1a, FLAG-tagged SK1b or Myc-tagged SK2 in LNCaP and LNCaP-AI cells

LNCaP and LNCaP-AI cells were transiently transfected with plasmid constructs encoding Myc-tagged SK1a, FLAG-tagged SK1b, Myc-tagged SK2 or with the vector plasmid (pcDNA3.1/pcDNA4.0) as a control. Plasmid DNA was delivered into the cells using the Lipofectamine™2000 Reagent, following the manufacturer's protocol. When plated for transfection, LNCaP and LNCaP-AI cells were grown in Opti-MEM®, supplemented with 10% (v/v) of the appropriate serum and 1% (v/v) L-glutamine, since the medium normally used for maintaining these cell lines, RPMI 1640, inhibits transfection mediated by cationic lipid reagents, such as Lipofectamine™2000 Reagent. The day before transfection, cells were trypsinized, resuspended in 10% serum Opti-MEM® and counted using a haemocytometer. 2.5 x $10^5$ cells were then plated in each well of a 12-well plate, to have approximately 50-60% confluent cells on the day of transfection. For each well of cells to be transfected, 0.5 μg of the appropriate plasmid DNA and 1 μl of Lipofectamine™2000 were diluted separately in 100 μl of Opti-MEM® each, and incubated for 5 minutes before being mixed and incubated for further 20 minutes, to allow for the formation of the DNA-transfection reagent complexes. For each well, 200 μl of the transfection mix were then added to the medium, and the plate was gently tilted to ensure an even distribution of the DNA-transfection reagent complexes. Cells were incubated at 37°C in 5% $CO_2$ for 24 hours before being treated. After the appropriate treatment, cells were harvested in boiling sample buffer and cell lysates were homogenized by repeated passages (10x) through a 0.24 mm gauge needle and syringe, and kept at -20°C if not used immediately for SDS-PAGE and western blotting analyis.

Cells were incubated for 48 hours after transfection if used for preparing lysates for SK1 activity assay (paragraph 2.12.1).

## 2.4 Reverse transcription-polymerase chain reaction (RT-PCR) assay

RNase-free tips and microcentrifuge tubes were used at any time when isolating RNA from cells and processing samples for RT-PCR assay or quantitative Real-Time PCR.

### 2.4.1 Isolation of total RNA

Total RNA was extracted from approximately 70-80% confluent cells using the NucleoSpin® RNA II extraction kit according to the manufacturer's protocol. For this purpose, cells were harvested in a lysis buffer which also inactivates RNases. The resulting cell lysate was then homogenized by passing it through a 0.20 mm gauge syringe needle (5x) and cleared from the membrane component by filtration through a NucleoSpin® Filter. Next, the flow-through was combined with 70% ethanol and filtered through a NucleoSpin® RNA II Column, containing a silica membrane which allows the adsorption of the nucleic acids, DNA and RNA, present in the sample. After a wash with a desalting buffer, the DNA bound to the silica membrane was digested by a 15 minute incubation with a DNase solution, followed by a wash with a buffer which inactivates this enzyme. After two further washes of the silica membrane, the RNA was eluted in RNase-free $H_2O$.
RNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer, and the ratio between the absorbance at 260 nm and at 280 nm (>1.9) was checked as an index of RNA purity.

### 2.4.2 Synthesis of cDNA by reverse transcription

1500 ng of total RNA were used for cDNA synthesis by the reaction of reverse transcription. Before this, RNA samples were subjected to a further treatment with 1 unit per sample of amplification grade DNase I, according to the protocol provided by the manufacturer, for degrading any contaminating DNA. DNase was then inactivated by heating (70°C for 10 minutes) in the presence of Stop solution before carrying on with cDNA synthesis. For this purpose, the RNA was incubated at 42°C for 2 minutes with 0.5 µl of 20 mM each dNTP Mix, 1 µl of 500 µg/ml Oligo$(dT)_{12\text{-}18}$ primers, 2 µl of 0.1 M DTT and 2 µl of 10X Reaction Buffer [50 mM Tris-HCl (pH 8.6), 40 mM KCl, 1 mM $MnSO_4$] in a final volume of 18 µl. Next, 2 µl (corresponding to 200 units) of the retrotranscriptase enzyme BioScript™ were added to the mixture, before incubating at 42°C for 50 minutes. The reaction of retrotranscription was terminated by heating the samples at 70°C for 15 minutes. For each RNA sample, a reaction was also set up lacking the retrotranscriptase enzyme (RT-) to be used in the subsequent PCR as a control for any contaminating genomic DNA.

### 2.4.3 Amplification of cDNA specific sequences by PCR

2 µl of cDNA resulting from RNA retrotranscription were used as a template for PCR. For each sample, a 50 µl PCR mixture was prepared containing cDNA, 5 µl of 10X PCR Reaction Buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween], 3 µl of 25 mM $MgCl_2$, 0.5 µl of 20 mM each dNTP mix, 10-50 pmol of each primer (forward and reverse) and 2 units of *Taq* DNA polymerase. RT- reactions and no-template reactions (with $dH_2O$ instead of cDNA) were set up in parallel, as a control for genomic DNA contamination and primer dimerization, respectively.

PCR consisted of an initial heating step at 94°C for 2 minutes, followed by 25-35 cycles of amplification (each featuring a denaturation step at 94°C for 1 minute and 30 seconds, an annealing step at 50-56°C for 30 seconds and a polymerization step at 72°C for 1 minute and 40 seconds) and a final extension step at 72°C for 5-10

minutes. The nucleotide sequence of the primers that were used for PCR and the size of the amplification product for each set of primers are reported in Table 2.1, along with the specific amplification conditions (annealing temperature and number of cycles) that were followed for each PCR assay.

| | | | | |
|---|---|---|---|---|
| **SK1** | For | 5'-ctgtcacccatgaacctgctgtc-3' | 480 bps | Ta, 55°C |
| | Rev | 5'-catggccaggaagaggcgcagca-3' | | 35 cycles |
| **SK2** | For | 5'-gcctacttctgcatctacacctacc-3' | 315 bps | Ta, 56°C |
| | Rev | 5'-gaggttgaaggacagcccagcttc-3' | | 30 cycles |
| **$S1P_1$** | For | 5'-gatatcatcgtccggcattac-3' | 1288 bps | Ta, 53°C |
| | Rev | 5'-acccttcccagtgcattgttc-3' | | 35 cycles |
| **$S1P_2$** | For | 5'-cactcggcaatgtacctgtttc-3' | 531 bps | Ta, 56°C |
| | Rev | 5'-gacgcctagcacgatggtgac-3' | | 30 cycles |
| **$S1P_3$** | For | 5'-gactgctctaccatcctgccc-3' | 345 bps | Ta, 56°C |
| | Rev | 5'-gtagatgaccgggttcatggc-3' | | 30 cycles |
| **LPP1** | For | 5'-cttcaaggcatacccccttccaac-3' | 502 bps | Ta, 52°C |
| | Rev | 5'-gcccagtctcccttcatcctg-3' | | 25 cycles |
| **LPP2** | For | 5'-ctgctgtatacaaggtgctgggg-3' | 301 bps | Ta, 54°C |
| | Rev | 5'-cgtgcccacttccaacagagtc-3' | | 30 cycles |
| **LPP3** | For | 5'-ctcgacctcttctgcctcttcatg-3' | 458 bps | Ta, 52°C |
| | Rev | 5'-gcttcctggactttgctgtcatcac-3' | | 30 cycles |
| **GAPDH** | For | 5'-tgaaggtcggtgtcaacggatttggc-3' | 983 bps | Ta, 55°C |
| | Rev | 5'-catgtaggccatgaggtccaccac-3' | | 25 cycles |

**Table 2.1** ***Nucleotide sequence of primers used in PCR assays and amplification conditions.*** *For each set of primers, the nucleotide sequence of the forward (For) and reverse (Rev) primers and the size (in base pairs, bps) of the amplification product are shown. The annealing temperature (Ta) and the number of amplification cycles are also shown.*

### 2.4.4 Agarose gel electrophoresis of PCR products

PCR products were resolved by electrophoresis on 1% agarose gels, prepared from agarose and TBE buffer [89 mM Tris-Base, pH 8.3, 89 mM boric acid, 2.2 mM EDTA], containing the nucleic acid stain SafeView to allow for DNA detection under UV light. The electrophoresis apparatus was filled with TBE buffer and the PCR samples, added with loading dye [0.25% (w/v) bromophemol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in sterile distilled water], were loaded into the wells of the gel and run at 65 V for ~30-45 minutes. PCR products were visualised under a UV transilluminator and photographed using a Kodak Digital Science EDAS120 camera. The molecular size of the amplification products was estimated by comparing their mobility on agarose gel to that of molecular markers of known size that were run along with the PCR samples.
For each sample, the cDNA corresponding to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in a parallel reaction as a control for equal cDNA loading.

## 2.5 Quantitative Real-Time PCR

Total RNA was extracted from LNCaP cells following the same procedure as described in paragraph 2.4.1 and quantified by a NanoDrop-2000C spectrophotometer. cDNA was synthesised from each RNA sample by retrotranscription catalysed by BioScript™ (200 units/reaction), using the same conditions described in paragraph 2.4.2. "RT-" reactions, lacking the retrotranscriptase enzyme, were also set up to be used as a control to assess any genomic DNA contamination. Quantitative Real-Time PCR (qPCR) was performed using a Solaris Human qPCR Gene Expression Assay kit specific for the gene of interest, following the manufacturer's protocol. Briefly, 100 ng of cDNA template, or the equivalent volume of RT- or $dH_2O$ (for no-template negative control), were used for each qPCR reaction, in the presence of the forward and reverse primers (final concentration 800 nM each), the gene-specific probe (final concentration 200 nM)

and Solaris qPCR Master Mix. PCR reactions were run in triplicate on a BioRad DNA Engine with Chromo4 Real-Time PCR Detection System (Bio-Rad, UK), according to the cycling conditions indicated by the manufacturer, which included an initial step at 95°C for 15 minutes for optimal enzyme activation and 40 cycles of amplification, each of which featured a denaturation step (95°C for 15 seconds) and an annealing/extension step (60°C for 1 minute). The data were collected using Opticon Monitor 3.1 (Bio-Rad, UK) and gene expression was quantified using the Comparative $C_t$ method ($\Delta\Delta C_t$ method) (Schefe et al., 2006). Results are expressed as relative SK1 or AR mRNA levels, normalized to GAPDH (reference gene) mRNA levels.

Table 2.2 reports the nucleotide sequence of the gene-specific primers and probes used for qPCR analysis.

| | | |
|---|---|---|
| **SK1** | For | 5'-accattatgctggctatgagc-3' |
| | Rev | 5'-cagcaatagcgtgcagtt-3' |
| | Probe | 5'-tgaagacctcctgacca-3' |
| **AR** | For | 5'-aagacctgcctgatctgtgga-3' |
| | Rev | 5'-gcgcacaggtacttctgtt-3' |
| | Probe | 5'-agccgctgaagggaaac-3' |
| **GAPDH** | For | 5'-gcctcaagatcatcagcaatg-3' |
| | Rev | 5'-cttccacgataccaaagttgtc-3' |
| | Probe | 5'-gccaaggtcatccatga-3' |

**Table 2.2** ***Nucleotide sequence of primers and probes used in qPCR assays.*** *For each gene, the nucleotide sequence of the forward (For) and reverse (Rev) primers and the gene-specific probe (complementary to a sequence in the amplification product) that were used for Real-Time quantitative PCR are shown.*

## 2.6 Preparation of cell lysates for western blotting analysis of protein expression

After the appropriate treatment (as detailed in each figure legend in the Results sections), adherent cells and any floating cells (present in different proportions depending on the treatment) were harvested in their culture medium using a cell scraper and transferred to a 15 ml tube. Cells were pelletted by centrifugation at 1,000 rpm for 3 minutes, washed with ice-cold phosphate buffer solution (PBS) and resuspended in ice-cold lysis buffer [137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) Nonidet P-40 (NP-40, IGEPAL), 10% (v/v) glycerol, 20 mM Tris-base, 0.5 mM $Na_3VO_4$, 0.2 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin; pH 8.0], keeping the tubes on ice. Cell lysates were then homogenized by repeated passages (10x) through a 0.24 mm gauge needle and syringe and then transferred into microcentrifuges tubes and mixed for 30 minutes at 4°C to complete the lysis. This was followed by a centrifugation at 15,300 rpm for 10 minutes at 4°C, after which the supernatant was transferred, without disturbing the pellet, into a fresh microcentrifuge tube, and stored at -20°C if not immediately used for protein concentration assay.

Protein concentration of the cell lysates was determined using the BCA assay (paragraph 2.7.1). For each sample, 10 µg of protein of cell lysate were used for SDS-PAGE and western blotting. Cell lysates were diluted with lysis buffer to normalise protein concentrations before the addition of the required volume of sample buffer. Samples were boiled for 5 minutes to completely denaturate the proteins before being loaded into polyacrylamide gels and assayed by western blotting analysis (paragraph 2.9).

For siRNA and plasmid transfection experiments, cells lysates were prepared by harvesting the cells directly in sample buffer, as described in paragraph 2.3.1.

When preparing samples for the detection of LC3 conversion (LC3-I to LC3-II) by immunoblotting for autophagy analysis, cells were washed with ice-cold PBS and resuspended in TNTE lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 50 µg/ml PMSF, Protease Inhibitor Cocktail; pH 7.5]. Samples were repeatedly (x10) passed through a 0.24 mm gauge needle and syringe and left

for 5 minutes at 4°C to allow for efficient lysis. Cell debris was then pelleted by centrifugation at 15,400 rpm for 10 min at 4°C and the supernatant was collected. The protein content was measured using the BCA Assay (paragraph 2.7.1). For each sample, 80 µg of protein were combined with sample buffer and subjected to SDS-PAGE and western blotting (paragraph 2.9).

## 2.7 Determination of protein concentration

Protein content in cell lysates was measured by two different spectrophotometric methods, the bicinchoninic acid (BCA) assay (Smith et al., 1985) or the BIO-RAD assay (Bradford et al., 1976), depending on the composition of the buffer that was used to obtain cell lysates. Buffers can in fact contain substances that by interfering with the reagents used for protein quantification do not allow to accurately measure the protein concentration of the samples. In this regard, the main difference between the two methods is that detergents do not affect the BCA assay whereas it is affected by reducing agents; conversely, the BIO-RAD assay is not compatible with detergent-containing buffers but can be used in the presence of reducing agents.

### 2.7.1 Bicinchoninic acid assay

The bicinchoninic acid (BCA) assay is a spectrophotometric method for the determination of protein concentration that relies on two different reactions: 1) the reduction of $Cu^{2+}$ to $Cu^{+}$ by proteins in an alkaline medium, and 2) the binding of BCA to $Cu^{+}$, which leads to the formation of a reaction product that absorbs at 562 nm. Absorbance at this wavelength ($OD_{562}$) is therefore proportional to the protein concentration of a sample, that can be determined by comparison with a standard curve constructed using known concentrations of bovine serum albumin (BSA).
The BCA assay was performed using the Pierce® BCA Protein Assay Kit according to the instructions provided by the manufacturers. For each sample, the assay was performed in duplicate, and the protein concentration calculated from the $OD_{562}$ mean value.

### 2.7.2 BIO-RAD protein assay (Bradford method)

This method for protein quantification is based on the use of a reagent which forms a complex with proteins that can be quantified by measuring absorbance at 595 nm. As above, a standard curve constructed using known concentrations of BSA is conducted in parallel.
The assay mixture was prepared with 10 µl of cell lysate, 800 µl of distilled $H_2O$ and 200 µl of BIO-RAD reagent. After a 5 minutes incubation, the absorbance at 595 nm ($OD_{595}$) was measured using a spectrophotometer. For each sample, the assay was performed in duplicate and the protein concentration calculated from the $OD_{595}$ mean value.

## 2.8 Analysis of SK1 interacting proteins by [$^{35}$S]-Methionine labelling and immunoprecipitation

LNCaP cells were transiently transfected with SK1a-Myc, SK1b-FLAG or vector plasmid following the protocol described in paragraph 2.3.2. 24 hours after transfection, [$^{35}$S]-Methionine was added to the culture medium (10 µCi/ml) and cells were incubated for further 24 hours before being treated as described under Results. Cell extracts for immunoprecipitation were prepared following the protocol described in paragraph 2.8.1 and the resulting immunocomplexes were subjected to SDS-PAGE (paragraph 2.9). [$^{35}$S]-Methionine-labelled proteins were electrophoretically transferred to a nitrocellulose membrane and detected by autoradiography, exposing the nitrocellulose for approximately 24 hours to an X-ray film, which was then developed using the X-Omat machine.

### 2.8.1 Immunoprecipitation

Cells were lysed in ice-cold lysis buffer (400 µl/well) [137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) Nonidet P-40 (NP-40, IGEPAL), 10% (v/v)

Glycerol, 20 mM Tris-Base, 1 mg/ml BSA, 0.5 mM $Na_3VO_4$, 0.2 mM PMSF, leupeptin and aprotinin (both at 10 µg/ml); pH 8.0] and left to shake at 4°C for 30 minutes, before transferring the cell lysates into microcentrifuge tubes and homogenizing them by repeated (10x) passages through a 0.24 mm gauge needle and syringe. Next, the cell lysates were mixed for further 60 minutes at 4°C, to allow for complete lysis, and then centrifuged at 15,300 rpm at 4°C for 10 minutes to pellet the membrane component. For each sample, 250 µl of supernatant were transferred into a new microcentrifuge tube and pre-cleared, in order to reduce aspecific signals, by adding 20 µl of 1 part lysis buffer and 1 part protein G Sepharose beads and mixing for 20 minutes at 4°C. The beads were then pulsed and the supernatant (200 µl per sample) was transferred into a new microcentrifuge tube and subjected to immunoprecipitation, by adding 2 µl of the appropriate primary antibody (anti-Myc and/or anti-FLAG antibodies) and 20 µl of 1 part lysis buffer and 1 part protein G Sepharose beads per sample and incubating overnight at 4°C, in mild agitation. The resulting immunoprecipitates were then collected by centrifugation at 15,300 rpm for 15 seconds at 4°C and washed once with Buffer A [containing 10 mM HEPES, 100 mM NaCl, 0.5% (v/v) NP-40 and 0.2 mM PMSF; pH 7.0] and once with Buffer A without NP-40. The beads were then pulsed and the supernatant removed, before adding 20 µl per sample of Laemmli buffer [0.125 M Tris-HCl, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 4% (w/v) SDS and 0.004% (w/v) bromophenol blue; pH 6.7] and heating at 100°C for 3 minutes. The samples were then subjected to SDS-PAGE (paragraph 2.9) and autoradiography (paragraph 2.8).

## 2.9 SDS-PAGE and western blotting analysis

### 2.9.1 Preparation of polyacrylamide gels

Each polyacrylamide gel used for resolving proteins by electrophoresis consists of two layers: the separating gel (lower layer), in which the resolution of proteins by electrophoresis occurs, and the stacking gel (upper layer), which allows the proteins to concentrate before entering the separating layer.

The separating gel was made by 10% (v/v) acrylamide:bis-acrylamide, 0.375 M Tris-Base (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.025% (w/v) tetramethylethylenediamine (TEMED), while the stacking gel was made by 4.5% (v/v) acrylamide-bis-acrylamide, 0.125 M Tris-Base (pH 6.7), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.1% (v/v) of TEMED.

### 2.9.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out using the Bio-Rad Mini-Protean II electrophoresis kit. Samples were loaded into the gel using a Hamilton syringe. Pre-stained molecular weight markers, whose molecular weight is known, were also loaded into the gel in order to identify the band corresponding to the protein of interest. Electrophoresis was carried out using a buffer containing 25 mM Tris-Base, 0.21 M glycine and 0.1% (w/v) SDS, at a voltage of 120 V and a current limit of 1.0 mA for about 2 hours.

### 2.9.3 Transfer to nitrocellulose membranes

The electrophoretic transfer of the resolved proteins from the gel to a nitrocellulose membrane was carried out using a Bio-Rad Mini Trans-Blot kit filled with a buffer containing 25 mM Tris-Base, 0.21 M glycine and 20% (v/v) methanol. The transfer occurs by applying a voltage of 100 V with a current limit of 0.6 mA for 60 minutes.

### 2.9.4 Western blotting

Before being incubated with the antibody specific for the protein to be detected, membranes were incubated for 60 minutes at room temperature in a blocking solution, in order to reduce the antibody binding to non-specific sites. The blocking solution consisted of 3% (w/v) non-fat dry milk in TBST [10 mM Tris-Base, 100 mM NaCl, 0.1% (v/v) Tween-20; pH 7.4] or 5% (w/v) bovine serum albumin (BSA) in TBST, depending on the primary antibody to be used. After blocking, membranes were incubated overnight with gentle agitation at 4°C with the primary antibody

specific for the detection of the protein of interest, diluted in 1% (w/v) BSA in TBST.

Incubation with primary antibody was followed by 3 washes (7 minutes each) of the membranes in TBST to remove any unbound antibody and by their incubation for 60 minutes at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, depending on the primary antibody origin) diluted 1:80,000 in 1% (w/v) non-fat dry milk in TBST. Excess antibody was then removed by 3 washes in TBST (7 minutes each), before incubating the membranes in the enhanced chemiluminescence (ECL) reagent, which provides the substrate for the peroxidase, thus allowing the detection of immunoreactive proteins. The reagent was freshly prepared before using by mixing equal volumes of a solution containing 0.04% (w/v) luminol, 0.1 M Tris-Base (pH 8.5) and 0.016% (w/v) *p*-coumaric acid, and a solution containing 2% (v/v) $H_2O_2$ and 0.1 M Tris-Base (pH 8.5). After a 2 minutes incubation at room temperature, membranes were dried from the excess of ECL reagent, placed between two transparent plastic sheets in a metal cassette and exposed to an X-ray film, which was then developed by passing it through the X-Omat machine. The exposure time was varied depending on the intensity of the chemiluminescence signal.

Immunoreactive proteins appeared on the film as dark bands and their molecular weight was estimated by comparing their mobility on SDS-PAGE to that of pre-stained molecular weight markers.

### 2.9.5 Stripping and reprobing of nitrocellulose membranes

Membranes can be stripped to remove bound antibodies before being reprobed with a different antibody, in order to detect other proteins. Stripping of the membranes was carried out by incubating them in a buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% (w/v) SDS and 100 mM β-mercaptoethanol for 1 hour at 70°C with gentle agitation. Membranes were then rinsed with distilled water and washed with TBST (3 washes, 15 minutes each) in mild agitation before incubating them overnight with the primary antibody specific for the protein of interest.

For each experiment, blots were stripped and reprobed with anti-ERK2 antibody to ensure comparable loading between samples. However, following the finding that ERK-2 protein levels were down-regulated by SKi treatment in some cell lines (unpublished data), the expression of actin was measured as a control for equal protein loading.

## 2.10 Proteasome activity assay

Proteasome activity was measured in cells in culture using a Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit (Promega). This assay is based on the use of an exogenous luminogenic proteasome substrate that, following the cleavage by the proteasome, generates a molecule that, in turn, is a substrate for luciferase, whose enzymatic activity leads to the generation of a luminescent signal. The intensity of the signal, measured using a luminometer, is therefore proportional to the proteasomal activity of cells against the exogenous luminogenic substrate.

Cells were seeded in 96 well-plates at a density of 8,000 cells/well, and incubated overnight at 37°C in 5% $CO_2$ before being treated as described in the Results sections. Proteasome activity was then assessed using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit, following the manufacturer's instructions. Briefly, the luminogenic substrate was resuspended in a buffer optimized for cell permeabilization and luciferase activity, and mixed to the luciferase enzyme, before being added to stimulated cells. After a 10 minute incubation at room temperature, luminescence (350-650 nm) was measured using a luminometer. For each experiment, a blank was also prepared, consisting of culture medium without cells and added with the vehicle, and the corresponding value of luminescence was subtracted from the values relative to cell samples.

## 2.11 Immunofluorescence microscopy

Cells were plated on autoclaved glass coverslips (13mm diameter), each of which was placed in one well of a 12-well plate, and transfected with Myc-tagged SK1a, FLAG-tagged SK1b or vector plasmid construct, as described in paragraph 2.3.2. 48 hours after transfection, cells were fixed by a 10 minute incubation in 3.7% (v/v) formaldehyde in PBS before being permeabilised using 0.1% (v/v) Triton X-100 in PBS for 3 minutes. Cells were then incubated with blocking solution [5% (v/v) FCS and 1% (w/v) BSA in PBS] for 30 minutes at room temperature, followed by overnight incubation at 4°C with polyclonal anti-Myc and monoclonal anti-FLAG antibodies (both at 1:100 dilution in blocking solution). Coverslips were then washed twice with PBS and incubated for 60 minutes at room temperature with the secondary antibodies (TRITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG) at 1:100 dilution in blocking solution. Next, coverslips were washed twice with PBS and mounted on glass slides using Vectashield® hard set mounting medium containing 4′,6-Diamidino-2-phenylindole (DAPI) to stain the nuclei. Fluorescence was visualised using a Nikon E600 epifluorescence microscope.

## 2.12 Sphingosine kinase activity assay

SK1 activity was assayed using cell lysates from LNCaP cells transiently over-expressing SK1a or SK1b.

### 2.12.1 Preparation of cell lysates from LNCaP cells transiently over-expressing SK1a or SK1b

LNCaP cells were transiently transfected with Myc-tagged SK1a, FLAG-tagged SK1b or vector plasmid constructs following the protocol described in paragraph 2.3.2. 48 hours after transfection, cells were harvested in ice-cold SK1 assay buffer [20 mM Tris-Base, 1 mM EDTA, 1 mM $Na_3VO_4$, 40 mM β-glycerophosphate, 1 mM NaF, 20% (v/v) glycerol, 10 μg/ml soyabean trypsin inhibitor, 1 mM PMSF, 0.5 mM

4-deoxypyridoxine, 10 μg/ml aprotinin, 0.007% (v/v) β-mercaptoethanol; pH 7.4] and cell lysates were homogenised by repeated passages (10x) through a 0.24 mm gauge needle and syringe. Protein concentration of the cell lysates was measured using the Bio-Rad protein assay system (paragraph 2.7.2). Samples were stored at -20°C if not immediately used for the assay.

An aliquot of each cell lysate, corresponding to 10 μg of protein, was combined with sample buffer and subjected to SDS-PAGE and western blotting analysis, to assess the expression of SK1a and SK1b in the cell lysates used for SK activity assay.

### 2.12.2 Measurement of SK activity

SK1 activity was assayed according to Delon et al. (2004) by measuring the quantity of radioactive [$^{32}$P]-S1P produced by phosphorylation of sphingosine in the presence of [$^{32}$P]-γ-ATP. For this purpose, 10 μg of protein of cell lysates in SK1 assay buffer (140 μl) were incubated with 10 μM sphingosine, previously resuspended in 40 μl of Triton X-100 (final concentration 0.063% w/v), and varying concentrations of the SK inhibitor, SKi (final concentration 10, 25, 50, 100 μM), or the vehicle alone as a control (DMSO, final concentration 5% v/v). Two blanks were also prepared omitting sphingosine. Since Triton X-100 suppresses SK2 activity (Liu et al., 2000a), the addition of this detergent to the assay buffer enables to separately measure SK1 activity in cell lysates.

The reaction was initiated by adding 10 μl per sample of the ATP mix [containing 50 nmoles of unlabelled ATP (final concentration 250 μM) in 10 mM $MgCl_2$ and 1 μCi of [$^{32}$P]-γ-ATP for each sample] and carried out for 10 minutes at 30°C. The reaction was then stopped by adding 500 μl of butan-1-ol and mixing thoroughly. Unreacted [$^{32}$P]-γ-ATP was afterward removed by three washes with 1 ml of 2 M KCl each. For this purpose, after adding KCl, samples were mixed and centrifuged in order to separate the organic phase, containing S1P, from the acqueous phase, containing the unreacted [$^{32}$P]-γ-ATP, which after each centrifugation was removed using a glass pasteur. The organic phase was then transferred to scintillation vials and the [$^{32}$P]-S1P content was quantified by counting the dpm (disintegrations per minute) using a scintillation counter.

For each sample, SK1 activity (expressed as pmoles of S1P formed per minute per μg of protein) was calculated as follows: (dpm of sample-dpm of blank)/(dpm of [$^{32}$P]-γ-ATP) x (nmoles unlabelled ATP) x 1/(time of incubation in minutes) x 1/(μg protein) x 1000.

## 2.13 Statistical analysis

Experiments were repeated at least three times, unless otherwise stated. Data are presented as mean with standard deviation (SD), unless otherwise stated. Densitometric quantification of western blots and RT-PCR results was performed using ScanImage program (Scion Corporation, Frederick, MD). Statistical analysis was performed using an unpaired Student's t test, and a p-value $<0.05$ was considered significant.

# CHAPTER 3:

# DIFFERENTIAL EFFECT OF SKi ON SK1 ISOFORMS, SK1a AND SK1b, IN LNCaP AND LNCaP-AI CELLS: MECHANISMS AND FUNCTIONAL RELEVANCE

# 3. DIFFERENTIAL EFFECT OF SKi ON SK1 ISOFORMS, SK1a AND SK1b, IN LNCaP AND LNCaP-AI CELLS: MECHANISMS AND FUNCTIONAL RELEVANCE

## 3.1 Introduction

There are currently a limited number of published reports investigating the role of SK1 in prostate cancer. The results of these studies, however, provide compelling evidence that SK1 has a crucial role in prostate cancer development and progression, being able to contribute to the major hallmarks of cancer. These findings provide a rationale for further investigating the role of SK1 in prostate cancer. The current knowledge on how alterations in SK1/S1P signalling are involved in prostate cancer biology and resistance to therapy has been extensively reviewed in Chapter 1 and will be only briefly recapitulated herein.

SK1 promotes proliferation and survival of prostate cancer cells, as suggested by the finding that SK1 inhibition (either pharmacological or genetic) reduces prostate cancer cell growth and viability (Nava et al., 2000b; Pchejetski et al., 2005; Akao et al., 2006; Brizuela et al., 2010) while enforced over-expression of SK1 in prostate cancer cells increases proliferation (Brizuela et al., 2010). Indeed, SK1 over-expressing prostate cancer cells develop significantly larger tumours when implanted in nude mice (Pchejetski et al., 2005; Brizuela et al., 2010), whereas SK1 inhibition is associated with a reduction in tumour size (Brizuela et al., 2010). Moreover, SK1/S1P signalling stimulates prostate cancer cell migration and invasiveness (Sekine et al., 2011; Beckham et al., 2012). Accordingly, SK1 inhibition is associated with a significant reduction of metastatic dissemination in prostate cancer animal models (Pchejetski et al., 2005; Brizuela et al., 2010).

Significantly, the progression of prostate cancer cells to androgen independence *in vitro* can be prevented by inhibiting SK1 (Dayon et al., 2009).

The relevance of SK1 in human prostate cancer is confirmed by a clinical study showing that SK1 expression/activity in patient tumour samples is increased compared with healthy counterparts and correlates with disease progression (e.g. increased tumour grade) (Malavaud et al., 2010).

SK1 has also a crucial role in the response of prostate cancer cells to therapeutic treatments, mediating the resistance to apoptosis induced by γ-irradiation and chemotherapeutic drugs. Indeed, *in vitro* and *in vivo* studies show that SK1 over-expression in prostate cancer cells confers resistance to radiotherapy and chemotherapy, whereas SK1 inhibition sensitises prostate cancer cells to these treatments (Nava et al., 2000b; Pchejetski et al., 2005; Pchejetski et al., 2008; Sauer et al., 2009; Pchejetski et al., 2010).

Taken together, these findings demonstrate strong evidence that SK1 represents an attractive therapeutic target for prostate cancer treatment and provide impetus for the development of effective SK1 inhibitors for anti-cancer clinical use.

The human prostate cancer cell lines LNCaP (androgen-dependent) and LNCaP-AI (androgen-independent) were used for this study as *in vitro* models of hormone-sensitive and hormone-refractory prostate cancer, respectively.

The LNCaP cell line has been established from a lymph node metastasis of human prostate carcinoma (Horoszewicz et al., 1980). These cells proliferate *in vitro* in the presence of androgens in the culture medium and, importantly, maintain the characteristic of human prostate cancer, i.e. expression of androgen receptor, responsiveness to hormone stimulation and secretion of organ-specific proteins (Horoszewicz et al., 1983). Additionally, LNCaP cells preserve malignant properties, being tumorigenic when injected in nude mice (Horoszewicz et al., 1980).
The androgen receptor in LNCaP cells contains a missense mutation in the ligand binding domain (Veldscholte et al., 1992). This results in broadened ligand specificity (that is, this receptor is inappropriately activated by other ligands, i.e.

other steroid hormones and antiandrogens) as well as in enhanced activation by androgens compared with wild type androgen receptor (Veldscholte et al., 1992).

LNCaP-AI cells have been selected by culturing LNCaP cells in androgen-deprivation conditions for prolonged periods of time (Halkidou et al., 2003). This recapitulates the development of a hormone-refractory tumour in prostate cancer patients treated with androgen ablation therapy. LNCaP-AI cells are not dependent upon androgen for proliferation as they can grow in hormone-free medium. However, these cells express androgen receptor and respond to androgens, being able to express androgen-regulated genes when stimulated (Halkidou et al., 2003). LNCaP-AI cells are therefore, considered as a good *in vitro* model for hormone-refractory prostate cancer.

Among the commercially available SK1 inhibitors, SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) (French et al., 2003) (Figure 1.5) was chosen for the current study as a tool to further investigate the role of SK1 in prostate cancer cells. Inhibition kinetic analysis of SK1a demonstrated that SKi is a mixed (competitive and uncompetitive) inhibitor against sphingosine ($K_{ic}$= 17 μM; $K_{iu}$= 48.3 μM) (Lim et al., 2011b) and a mixed inhibitor against ATP ($K_{ic}$= 60 μM; $K_{iu}$= 35 μM) (Lim et al., 2012b). Importantly, French and colleagues demonstrated that SKi inhibits endogenous SK1 activity in intact cancer cells (French et al., 2003).

Having established that the chronic treatment of human pulmonary aortic smooth muscle cells (hPASMC) with SKi down-regulates SK1, the first part of the current study focused on investigating the effect of this inhibitor on SK1 expression in LNCaP and LNCaP-AI cells and the mechanism involved. Next, based on the ability of SKi to induce a chemical knock-down of SK1, this inhibitor was used to interrogate the role of SK1 in regulating prostate cancer cell survival.

## 3.2 Results

### 3.2.1 Analysis of SK1 expression in LNCaP and LNCaP-AI cells

Three N-terminal variants of SK1 have been identified, herein referred to as SK1a (transcript variant 3, GenBank number: NM_001142601; molecular mass of 42.5 kDa), SK1b (transcript variant 2, GenBank number: NM_182965; molecular mass of 51 kDa), which differs from SK1a for an N-terminal extension of 86 amino acids, and SK1a+14 (transcript variant 1, GenBank number: NM_021972; molecular mass of 43.9 kDa), which is identical to SK1a except for a 14 amino acid N-terminal extension.
The annotation used in the current study should not be confused with that used by other authors, that refer to transcript variant 2 and transcript variant 1 as "SK1c" (51 kDa) and "SK1b" (43.9 kDa), respectively (Pitson, 2011).

To establish whether there were any differences in the expression of SK1 isoform variants in LNCaP and LNCaP-AI cells we performed RT-PCR with gene-specific primers. The primers that were used for amplifying SK1 are designed to a region that is common to SK1a, SK1a+14 and SK1b, so that the product of amplification potentially includes the mRNA for all the three isoforms. These primers amplified a single product, of the predicted size (480 bp) (Figure 3.1a). As shown in Figure 3.1a, RT-PCR analysis revealed that SK1 mRNA levels are significantly increased in LNCaP-AI cells compared with LNCaP cells (LNCaP-AI cells *vs* LNCaP cells, p value $<0.01$).

SK1 protein expression levels were also examined. For this purpose, western blotting analysis of lysates from LNCaP and LNCaP-AI cells was carried out using specific antibodies that recognize the two different isoforms SK1a and SK1b. Since SK1a+14 migrates on SDS-PAGE with similar mobility as SK1a, the annotation "SK1a" is used herein to indicate the possibility that one or either isoform is expressed. Figure 3.1b shows that SK1a and SK1b proteins are expressed in the two cell lines and that the expression of both isoforms is increased in LNCaP-AI cells compared with

LNCaP cells (SK1a, 74±28% increase in LNCaP-AI *vs* LNCaP cells, p value <0.01; SK1b, 55±14% increase in LNCaP-AI *vs* LNCaP cells, p value <0.001).

To our knowledge, this is the first study to report that LNCaP and LNCaP-AI cells express both SK1a and SK1b isoforms. It is important to note that the annotation "SK1b" has been used by other authors to indicate a highly unstable 34 kDa version of SK1 (Kihara et al., 2006), which is different from the 51 kDa SK1b isoform referred to in the present study. The expression of 34 kDa "SK1b" was not detected in LNCaP nor LNCaP-AI cells.

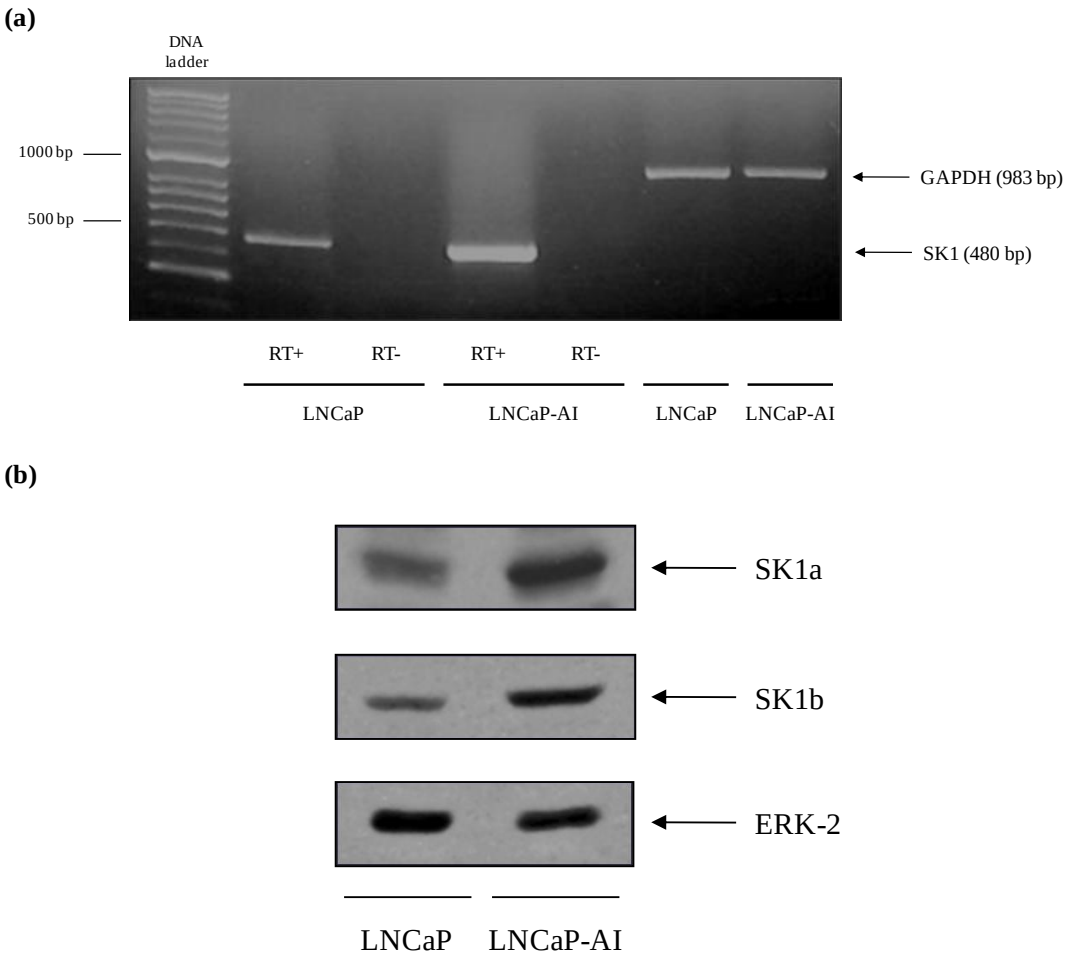



***Figure 3.1 Expression of SK1 in LNCaP and LNCaP-AI cells.*** *(a) SK1 mRNA levels were examined by RT-PCR using gene-specific primers, followed by agarose gel electrophoresis of the amplification products. A negative control with omission of reverse transcriptase (RT-) was included to establish a lack of genomic DNA contamination. The expression of the housekeeping gene GAPDH was analysed using gene-specific primers to ensure that comparable amounts of cDNA template were used for the two cell lines. The results shown are representative of three independent experiments. (b) SK1a and SK1b protein levels were examined by western blotting analysis of lysates from LNCaP and LNCaP-AI cells using antibodies that specifically recognize the two isoforms. Blots were then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between the two cell lines. Results are representative of at least three independent experiments.*

### 3.2.2 Effect of the SK1 inhibitor SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) on SK1 expression in LNCaP and LNCaP-AI cells

The chronic treatment of LNCaP cells with SKi reduced the expression of SK1a and SK1b (Figure 3.2; supplementary data, Figure S1). The reduction in SK1a and SK1b expression was detectable within 24 hours of treatment with the inhibitor (Figure 3.15 for SK1a; Figure 3.3 for SK1b; supplementary data, Figure S1). In contrast, SKi treatment of LNCaP-AI cells reduced the expression of SK1a but not SK1b (Figure 3.2; supplementary data, Figure S3).

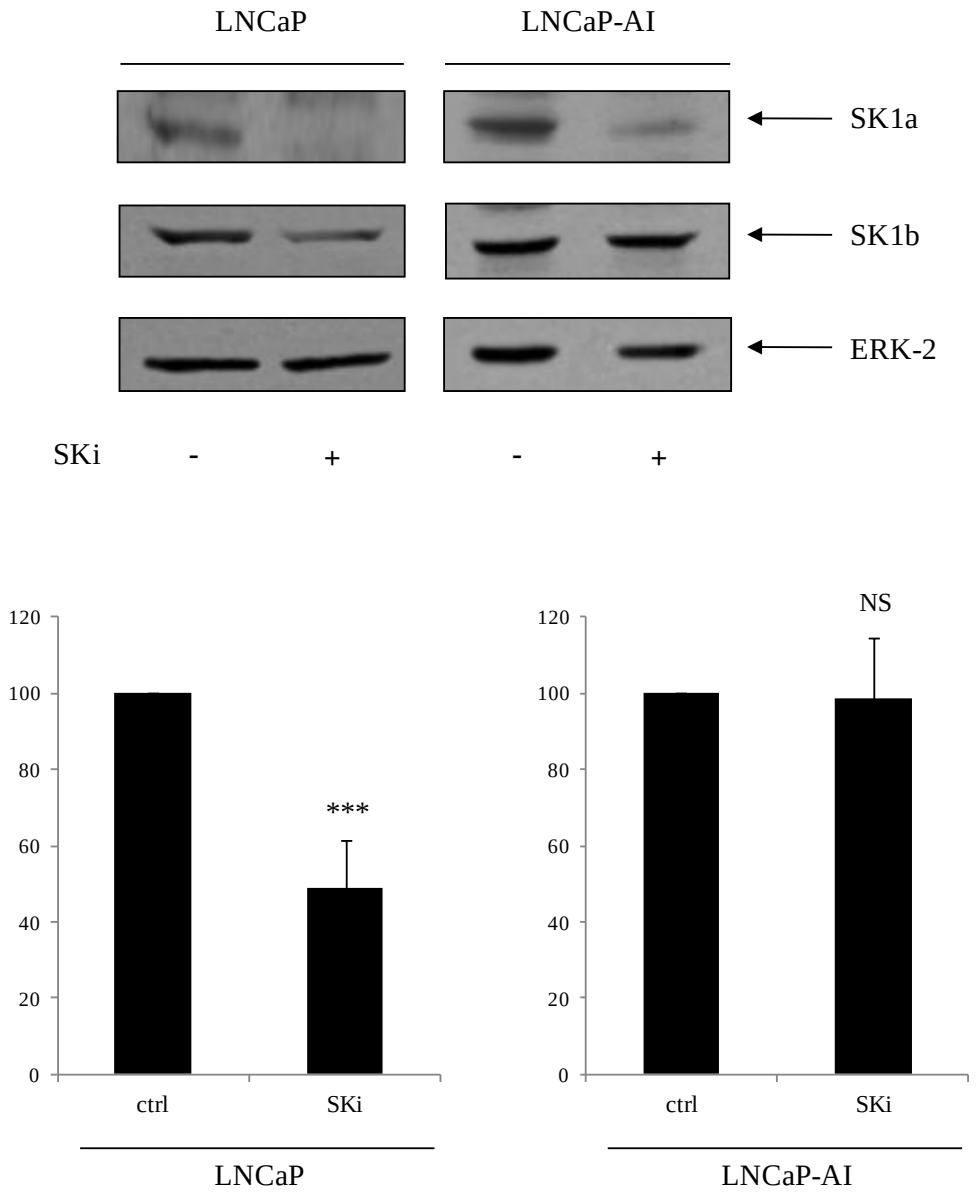



**Figure 3.2** ***Effect of SKi on SK1 protein expression in LNCaP and LNCaP-AI cells.*** *Cells were treated with SKi (10 μM, 48 hours) or with the vehicle alone (DMSO, 0.1% v/v). SK1a and SK1b protein levels were then examined by western blotting analysis using specific antibodies for the two different SK1 isoforms. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of at least three separate experiments. Also shown is the densitometric quantification of SK1b immunoreactivity, expressed as a percentage of the control (ctrl=100%).*** denotes a p value <0.001; NS denotes not statistically significant (p value >0.05).*

To assess whether SKi down-regulates SK1 by modulating *SK1* gene expression, cells were pre-treated with cycloheximide, an inhibitor of protein translation, before being challenged with SKi. As shown in Figure 3.3, cycloheximide did not block SKi-induced down-regulation of SK1b expression in LNCaP cells (supplementary data, Figure S1), suggesting that the effect of SKi is not on SK1 protein synthesis.

Quantitative RT-PCR analysis of SK1 mRNA expression in LNCaP cells treated with SKi confirmed that this inhibitor does not affect *SK1* gene expression. SK1 mRNA levels in SKi-treated LNCaP cells were not significantly different from those in control cells (Figure 3.4).

Taken together, these data indicate that the effect of SKi on SK1 expression in LNCaP cells is post-translational, i.e. SKi promotes degradation of SK1.

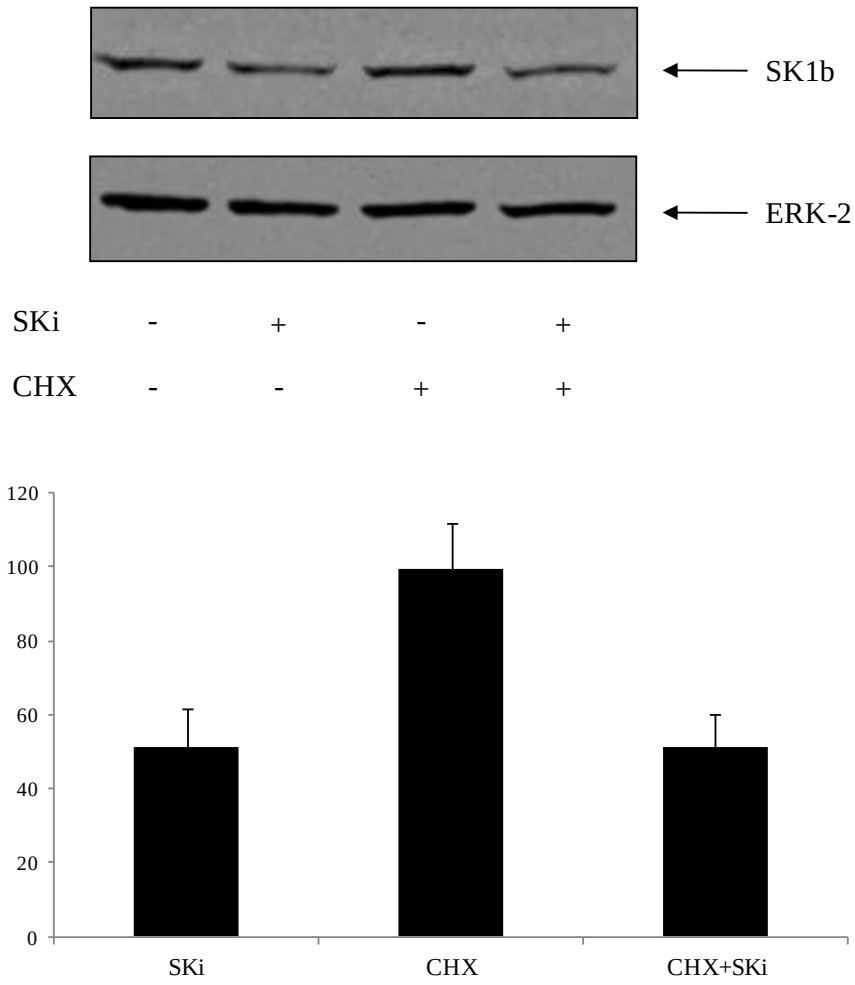



**Figure 3.3** ***Effect of protein synthesis inhibition on SKi-induced down-regulation of SK1 in LNCaP cells.*** *Cells were treated with SKi (10 μM, 24 hours) or with the vehicle alone (DMSO 0.1% v/v), in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX) (5 μg/ml, 30 minutes pre-treatment). SK1b protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1b immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001); CHX vs control, N.S.; CHX+SKi vs SKi, N.S. NS denotes not statistically significant (p value >0.05).*

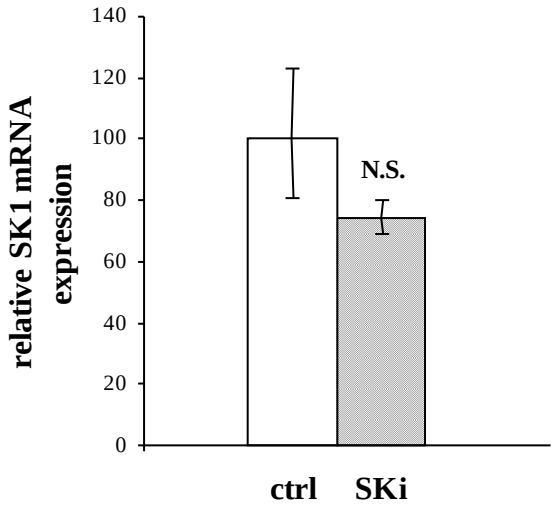



**Figure 3.4** ***Effect of SKi on SK1 mRNA expression in LNCaP cells.*** *Quantitative Real Time-PCR analysis of SK1 mRNA levels in LNCaP cells treated with SKi (10 μM, 24 hours) or with the vehicle (DMSO 0.1% v/v, ctrl). GAPDH was used as a reference gene. Data are expressed as relative SK1 mRNA expression, normalized to GAPDH mRNA levels. Bars represent mean of triplicate values ± S.E. and are expressed as % of control. N.S. denotes a p-value >0.05 compared with control (not statistically significant).*

The degradation of cellular proteins occurs through two main routes: the proteasomal pathway and the lysosomal pathway. In order to test whether SKi induces SK1 degradation by either of these routes, specific inhibitors of proteasomal and lysosomal degradative pathways were used.

The proteasome inhibitor MG132 reduced the effect of SKi on SK1a protein levels in LNCaP and LNCaP-AI cells and on SK1b protein levels in LNCaP cells (Figure 3.5a; supplementary data, Figure S1), indicating that SKi reduces SK1 expression via the proteasomal degradation pathway. In contrast, the treatment with a specific inhibitor of the lysosomal protease cathepsin B (CA074Me) did not prevent SK1b down-regulation in response to SKi (Figure 3.5b; supplementary data, Figure S1), indicating that the lysosomal proteolytic pathway is not involved.

The removal of SK1a and SK1b is associated with the induction of apoptosis as indicated by the auto-cleavage of caspase-3 (Figure 3.23a). To evaluate whether caspase-3 is responsible for causing proteolytic degradation of SK1, we used a specific caspase-3/7 inhibitor, Ac-DEVD-CHO. However, pre-treatment with this inhibitor did not block SKi-induced degradation of SK1a nor SK1b in LNCaP cells (Figure 3.6; supplementary data, Figure S1), suggesting that the activation of apoptosis executioner caspases-3 and -7 is not upstream of the degradation of SK1 in SKi-treated cells.

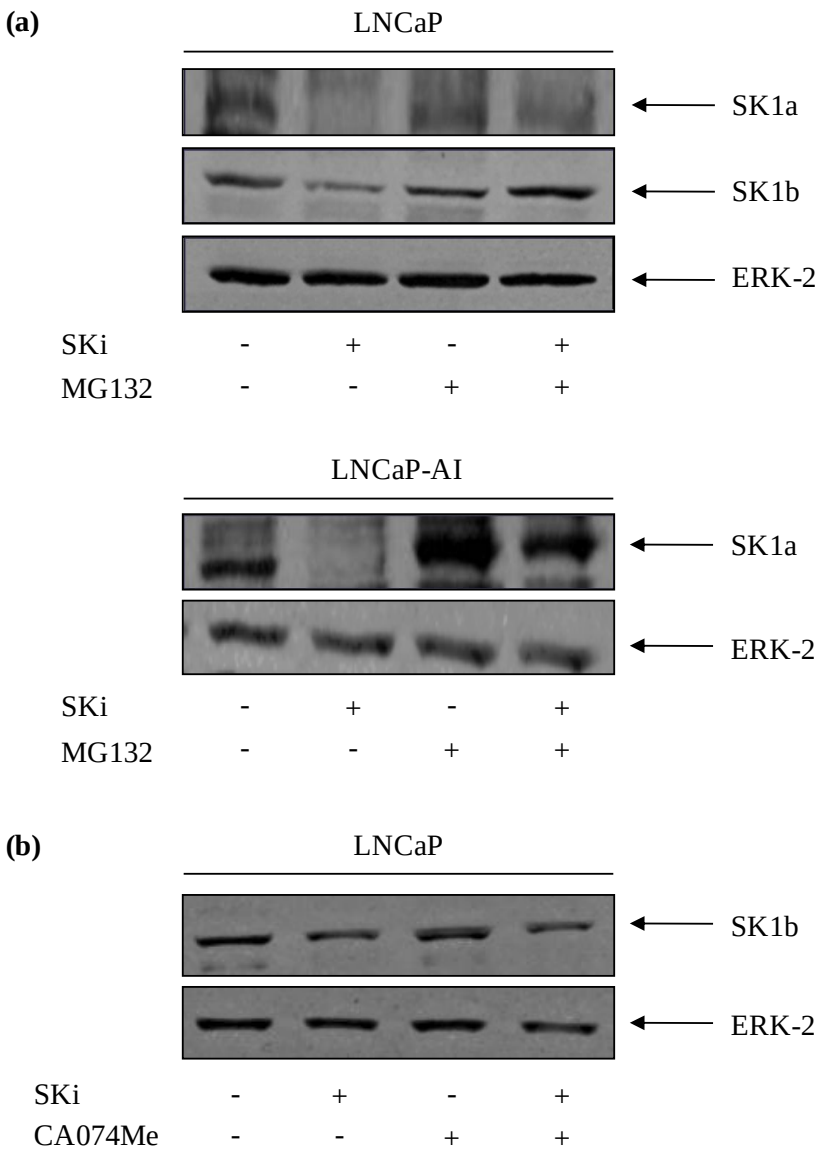



**Figure 3.5** ***Effect of proteasomal or lysosomal degradative pathway inhibition on SKi-induced down-regulation of SK1 expression in LNCaP and LNCaP-AI cells.***

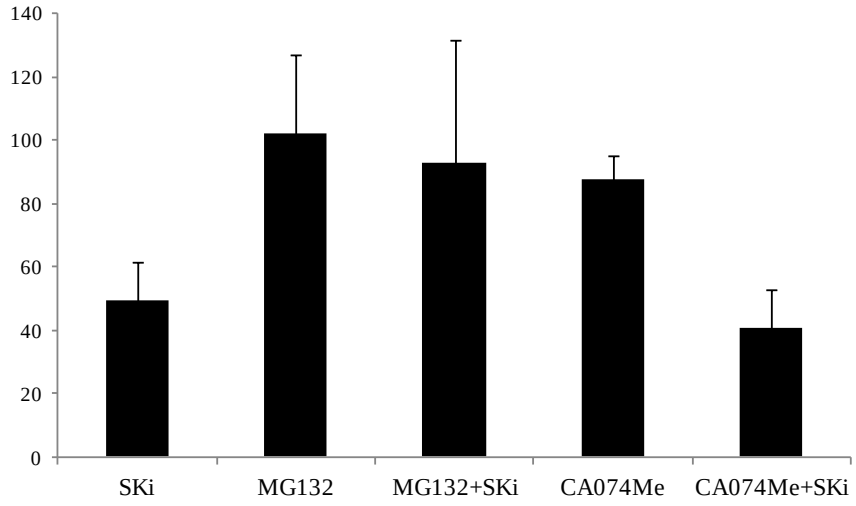



**Figure 3.5** ***Effect of proteasomal or lysosomal degradative pathway inhibition on SKi-induced down-regulation of SK1 expression in LNCaP and LNCaP-AI cells (continued).*** *(a) LNCaP and LNCaP-AI cells were pre-treated with the proteasome inhibitor MG132 (10 μM, 30 minutes) before being treated with SKi (10 μM, 48 hours) or with the vehicle (DMSO, 0.1% v/v). The levels of SK1a (in LNCaP and LNCaP-AI cells) or SK1b (in LNCaP cells) were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments. (b) LNCaP cells were pre-treated with the cathepsin B inhibitor CA074Me (10 μM, 30 minutes) before being treated with SKi (10 μM, 48 hours) or with the vehicle (DMSO, 0.1% v/v). SK1b protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1b immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001; MG132 vs control, N.S.; MG132+SKi vs SKi, <0.01; CA074Me vs control, <0.05; CA074Me+SKi vs SKi, N.S. N.S. denotes not statistically significant (p value >0.05).*

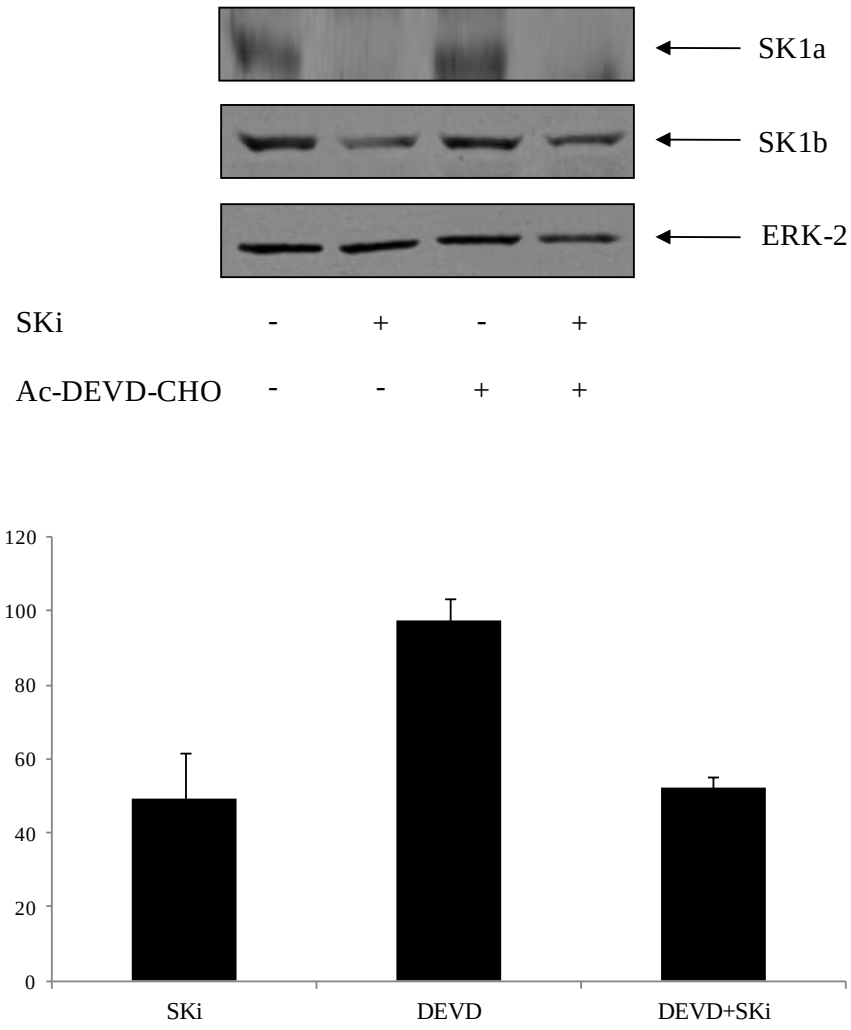



**Figure 3.6** ***Effect of caspase-3/7 inhibition on SKi-induced down-regulation of SK1 in LNCaP cells.*** *Cells were incubated with the caspase-3/7 inhibitor Ac-DEVD-CHO (100 μM, 30 minutes) before being treated with SKi (10 μM, 48 hours). SK1a and SK1b protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1b immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001; Ac-DEVD-CHO vs control, N.S.; Ac-DEVD-CHO+SKi vs SKi, N.S. N.S. denotes not statistically significant (p value >0.05).*

### 3.2.3 Effect of SKi on proteasome activity in LNCaP and LNCaP-AI cells

The study next focused on investigating the mechanism by which SKi promotes the proteasomal degradation of SK1 in LNCaP and LNCaP-AI cells.

The first step was to assess whether treatment with SKi stimulated proteasome activity in these cells. For this purpose, a Proteasome-Glo™ Cell-Based Assay was used. This assay measures the proteasome activity in cultured cells, by monitoring the luminescence generated by the proteasomal cleavage of a luminogenic substrate. Since this exogenous substrate competes with endogenous proteins for the proteasome, an increase in the proteasomal degradation of endogenous proteins, i.e. an activation of the proteasome results in a reduction in the proteasomal activity against the luminogenic substrate. To reflect the competition nature of this assay, data are presented as 100-% of the basal proteasome activity (i.e. in vehicle-treated cells), which is a measure of the proteasomal activity against endogenous proteins.

As shown in Figure 3.7, treatment of LNCaP and LNCaP-AI cells with SKi resulted in an increase in the proteasomal activity against endogenous proteins, indicating that SKi activates the proteasome in these cells. Interestingly, the extent to which the proteasome was activated in response to SKi stimulation was similar in LNCaP and LNCaP-AI cells. SKi increased proteasome activity within 8 hours of treatment (Chapter 5, Figure 5.7).

To confirm the competition nature of the assay, a titration experiment was carried out, which demonstrated that the stimulatory effect of SKi on the proteasomal degradation of endogenous proteins was reduced by increasing the concentration of the exogenous substrate (Figure 3.8).

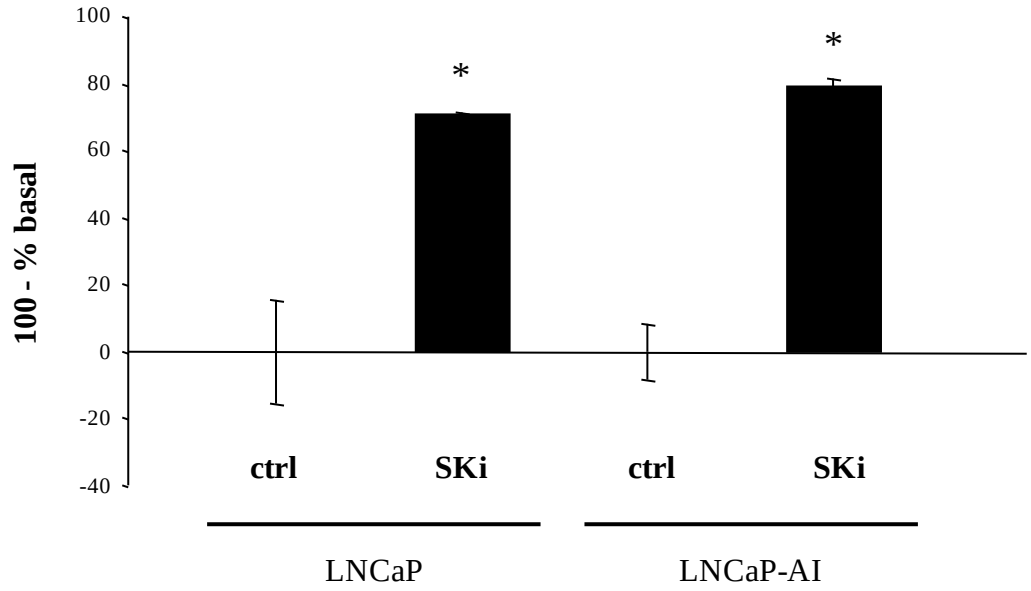



**Figure 3.7** ***Effect of SKi on proteasome activity in LNCaP and LNCaP-AI cells.*** *Cells were treated with SKi (10 μM), or with the vehicle alone (DMSO 0.1% v/v), for 24 hours, before measuring proteasomal activity using a specific assay, as described under "Methods". For each cell line, results are expressed as 100-% of proteasomal activity against luminogenic substrate in cells treated with the vehicle alone (which corresponds to SKi-stimulated activity against endogenous proteins), and are the mean with standard deviation of triplicate assays (n=3). * denotes a p-value <0.05 compared with control.*

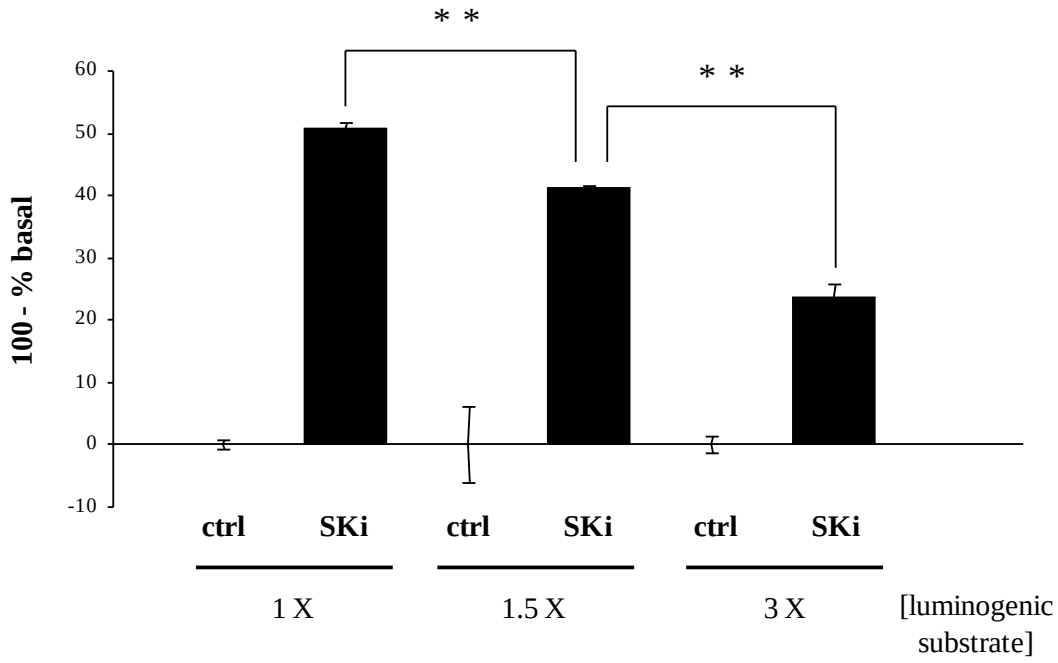



**Figure 3.8** ***Proteasome activity assay: effect of increasing concentrations of luminogenic substrate on SKi-stimulated proteasomal activity against endogenous proteins in LNCaP cells.*** *Cells were treated with SKi (10 μM, 24 hours), or with the vehicle alone (DMSO 0.1% v/v), before measuring proteasomal activity with a specific assay (as described under "Methods"), using increasing concentrations of the luminogenic substrate (corresponding to 1X, 1.5X, and 3X the concentration used for experiment reported in Figure 3.7). For each concentration of the luminogenic substrate, results are expressed as 100-% of proteasomal activity against luminogenic substrate in cells treated with the vehicle alone (which corresponds to SKi-stimulated activity against endogenous proteins), and are the mean with standard deviation of triplicate assays (n=3). ** denotes a p-value <0.01 compared with control.*

To confirm the ability of SKi to stimulate proteasomal activity, the effect of this inhibitor was also measured on the expression of cyclin D1 and c-Myc, two proteins that are known to be degraded via the ubiquitin-proteasome pathway.

Figure 3.9a and Figure 3.9c show that cyclin D1 and c-Myc protein levels were markedly down-regulated in LNCaP cells treated with SKi, an effect that was reversed by pre-treatment with the proteasome inhibitor MG132. Similarly, cyclin D1 expression was substantially reduced in SKi-treated LNCaP-AI cells (Figure 3.9b). The effect of SKi on cyclin D1 expression was also tested in a different cell line, MCF-7 breast cancer cells. As shown in Figure 3.10, cyclin D1 protein levels were significantly reduced in these cells in response to SKi treatment.

Consistent with the possibility that SKi activates the proteasome, treatment of LNCaP and LNCaP-AI cells with SKi reduced MG132-induced apoptosis, as evidenced by a decrease in the levels of 89 kDa cleaved PARP (a marker of apoptosis) compared with cells treated with MG132 alone (Figure 3.11). These findings suggest that SKi can partially override inhibition of the proteasome by MG132.

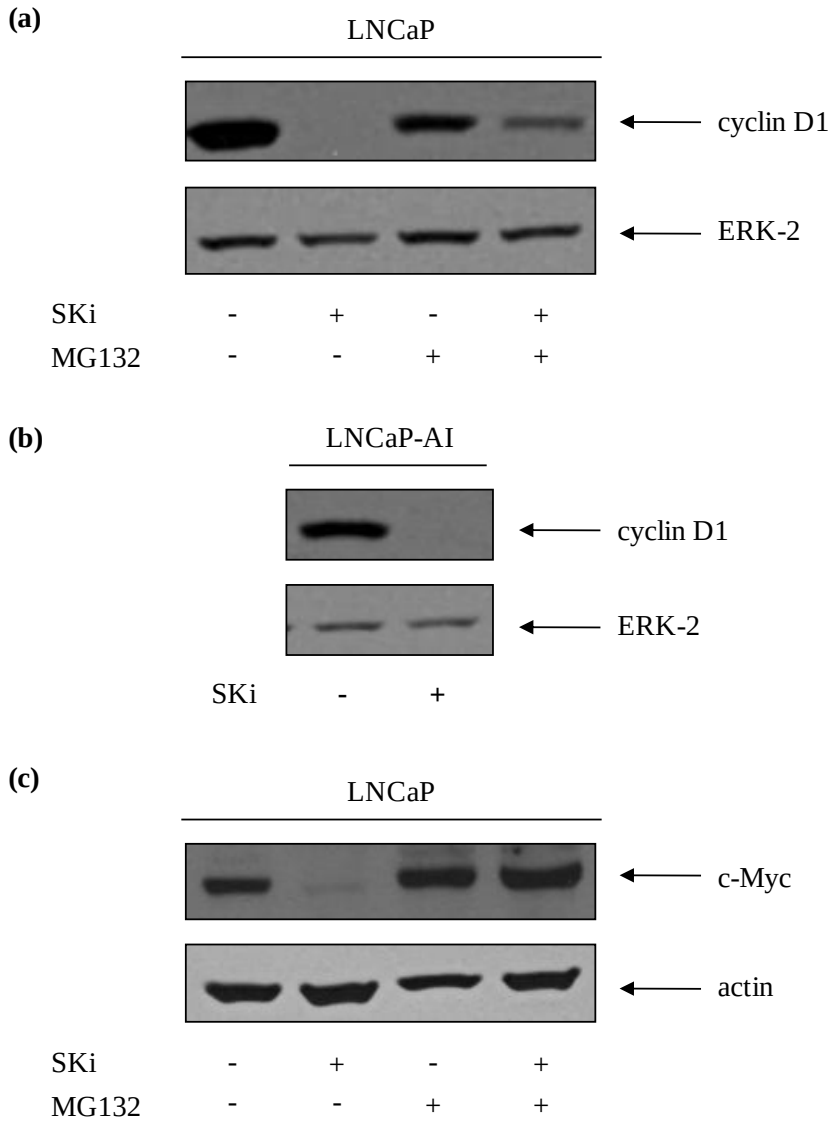



**Figure 3.9** ***Effect of SKi on cyclin D1 and c-Myc expression in LNCaP and LNCaP-AI cells.*** *(a) LNCaP cells were pre-treated with MG132 (10 μM, 30 minutes) before being treated with SKi (10 μM, 48 hours) or with the vehicle alone (DMSO 0.1% v/v). Cyclin D1 protein levels were measured by western blotting analysis. Blots were then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. (b) LNCaP-AI cells were treated with SKi (10 μM, 48 hours) or with the vehicle alone (DMSO 0.1% v/v) and cyclin D1 protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. (c) LNCaP cells were pre-treated with MG132 (10 μM, 30 minutes) before being treated with SKi (10 μM, 24 hours) or with the vehicle alone (DMSO 0.1% v/v). c-Myc protein levels were measured by western blotting analysis. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

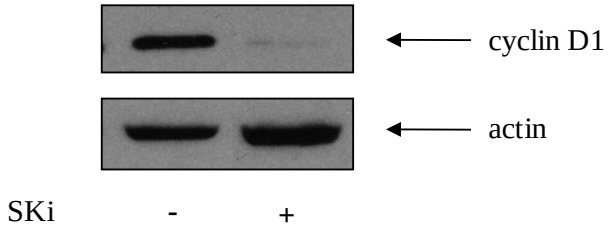



**Figure 3.10** ***Effect of SKi on cyclin D1 expression in MCF-7 breast cancer cells.*** *Cells were treated with SKi (10 μM, 24 hours) or with the vehicle alone (DMSO 0.1% v/v) and cyclin D1 protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments.*

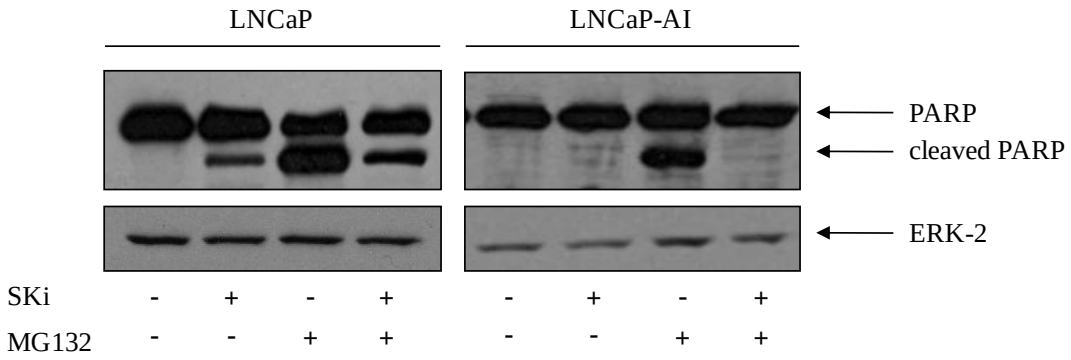



**Figure 3.11** ***Effect of SKi on MG132-induced apoptosis in LNCaP and LNCaP-AI cells.*** *Cells were treated with SKi (10 μM, 48 hours) or with the vehicle alone (DMSO 0.1% v/v) in the absence or presence of MG132 (10 μM, 30 minutes pre-treatment). Apoptosis was assessed by western blotting analysis of cleaved PARP levels using an antibody that detects both full-length (116 kDa) and cleaved (89 kDa) PARP. Blots were then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments.*

### 3.2.4 Effect of the ceramide synthase inhibitor fumonisin B1 on SKi-induced SK1 and cyclin D1 degradation in LNCaP cells

A possible model for SKi-induced proteasomal degradation of SK1 might involve an initial inhibition of SK1 activity which disrupts the ceramide-sphingosine-S1P rheostat and results in the accumulation of ceramide and the subsequent ceramide-dependent activation of the proteasome. To test this possibility, we used fumonisin B1 (FB1), which inhibits the activity of ceramide synthase and therefore the back-conversion of sphingosine to ceramide (Merrill et al., 1993; Merrill et al., 2001). FB1 reduced the effect of SKi on SK1b expression in LNCaP cells (Figure 3.12; supplementary data, Figure S1). Similarly, cyclin D1 protein levels were partially restored in LNCaP cells pre-treated with FB1 before being treated with SKi (Figure 3.12).

Higher concentrations of FB1 could not be used, as they were toxic for LNCaP cells and resulted in cell death (not shown).

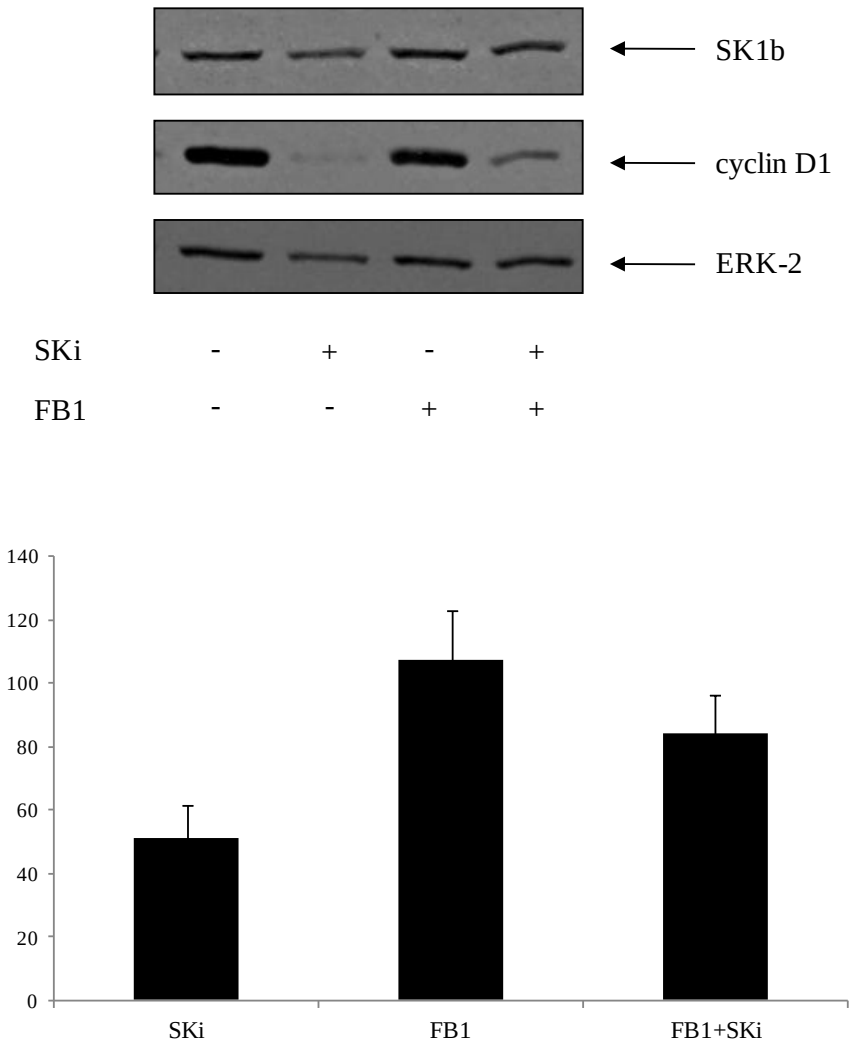



**Figure 3.12** ***Effect of ceramide synthase inhibition on SKi-induced down-regulation of SK1b and cyclin D1 in LNCaP cells.*** *Cells were pre-treated with fumonisin B1 (FB1) (100 μM, 1 hour) before being treated with SKi (10 μM, 24 hours). SK1b and cyclin D1 protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1b immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001; FB1 vs control, N.S.; FB1+SKi vs SKi, <0.01. N.S. denotes not statistically significant (p value >0.05).*

### 3.2.5 Effect of C2-ceramide, C2-dihydroceramide and dihydrosphingosine on SK1 expression in LNCaP and LNCaP-AI cells

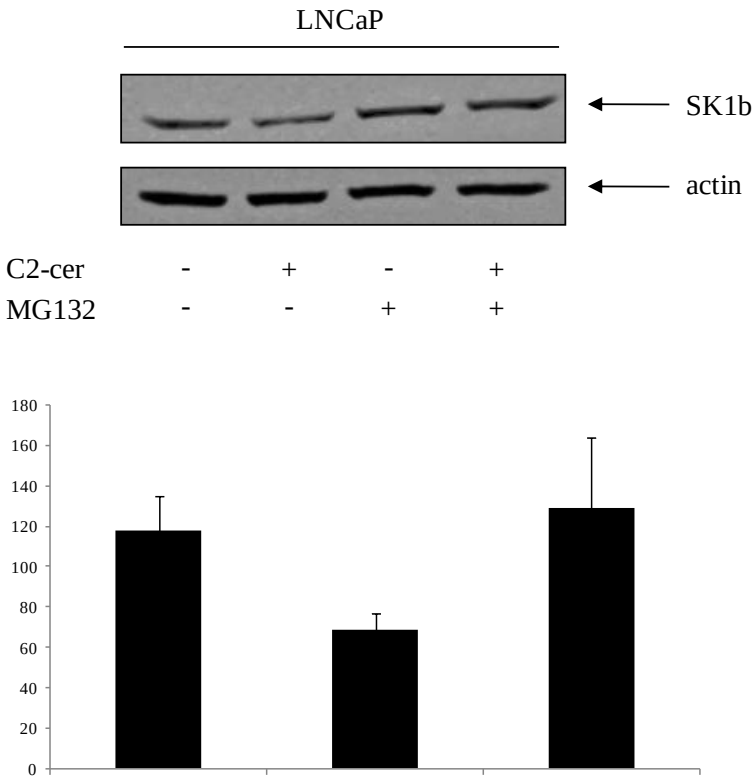
FB1 inhibits the back-conversion of sphingosine to ceramide. However, ceramide synthase is also involved in the *de novo* ceramide formation as it catalyses the conversion of dihydrosphingosine to dihydroceramide. Therefore, treatment with FB1 also reduces the levels of dihydroceramide and ceramide formed via the *de novo* pathway. To establish whether these sphingolipids are involved in the proteasomal degradation of SK1, we tested the effect of C2-dihydroceramide and C2-ceramide (short chain cell-permeable analogues of dihydroceramide and ceramide, respectively) on SK1 expression. As shown in Figure 3.14, treatment of LNCaP cells with C2-dihydroceramide did not significantly reduce SK1b protein levels (control *vs* treated cells, p value >0.05). In contrast, C2-ceramide mimicked the effect of SKi, inducing the down-regulation of SK1b expression in LNCaP cells, that was reversed in the presence of the proteasome inhibitor MG132 (Figure 3.13a; supplementary data, Figure S1). Similarly, treatment of LNCaP-AI cells with C2-ceramide induced the MG132-sensitive down-regulation of SK1a protein levels (Figure 3.13a; supplementary data, Figure S2). In contrast with SKi, C2-ceramide induced the down-regulation of SK1b in LNCaP-AI cells (Figure 3.13b; supplementary data, Figure S3).

These findings suggest that ceramide is the most likely sphingolipid metabolite responsible for inducing SK1 proteasomal degradation in cells treated with SKi.

To further investigate whether ceramide formed via the *de novo* pathway participates in the regulation of SK1 expression, cells were pre-treated with myriocin, which inhibits serine palmitoyltransferase and therefore the *de novo* ceramide synthesis. Interestingly, myriocin failed to prevent the reduction in SK1a and cyclin D1 protein levels in LNCaP cells treated with SKi (Figure 3.15), suggesting that *de novo*-synthesised ceramide is not involved in SKi-induced activation of the proteasome. Therefore, the ceramide involved in the proteasomal degradation of SK1 appears to be formed by the salvage pathway. Treatment of LNCaP cells with

dihydrosphingosine did not significantly reduce SK1a expression (control *vs* treated cells, p value >0.05) (Figure 3.14).

**(a)**


LNCaP
SK1b
actin
C2-cer - + - +
MG132 - - + +
180
160
140
120
100
80
60
40
20
0
MG132
CER
MG132+CER
LNCaP-AI
SK1a
actin
C2-cer - + - +
MG132 - - + +
160
140
120
100
80
60
40
20
0
C2-cer
MG132
MG132+C2-cer

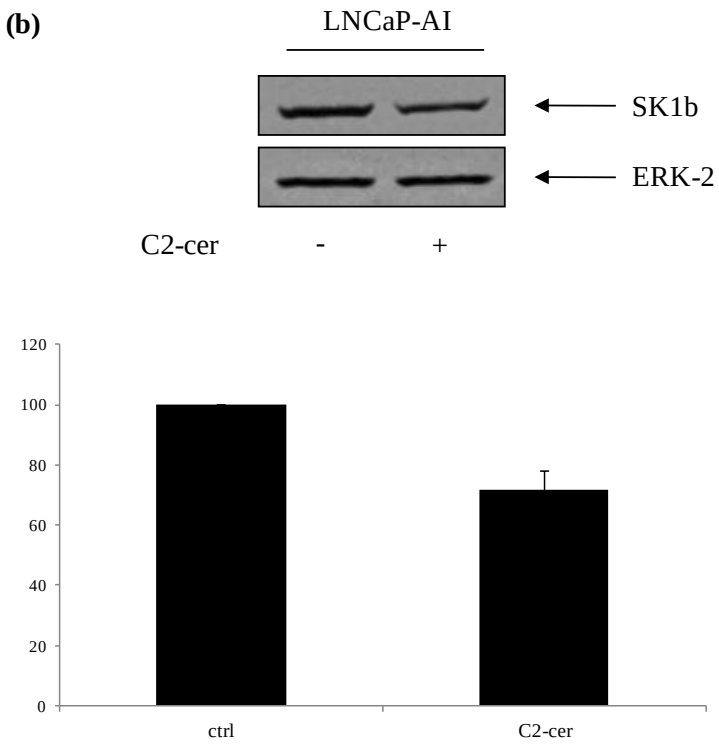



**Figure 3.13** ***Effect of C2-ceramide on SK1a and SK1b expression in LNCaP and LNCaP-AI cells.*** *(a) Western blots showing the reversal by MG132 (10 µM, 30 minutes pre-treatment) of the C2-ceramide (C2-cer) (50 µM, 24 h)-induced reduction of SK1b levels in LNCaP cells and SK1a levels in LNCaP-AI. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments. (b) Western blot showing the effect of C2-ceramide (C2-cer) (50 µM, 24 h) on SK1b expression in LNCaP-AI cells. The blot was then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1a and SK1b immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: LNCaP cells: C2-ceramide vs control, <0.001; MG132 vs control, N.S.; MG132+C2-ceramide vs C2-ceramide, <0.01. LNCaP-AI cells: SK1a: C2-ceramide vs control, <0.001; MG132 vs control, <0.001; MG132+C2-ceramide vs C2-ceramide, <0.001; SK1b: C2-ceramide vs control, <0.01. N.S. denotes not statistically significant (p value >0.05).*

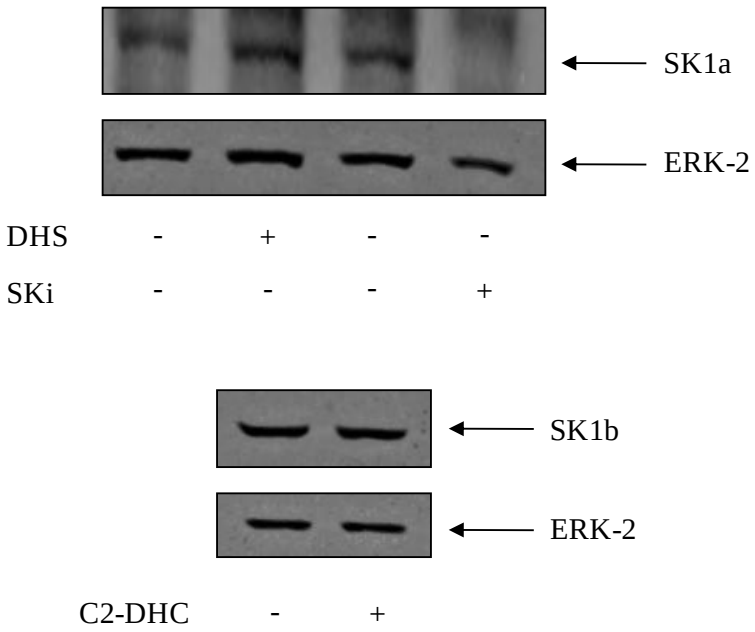



**Figure 3.14** ***Lack of effect of dihydrosphingosine or C2-dihydroceramide on SK1 expression in LNCaP cells.*** *LNCaP cells were treated with dihydrosphingosine (DHS) (10 μM, 48 hours) or C2-dihydroceramide (C2-DHC) (50 μM, 24 hours) or with the vehicle alone (DMSO 0.1% v/v) and SK1a or SK1b protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments. SKi (10 μM) was used as a reference compound for SK1a immunoblotting.*

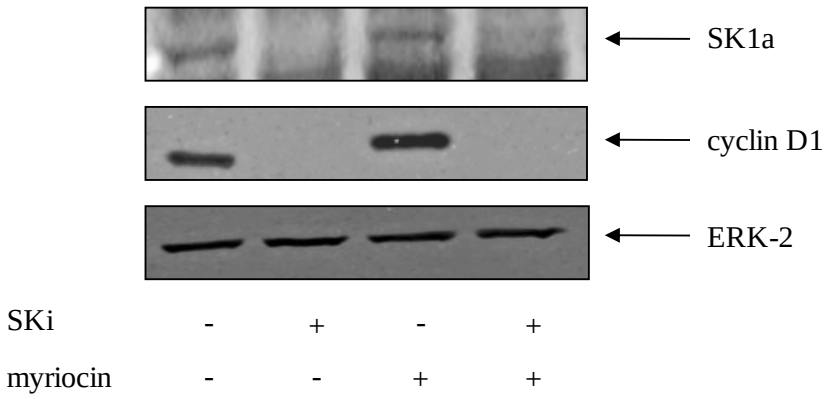



**Figure 3.15** ***Lack of effect of de novo ceramide synthesis inhibition on SKi-induced down-regulation of SK1a and cyclin D1 in LNCaP cells.*** *Cells were incubated with myriocin (100 nM, 2 hours pre-treatment) before being treated with SKi (10 μM, 24 hours). SK1a and cyclin D1 protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of two separate experiments.*

### 3.2.6 Effect of C2-ceramide on proteasome activity in LNCaP and LNCaP-AI cells

We next tested whether C2-ceramide activates the proteasome in LNCaP and LNCaP-AI cells. Treatment with C2-ceramide reduced cyclin D1 protein levels in LNCaP cells (Figure 3.16a) and increased proteasomal activity against endogenous proteins in LNCaP-AI cells (Figure 3.16b). In addition, C2-ceramide reduced MG132-induced PARP cleavage (Figure 3.17), suggesting that, as was observed for SKi, C2-ceramide counters the inhibition of the proteasome by MG132. These results provide evidence that C2-ceramide stimulates proteasomal activity in LNCaP and LNCaP-AI cells.

Pre-treatment of LNCaP cells with okadaic acid, an inhibitor of protein phosphatase 2A (PP2A, an intracellular target of ceramide: Dobrowsky et al., 1993; Chalfant et al., 1999), partially reversed SKi-induced down-regulation of cyclin D1 (Figure 3.18).

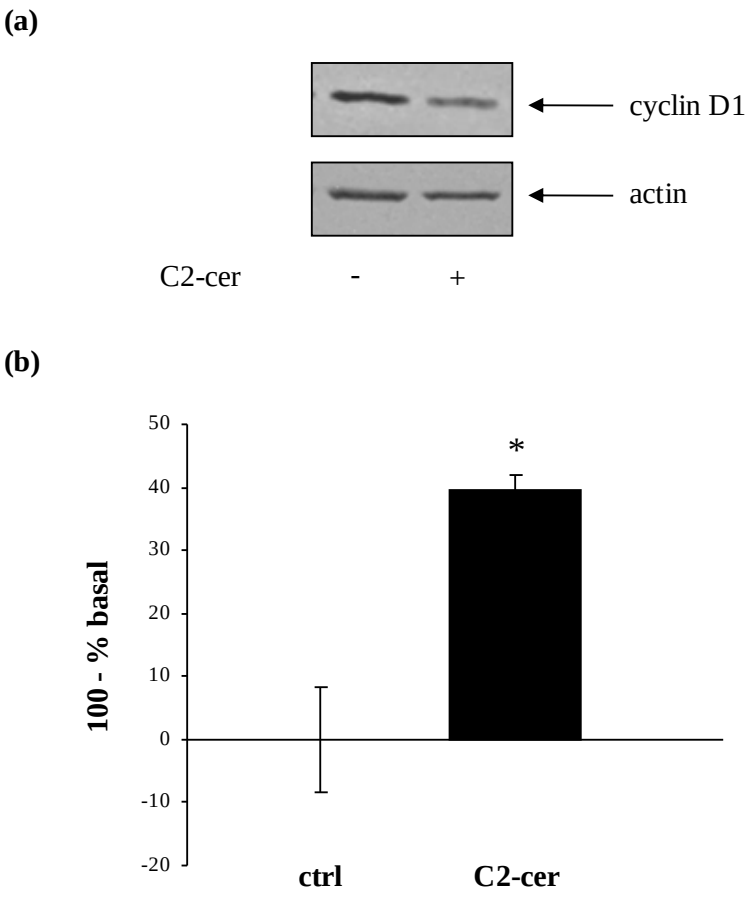



**Figure 3.16** ***Effect of C2-ceramide on proteasome activity in LNCaP and LNCaP-AI cells.*** *(a) Western blot showing the effect of C2-ceramide (C2-cer) (50 μM, 24 hours) on cyclin D1 protein levels in LNCaP cells. Blot was stripped and reprobed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of two separate experiments. (b) Proteasomal luminogenic assay showing the effect of C2-ceramide (C2-cer) (50 μM, 24 hours) on proteasome activity in LNCaP-AI cells. Results are expressed as 100-% of proteasomal activity against luminogenic substrate in vehicle (DMSO 0.1% v/v)-treated cells (which corresponds to inhibitor-stimulated activity against endogenous proteins) and are the mean ± standard deviation of triplicate assays (n=3). * denotes a p-value <0.05 compared with control.*

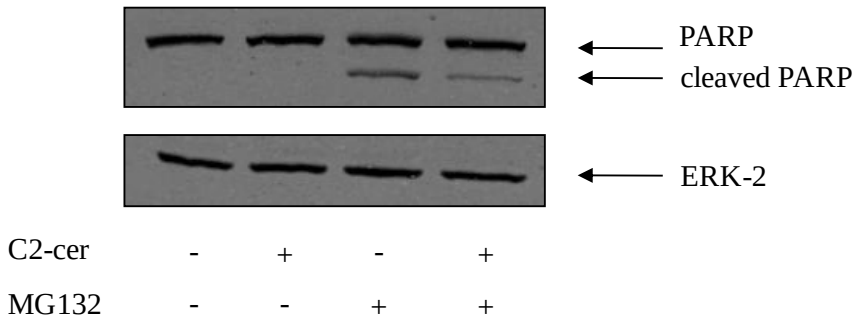



**Figure 3.17** ***Effect of C2-ceramide on MG132-induced apoptosis in LNCaP cells.*** *Cells were treated with C2-ceramide (C2-cer) (50 μM, 24 hours) or with the vehicle (DMSO 0.1% v/v) in the absence or presence of MG132 (10 μM, 30 minutes pre-treatment). Apoptosis was assessed by western blotting analysis of cleaved PARP levels, using an antibody that detects both full-length (116 kDa) and cleaved (89 kDa) PARP. Blots were then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading between samples. Results are representative of two separate experiments.*

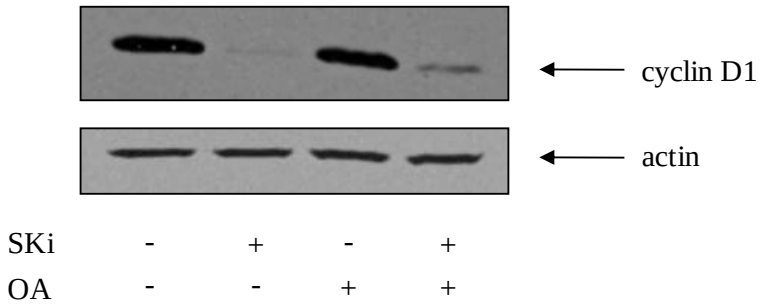



**Figure 3.18** ***Effect of PP2A inhibition on SKi-induced down-regulation of cyclin D1 in LNCaP cells.*** *Cells were incubated with okadaic acid (OA) (20 nM, 30 minutes) before being treated with SKi (10 μM, 24 hours). Cyclin D1 protein levels were measured by western blotting analysis. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading between samples.*

### 3.2.7 Effect of SKi on over-expressed SK1a and SK1b in LNCaP cells

Having established that SK1b is resistant to SKi-induced down-regulation in LNCaP-AI cells, the mechanism(s) that underlie the ability of SK1b to evade proteasomal degradation in response to SKi in LNCaP-AI cells was next investigated.

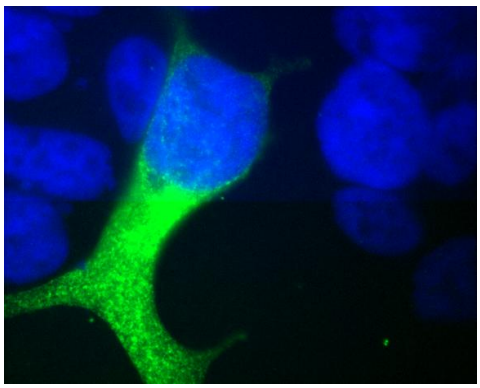
To assess whether the susceptibility of SK1 to SKi-induced degradation is dependent on its expression level, recombinant SK1a and SK1b were over-expressed in LNCaP cells and their sensitivity to SKi determined.

Transfection efficiency was assessed by immunofluorescence cell imaging using anti-Myc or anti-FLAG antibodies and the corresponding fluorophore-tagged secondary antibodies, as described under the Methods section. When viewed under the epifluorescence microscope, successfully transfected cells appeared green (if expressing Myc-tagged SK1a) or red (if expressing FLAG-tagged SK1b). Transfection efficiency was estimated by calculating the ratio between fluorescence-emitting cells and the total number of cells, determined by counting DAPI-stained nuclei. Immunofluorescence microscopy analysis revealed that Myc-SK1a and FLAG-SK1b transient transfection efficiency in LNCaP cells was approximately 10% (Figure 3.19). LNCaP cells transfected with the vector plasmid were used as a control for the specificity of the signal (not shown).

Despite the low transfection efficiency, recombinant SK1a and SK1b could be detected in lysates prepared from transiently transfected LNCaP cells by western blotting analysis using anti-Myc or anti-FLAG antibodies (Figure 3.20; Figure 3.21). Therefore, this system was suitable for testing the effect of SKi on over-expressed SK1a and SK1b levels. As shown in Figure 3.20 and Figure 3.21, no bands corresponding to proteins of the expected size were detected when lysates from vector-transfected LNCaP cells were immunoblotted using anti-Myc or anti-FLAG antibodies, confirming the specificity of the antibodies used to detect recombinant SK1.

SK1 activity assay of LNCaP cell lysates containing over-expressed SK1a or SK1b showed a 215-fold increase in SK1a activity and a 90-fold increase in SK1b activity compared with vector-transfected LNCaP cells (SK1 activity in vector-transfected cells: 0.004±0.002 pmol/min/ug protein; SK1a-transfected cells: 0.929±0.080 pmol/min/ug protein; SK1b-transfected cells: 0.387±0.020 pmol/min/ug protein). This data confirms that recombinant SK1a and SK1b ectopically expressed in LNCaP cells are catalytically active.

**(a)**



**(b)**

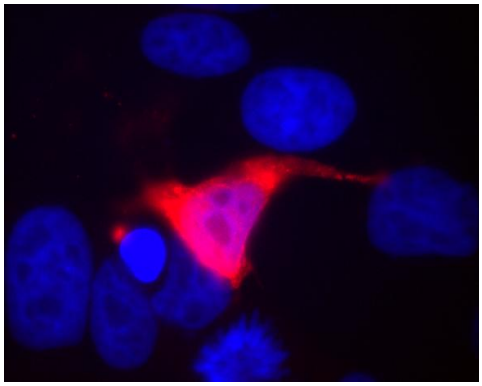


**Figure 3.19** ***Immunofluorescence microscopy images showing the transfection efficiency of Myc-tagged SK1a and FLAG-tagged SK1b in LNCaP cells.*** *Cells transiently transfected with (a) Myc-SK1a plasmid or (b) FLAG-SK1b plasmid were processed for immunofluorescence (as described under the Methods section) using (a) anti-Myc primary antibody and FITC-tagged secondary antibody (green signal) or (b) anti-FLAG primary antibody and TRITC-tagged secondary antibody (red signal). Cell nuclei were stained with DAPI (blue signal).*

To evaluate the effect of SKi on over-expressed recombinant SK1a and SK1b levels we used anti-Myc and anti-FLAG antibodies, so that we were only assessing cells in which these recombinant proteins had been successfully over-expressed.

In contrast with endogenous SK1a and SK1b (Figure 3.3; Figure 3.15), SKi (10 μM) failed to down-regulate ectopically expressed SK1a and SK1b in LNCaP cells (Figure 3.20). The effect of higher concentrations of the inhibitor was therefore tested. As shown in Figure 3.20, 50 μM SKi reduced the expression of both isoforms. Interestingly, the treatment of LNCaP cells with 25 μM SKi induced the down-regulation of recombinant SK1a, but not of SK1b (Figure 3.20). Thus, this system recapitulates the scenario observed for endogenous SK1 in LNCaP-AI cells, where SK1a is sensitive to SKi-induced proteasomal degradation while SK1b evades the proteasome (Figure 3.2; Figure 3.5).

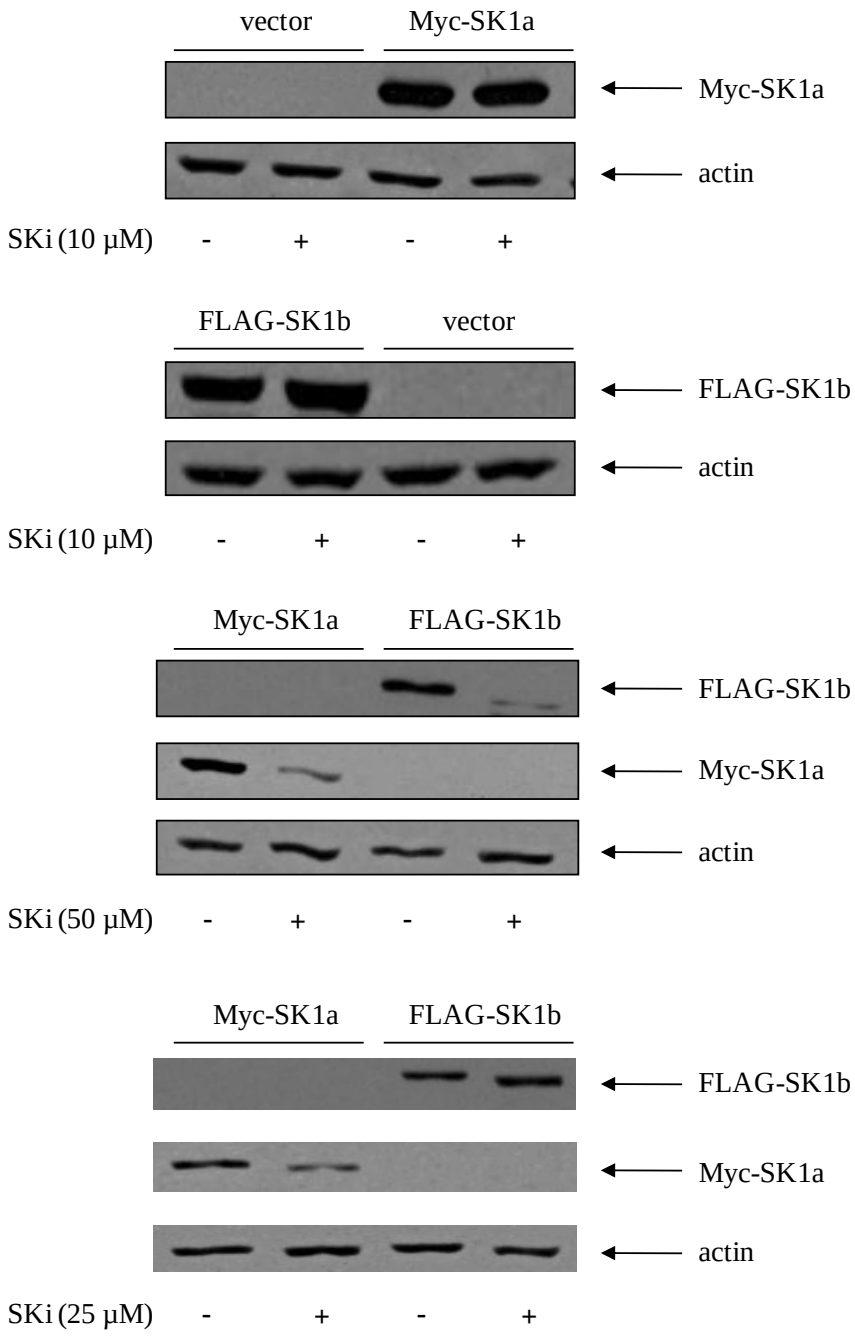



**Figure 3.20** ***Effect of SKi on recombinant SK1a and SK1b expression in LNCaP cells.*** *LNCaP cells transiently over-expressing Myc-SK1a or FLAG-SK1b were treated with the indicated concentrations of SKi or with the vehicle alone (DMSO, 0.1% v/v) for 24 hours. Myc-SK1a and FLAG-SK1b levels were measured by western blotting analysis using anti-Myc or anti-FLAG antibodies, respectively. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Lysates from LNCaP cells transfected with vector plasmid were used as a control of antibody specificity.*

### 3.2.8 Analysis of the inhibition kinetics of SK1a and SK1b in response to SKi

To assess whether the reduced sensitivity of SK1b to SKi-induced degradation was due to a defective binding of this enzyme to the inhibitor, the concentration-dependent inhibition of SK1a and SK1b by SKi was assessed, using lysates prepared from LNCaP cells transiently over-expressing SK1a or SK1b. The expression of recombinant SK1a or SK1b in the cell lysates used for the activity assay was confirmed by western blotting analysis (Figure 3.21b).

SK1 activity in lysates from LNCaP cells transiently transfected with the vector plasmid was very low (215-fold and 90-fold lower than in lysates from SK1a- and from SK1b-transfected cells, respectively) (section 3.2.7), indicating that the activity of endogenous SK1 is negligible compared to the activity of ectopically expressed SK1 (~0.5-1% of the activity in SK1a- and SK1b-transfected cells). Therefore, the activity measured in lysates from LNCaP cells transiently over-expressing SK1a or SK1b can be attributed to the ectopically expressed enzymes, and the observed % inhibition at each concentration of SKi can be regarded as due to inhibition of recombinant SK1a or SK1b activity. This enabled us to analyse the concentration-dependent inhibition for the two different SK1 isoforms separately.

As shown in Figure 3.21a, SKi inhibits SK1a and SK1b catalytic activity in a concentration-dependent manner and with similar potency for the two isoforms (extrapolated $IC_{50}$ was approximately 100 µM).

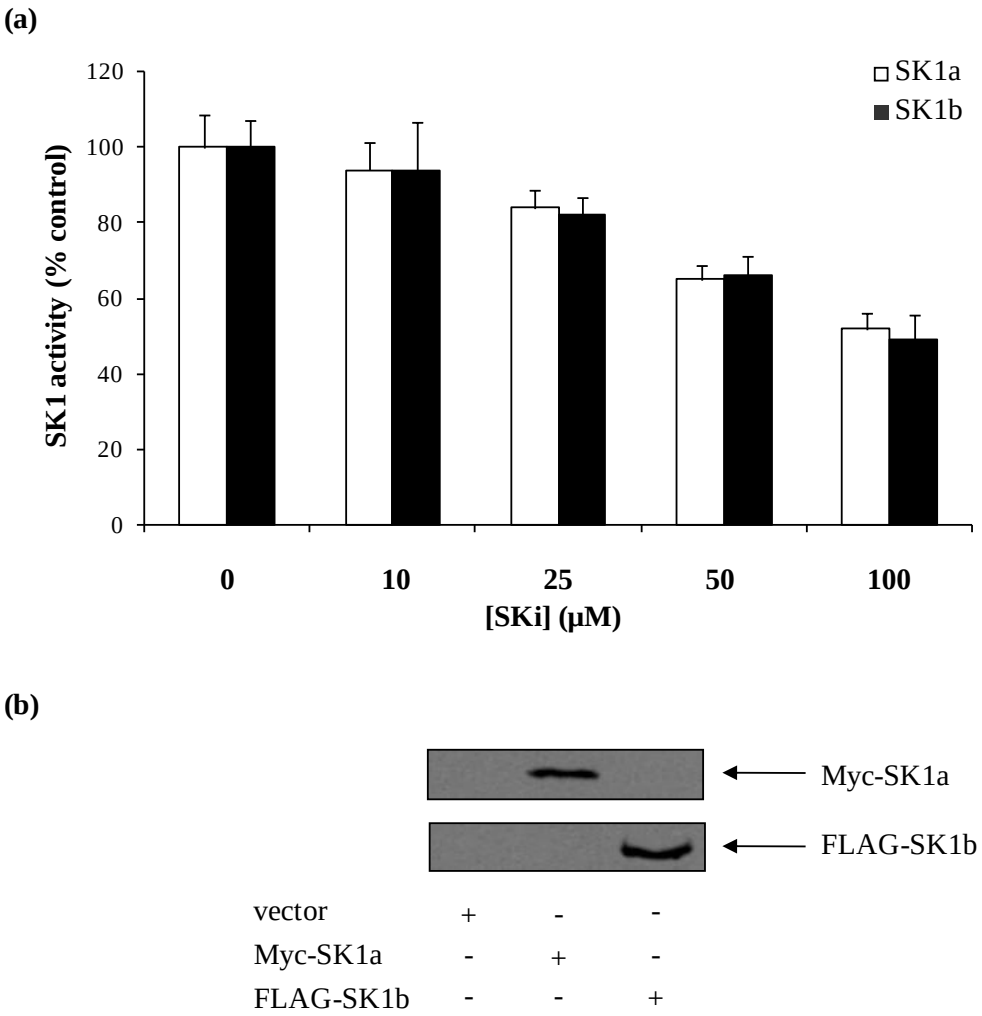



***Figure 3.21 Effect of SKi on SK1a and SK1b activity.*** *(a) SK1 activity was measured in lysates from LNCaP cells transiently transfected with Myc-SK1a or FLAG-SK1b plasmids, in the presence of the indicated concentrations of SKi or the vehicle (control, DMSO 5% v/v). For each isoform and for each concentration of SKi, results are expressed as % of SK1 activity in the absence of SKi (control=100% activity) and are the mean with standard deviation of n=4 assays. p values for SK1a or SK1b activity in the presence of SKi versus control are: SK1a, N.S. (10 µM), <0.05 (25 µM), <0.001 (50 µM), <0.001 (100 µM); SK1b, N.S. (10 µM), <0.01 (25 µM), <0.001 (50 µM), <0.001 (100 µM). N.S. denotes a p value >0.05 (not statistically significant). In the presence of each concentration of SKi, the activity of SK1a was not significantly different from the activity of SK1b (p value>0.05). (b) Western blot showing the over-expression of recombinant Myc-SK1a or FLAG-SK1b in the lysates used for SK1 activity assays. Blot was probed with anti-Myc antibody and then stripped and re-probed with anti-FLAG antibody.*

### 3.2.9 Investigation of potential SK1-interacting proteins in LNCaP cells

We next tested whether the different sensitivity of SK1a and SK1b to SKi-induced degradation was associated with a distinct pattern of interacting proteins. To address this issue, we used a combination of protein labelling, immunoprecipitation and autoradiography, as described in the Methods section.

LNCaP cells over-expressing recombinant SK1a or SK1b were treated with 25 μM SKi, that exerts a differential effect on SK1a and SK1b expression in terms of down-regulation (Figure 3.20). For this experiment, cells were challenged with a short-time treatment (3 hours) with SKi, to immunoprecipitate SK1a/SK1b before these proteins were proteasomally degraded.

However, there were no detectable differences between SK1a and SK1b in terms of interacting proteins in response to treatment with SKi. Indeed, the only [$^{35}$S]-labelled proteins that could be detected by autoradiography were those corresponding to SK1a (in LNCaP cells over-expressing SK1a-Myc) or SK1b (in LNCaP cells over-expressing SK1b-FLAG) (as assessed from their apparent molecular weight), and the treatment with SKi did not induce the appearance of any additional [$^{35}$S]-labelled proteins (Figure 3.22).

A parallel experiment was performed using lysates from LNCaP cells transfected with vector plasmid as a control.

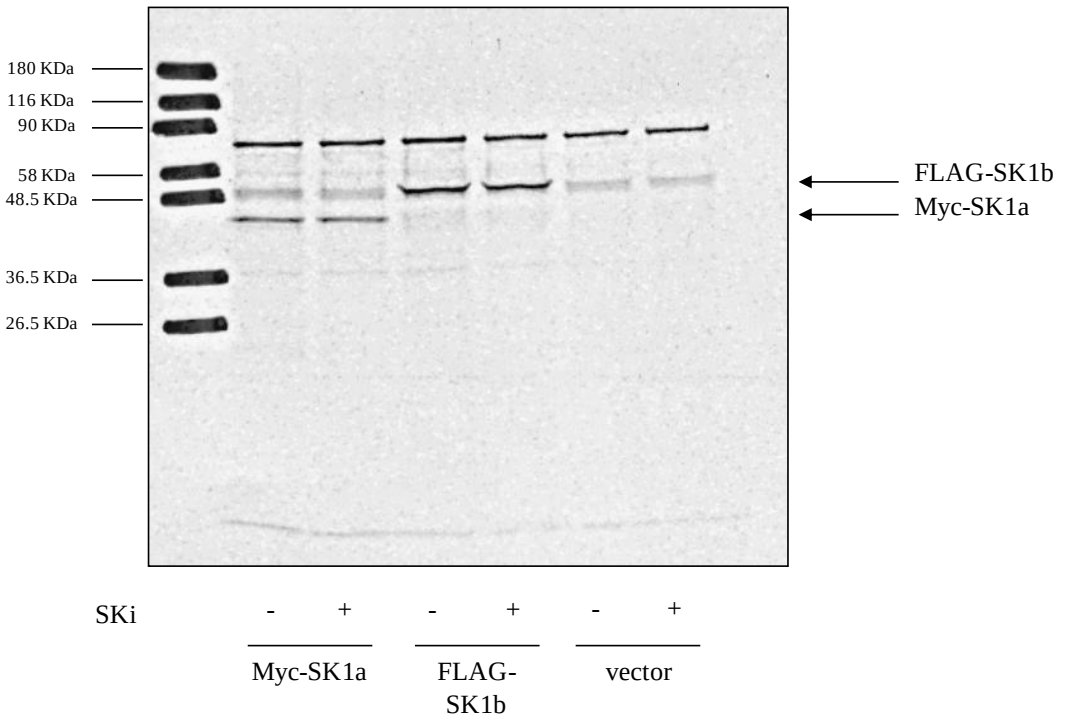



**Figure 3.22** ***Analysis of SK1-interacting proteins in LNCaP cells by autoradiography.*** *LNCaP cells transiently over-expressing recombinant SK1a or SK1b were incubated for 24 hours with [$^{35}$S]methionine (to label de novo-synthesised proteins, rendering them detectable by autoradiography) and then treated with SKi (25 μM) or the vehicle alone (DMSO, 0.1% v/v) for 3 hours. Recombinant SK1a and SK1b were immunoprecipitated (using anti-Myc or anti-FLAG antibodies, respectively) and the resulting immunocomplexes were resolved by SDS-PAGE and detected by autoradiography. Results are representative of three separate experiments. On the left is reported the position and the size of pre-stained molecular weight markers run on the polyacrylamide gel alongside the samples.*

### 3.2.10 Effect of SKi on LNCaP and LNCaP-AI cell survival

We next evaluated the functional significance of SKi-induced down-regulation of SK1 by assessing the apoptotic status of LNCaP and LNCaP-AI cells treated with this inhibitor.

Treatment with SKi induced cell death by apoptosis in LNCaP cells, as assessed by the detection of cleaved caspase-3 (17/19 kDa) and cleaved PARP (apoptosis-specific 89 kDa fragment), two markers of the apoptotic process (Figure 3.23a). The activation of apoptosis in LNCaP cells treated with SKi occurred within 24 hours, as assessed by the appearance of cleaved PARP (Figure 3.23b). In contrast, cleaved caspase-3 and cleaved PARP were not detected in SKi-treated LNCaP-AI cells, indicating that SKi did not induce the onset of apoptosis in these cells (Figure 3.23a).

The treatment of LNCaP cells with SKi was also associated with an increase in the phosphorylation of ERK-1/2, an enzyme which is involved in the regulation of cell survival, while this pro-survival pathway was not activated in LNCaP-AI cells (Figure 3.24a). Pre-treatment of LNCaP cells with PD98059, an inhibitor of MEK1 activation (an upstream regulator of ERK-1/2), enhanced PARP cleavage induced by SKi while blocking ERK-1/2 phosphorylation (Figure 3.24a; Figure 3.24b). This finding confirms that the activation of ERK-1/2 pathway represents a re-bound mechanism that affords some degree of protection against apoptotic stimuli in LNCaP cells.

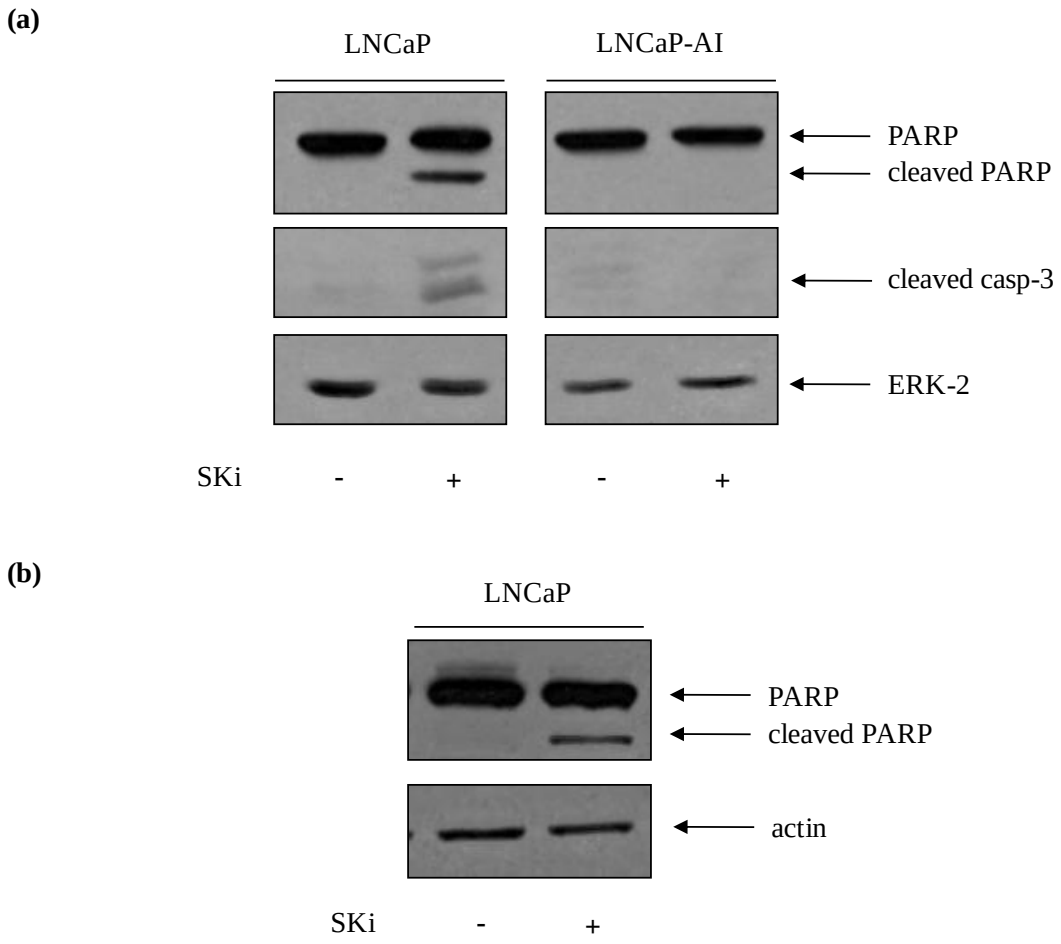



**Figure 3.23** ***Effect of SKi on cell survival in LNCaP and LNCaP-AI cells.*** *(a) Cells were treated for 48 hours with SKi (10 μM) or with the vehicle (DMSO, 0.1% v/v) and apoptosis was then evaluated by western blotting analysis of the expression of apoptosis-related markers: PARP cleavage, using an antibody detecting both full-lenght (116 kDa) and cleaved (89 kDa) PARP, and caspase-3 cleavage (17/19 kDa). Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of at least three separate experiments. (b) LNCaP cells were treated for 24 hours with SKi (10 μM) or with the vehicle (DMSO, 0.1% v/v) and apoptosis was then evaluated by western blotting analysis of the expression of PARP cleavage. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of at least three separate experiments.*

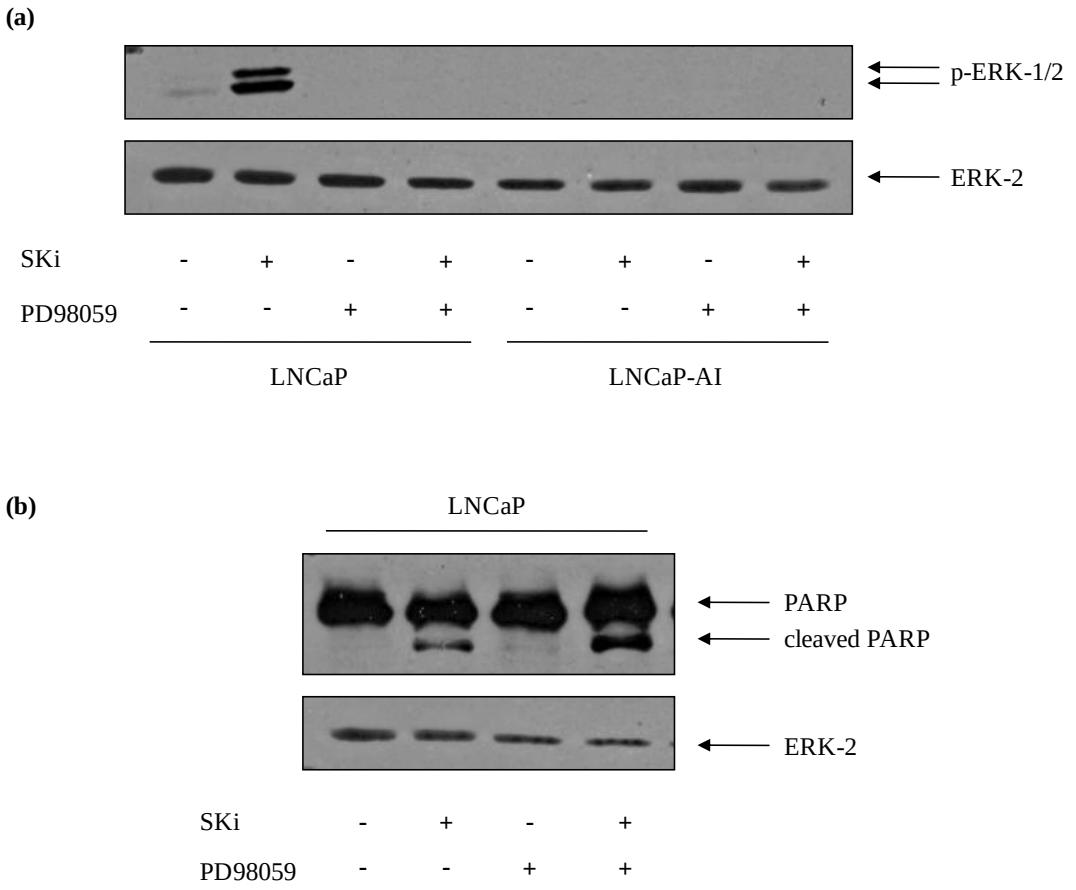



**Figure 3.24** ***Effect of MEK inhibition on SKi-induced ERK-1/2 activation and PARP cleavage.*** *LNCaP and LNCaP-AI cells were treated with SKi (10 μM, 48 hours) or with the vehicle alone (DMSO, 0.1% v/v) in the absence or presence of PD98059 (10 μM, 30 minutes pre-treatment), and (a) ERK-1/2 activation (in LNCaP and LNCaP-AI cells) or (b) apoptosis (in LNCaP cells) were then assessed by western blotting analysis, using an anti-phospho-ERK-1/2 or an anti-PARP/cleaved PARP antibody, respectively. Blots were then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments. p-ERK-1/2: phospho-ERK-1/2.*

### 3.2.11 Effect of SK1 siRNA on LNCaP and LNCaP-AI cell survival

The failure of SKi to induce apoptosis in LNCaP-AI cells is associated with the reduced sensitivity of SK1b to SKi-induced proteasomal degradation. In order to further investigate the role of SK1a and SK1b in regulating prostate cancer cell survival, we used specific siRNA to knock-down the expression of these enzymes. The SK1 siRNA used for these experiments is targeted at a common region in SK1a and SK1b mRNA and therefore is potentially able to knock-down the expression of both isoforms. In parallel, transfection with a "scrambled" siRNA, not targeting the gene of interest, was performed as a control.

The transfection of LNCaP cells with SK1 siRNA resulted in a marked reduction in SK1 mRNA levels compared to cells transfected with scrambled siRNA, as assessed by RT-PCR using gene-specific primers that amplify both SK1a and SK1b mRNA (Figure 3.25a). SK1 protein expression in LNCaP and LNCaP-AI cells transfected with SK1 siRNA was also measured. As shown in Figure 3.25b, SK1a protein levels were substantially reduced by siRNA treatment in both cell lines. However, SK1 siRNA failed to knock-down SK1b protein expression (SK1 siRNA *vs* scrambled siRNA, p value >0.05 for both cell lines) (Figure 3.25b; supplementary data, Figure S3).

siRNA knock down of SK1a did not result in apoptosis. Indeed, the treatment of LNCaP and LNCaP-AI cells with SK1 siRNA did not result in enhanced PARP cleavage compared with scrambled siRNA-treated cells (Figure 3.25b; Figure 3.26).

The lack of effect of SK1 siRNA in reducing SK1b protein levels is likely due to a slow rate of turnover of this protein. This hypothesis is supported by the finding that a 24.5 hours treatment of LNCaP cells with the protein synthesis inhibitor cycloheximide did not affect SK1b protein levels (Figure 3.3; supplementary data, Figure S1).
It is reasonable to suppose that a combination of SK1 siRNA (which acts at the mRNA level, thereby preventing the *de novo* protein synthesis) and SKi (which acts

at the protein level to induce proteasomal degradation) might overcome the resistance of SK1b to proteasomal degradation induced by SKi. To test this hypothesis, LNCaP-AI cells were transfected with SK1 siRNA or scrambled siRNA and challenged with SKi for the last 24 hours of treatment. Consistent with the results presented above, SKi failed to down-regulate SK1b in scrambled siRNA-transfected cells (Figure 3.26; supplementary data, Figure S3). In contrast, SK1b expression was reduced in response to the combined treatment with SKi and SK1 siRNA (Figure 3.26; supplementary data, Figure S3).

In line with the data presented in paragraph 3.2.10, SKi did not induce an increase in cleaved PARP levels in scrambled siRNA-transfected LNCaP-AI cells (Figure 3.26). However, the treatment with SKi enhanced PARP cleavage in LNCaP-AI cells transfected with SK1 siRNA (cleaved PARP, SK1 siRNA+SKi *vs* SK1 siRNA, p value $<0.05$) (Figure 3.26).

The effect of siRNA-mediated SK1 silencing on proteasome activity was also assessed. As shown in Figure 3.25c, treatment with SK1 siRNA, which knocks-down SK1a without however reducing SK1b expression (Figure 3.25b), did not down-regulate cyclin D1 nor c-Myc protein levels in LNCaP cells, indicating that the proteasome is not activated in these cells.

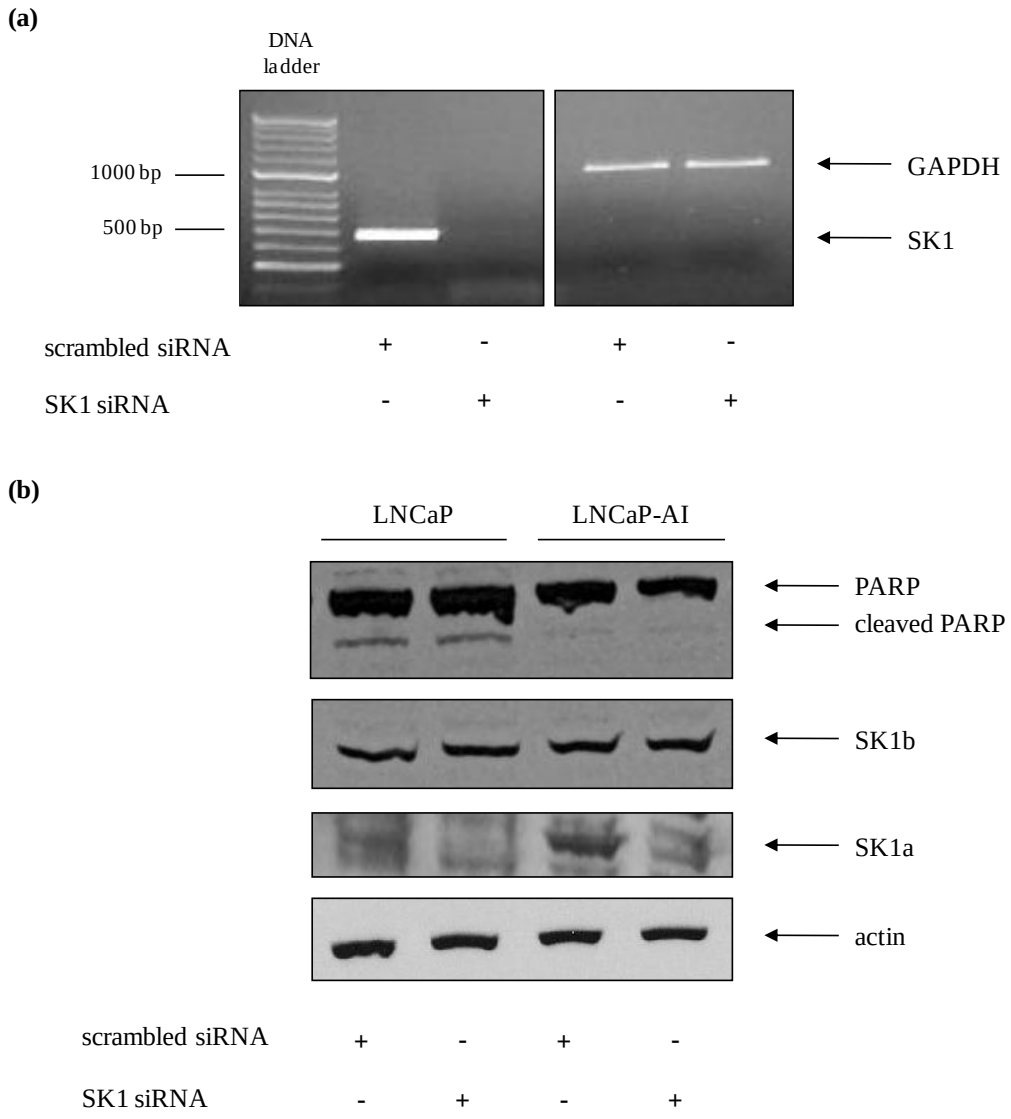



**Figure 3.25** ***Effect of transfection with siRNA oligonucleotides targeting SK1 on SK1 expression and PARP cleavage in LNCaP and LNCaP-AI cells.*** *(a) RT-PCR analysis of SK1 mRNA levels in LNCaP cells transfected with siRNA oligonucleotides targeting SK1 (200 nM) or scrambled siRNA (200 nM) for 48 hours. The expression of the housekeeping gene GAPDH was analysed using gene-specific primers to ensure that comparable amounts of cDNA template were used for the two cell lines. Shown is an agarose electrophoresis gel of the RT-PCR products. (b) Western blotting analysis of SK1a, SK1b or PARP/cleaved PARP levels in LNCaP and LNCaP-AI cells transfected with siRNA oligonucleotides targeting SK1 (200 nM) or scrambled siRNA (200 nM) for 72 hours. (c) Western blots showing the lack of effect of siRNA oligonucleotides targeting SK1 on cyclin D1 and c-Myc expression in LNCaP cells. Samples used for western blotting analysis are the same as for (b). Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

**(c)**

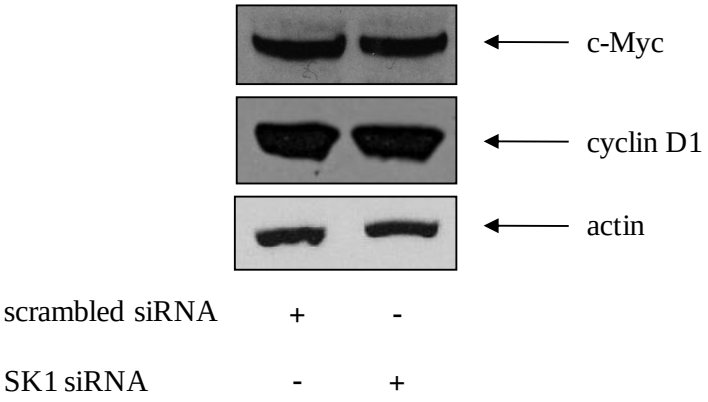



**Figure 3.25** ***Effect of transfection with siRNA oligonucleotides targeting SK1 on SK1 expression and PARP cleavage in LNCaP and LNCaP-AI cells (continued).***

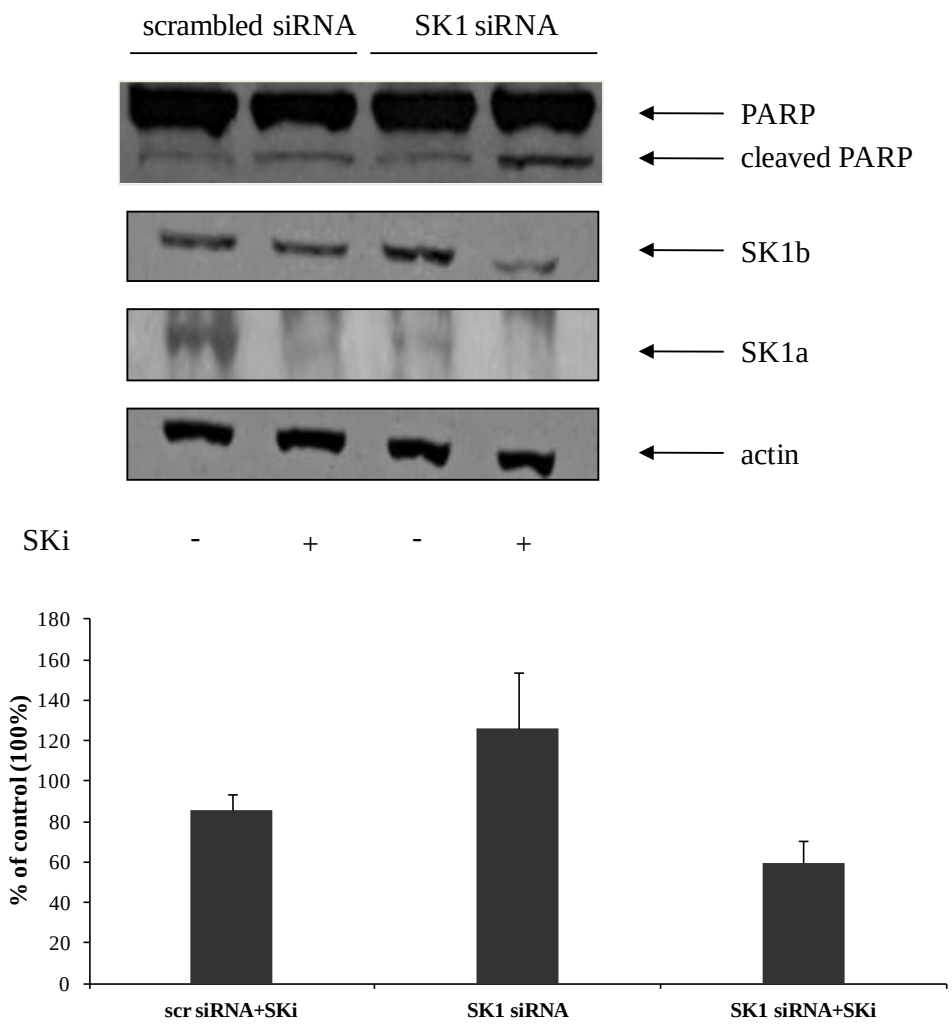



**Figure 3.26** ***Effect of SKi on SK1 expression and PARP cleavage in SK1 siRNA-transfected LNCaP-AI cells.*** *Cells were transfected with siRNA oligonucleotides targeting SK1 (200 nM) or scrambled siRNA (200 nM) and incubated for 48 hours before being treated with SKi (10 μM) or with the vehicle alone (DMSO, 0.1% v/v) for further 24 hours. SK1a, SK1b and PARP/cleaved PARP levels were measured by western blotting analysis. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1b immunoreactivity, expressed as a percentage of the control (scrambled siRNA) (ctrl=100%). p values were as follows: SK1 siRNA vs control, N.S.; scrambled siRNA+SKi vs control, N.S.; SK1 siRNA+SKi vs SK1 siRNA, <0.05; SKi siRNA+SKi vs scrambled siRNA+SKi, <0.05. N.S. denotes not statistically significant (p value >0.05).*

### 3.2.12 Effect of SK inhibitors on autophagy in LNCaP and LNCaP-AI cells

Besides regulating the apoptotic status of cells, the ceramide/S1P rheostat is also implicated in autophagy (Scarlatti et al., 2004; Lavieu et al., 2006; Lavieu et al., 2008). Therefore, we assessed whether SK1 inhibition/down-regulation in response to SKi modulates the autophagic process in LNCaP and LNCaP-AI cells (in collaboration with Dr E. Chan, University of Strathclyde).

LC3 (microtubule-associated protein light chain 3) is a widely used autophagic marker in mammalian cells (Kabeya et al., 2000; Mizushima, 2004). During the autophagic process, the cytosolic form of LC3 (LC3-I) is converted to LC3-II (the autophagosome-bound form) by conjugation to phosphatidylethanolamine. Therefore, the amount of LC3-II in a cell is proportional to the number of autophagosomes (the autophagic vacuoles), i.e. is an index of the cellular autophagic activity (Mizushima et al., 2010). The conversion from LC3-I to LC3-II can be monitored by immunoblotting using an antibody against LC3 (Mizushima et al., 2010). As shown in Figure 3.27, treatment with SKi induced a marked accumulation of LC3-I in both LNCaP and LNCaP-AI cells without however increasing the amount of LC3-II. This finding suggests that SKi inhibits the autophagic pathway in both cell lines.

Figure 3.27 also shows that the SK2-specific inhibitor (*R*)-FTY720-OMe induced a typical autophagy response in LNCaP and LNCaP-AI cells, characterized by the increase in the amount of LC3-II.

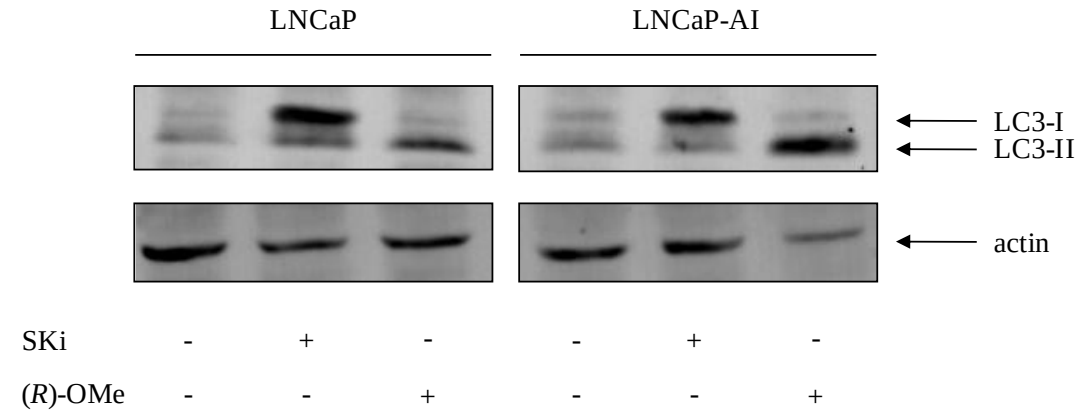



**Figure 3.27** ***Effect of SK inhibitors on autophagy in LNCaP and LNCaP-AI cells.*** *Cells were treated with SKi (10 µM), (R)-FTY720-OMe ((R)-OMe) (10 µM) or the vehicle alone (DMSO, 0.1% v/v) for 48 hours. LC3-I and LC3-II levels were measured by western blotting analysis using an anti-LC3 antibody that detects both forms. Blots were then stripped and re-probed for actin to ensure comparable protein loading between samples. Results are representative of three separate experiments.*

## 3.3 Discussion

### 3.3.1 SK1 expression is up-regulated in LNCaP-AI cells compared with LNCaP cells

The transition of androgen sensitive LNCaP cells to androgen-independent LNCaP-AI cells is associated with an up-regulation of SK1 expression. This finding is in line with a previous report showing that SK1 protein levels and activity are progressively increased in LNCaP cells subjected to chronic androgen deprivation (Dayon et al., 2009).

RT-PCR analysis of SK1 mRNA showed that SK1 up-regulation occurs at the transcriptional level. The molecular basis of SK1 transcriptional up-regulation remains to be elucidated. In this regard, it has been shown that prostate cancer progression to androgen independence is associated with increased expression of human EGF receptor 2 (HER2) *in vitro* (Craft et al., 1999; Shi et al., 2004) and in clinical samples from prostate cancer patients (Mukherjee et al., 2011). This might be significant because a functional association between HER2 and SK1 expression has been reported in MCF-7 breast cancer cells. In this case, the enforced over-expression of HER2 induces an increase in SK1 mRNA, protein and activity levels (Long et al., 2010a). Therefore, the transcriptional up-regulation of SK1 in LNCaP-AI cells might be dependent on HER2 signalling.

Intracellular pathways downstream of HER2 include ERK-1/2, protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K)/Akt (Roy and Perez, 2009). Of these, ERK-1/2 and PKC have been shown to positively regulate *SK1* gene expression via the activation of two transcription factors (Sp1 and AP-2) that interact with *SK1* promoter (Nakade et al., 2003). Indeed, ERK-1/2 activity is increased in LNCaP cells that have progressed to androgen independence (Shi et al., 2004). Whether PKC activity is up-regulated in androgen-independent prostate cancer cells compared with their androgen-dependent counterparts has not been reported. PI3K/Akt activity is also elevated during the progression of LNCaP cells to the androgen refractory state (Murillo et al., 2001; Shi et al., 2004; Dayon et al., 2009).

Interestingly, pharmacological inhibition of the PI3K/Akt pathway in LNCaP cells subjected to androgen withdrawal is associated with a reduction in SK1 activity (Dayon et al., 2009), providing evidence that PI3K/Akt regulates SK1 in prostate cancer cells.

It would be therefore interesting to evaluate the contribution of these pathways to SK1 up-regulation in prostate cancer cells, by assessing whether interfering with these signalling cascades reduces SK1 expression in LNCaP-AI cells.

The finding that SK1 expression is up-regulated in LNCaP cells that have progressed to androgen independence suggests that SK1 might be involved in androgen escape. Indeed, Dayon and colleagues have demonstrated that SK1 inhibition prevents the transition of LNCaP cells to a hormone-refractory state (Dayon et al., 2009). This finding provides evidence that SK1 is necessary for androgen escape and suggests that targeting SK1 might be a promising strategy to block the development of hormone-refractory prostate cancer. This finding is particularly significant, given the lack of effective therapeutic options for the treatment of androgen-independent prostate cancer.

The mechanism(s) by which SK1 promotes the acquisition of an androgen-independent phenotype have not been investigated. It might be speculated that the up-regulation of SK1 expression might confer an advantage to prostate cancer cells in terms of survival and proliferation in an environment depleted of an essential pro-growth signal. Indeed, the data presented in this Chapter and previous reports demonstrate that SK1 has a key role in promoting prostate cancer cell survival, as will be discussed below. In addition, SK1 promotes prostate cancer cell proliferation (Akao et al., 2006; Dayon et al., 2009). Significantly, the over-expression of SK1 sustains LNCaP cell proliferation under androgen deprivation conditions (Dayon et al., 2009).

Another mechanism by which SK1 might contribute to androgen escape is through the modulation of the androgen receptor (AR) pathway. In particular, S1P acts via its cognate receptors to stimulate intracellular signalling cascades (e.g. ERK-1/2, Akt)

that have been shown to phosphorylate AR, resulting in ligand-independent activation of AR and consequent expression of genes that promote cell proliferation (Ghosh et al., 2003; Lin et al., 2003). In addition, evidence exists that SK1 might also regulate AR expression in prostate cancer cells (Chapter 6).

Finally, the data presented in this Chapter indicate that SK1 might promote autophagy in LNCaP cells. Interestingly, it has been shown that androgen withdrawal stimulates autophagy in LNCaP cells and this protects cells from hormone ablation-induced apoptosis (Li et al., 2008a; Chhipa et al., 2011). Thus, the activation of autophagy might represent an additional mechanism by which SK1 sustains LNCaP cell survival during the transition to androgen independence.

Assessing the actual contribution of the mechanisms listed above to androgen escape requires formal investigation.

Importantly, the up-regulation of SK1b expression in LNCaP-AI cells is a critical element in the acquisition of resistance to SKi-induced proteasomal degradation and consequently to apoptosis, as will be discussed below.

Although SK1 expression is markedly up-regulated, S1P intracellular levels in LNCaP-AI cells are lower than in LNCaP cells, as assessed by Liquid Chromatography-Mass Spectrometry (LC-MS) (in collaboration with Dr E. Berdyshev, University of Illinois at Chicago) (Lim et al., 2012b) (Supplementary data, Figure S6). Since SK1 appears to be functionally coupled to S1P lyase in these cells (see below for discussion), this finding suggests that S1P lyase activity might be higher in LNCaP-AI cells compared with LNCaP cells.
mRNA expression of S1P receptors $S1P_2$ and $S1P_3$ is also increased in LNCaP-AI cells compared with LNCaP cells (Supplementary data, Figure S4). In addition, mRNA levels of LPP2, one of the enzymes that catalyse S1P dephosphorylation (Pyne et al., 2004), are substantially reduced in LNCaP-AI cells compared with LNCaP cells (Supplementary data, Figure S5). Thus, the bioavailability of S1P at its receptors (and therefore the activation of intracellular signalling pathways) might

also be increased in LNCaP-AI cells. By activating its cognate surface receptors, S1P modulates intracellular signalling pathways (ERK-1/2, Akt, JNK/p38) to promote cell survival (Cuvillier et al., 1996; Radeff-Huang et al., 2004). In addition, extracellular S1P has been shown to protect against apoptosis by modulating the expression of pro-apoptotic (Bcl-2) and anti-apoptotic (Bad, Bax) members of the Bcl-2 family (Pyne and Pyne, 2010). Thus, it is tempting to speculate that a more active S1P inside-out signalling in LNCaP-AI cells might result in increased resistance to apoptotic stimuli and survival in the absence of androgen stimulation. Interestingly, the progression of LNCaP cells to androgen independence is associated with the up-regulation of anti-apoptotic Bcl-2 and the down-regulation of pro-apoptotic Bax, which confer protection from androgen ablation-induced apoptosis (McDonnel et al., 1992; Shi et al., 2004; Raffo et al., 1995).

The functional significance of S1P inside-out signalling in LNCaP-AI cells remains to be defined. In particular, it would be interesting to assess whether interfering with this signalling system restores the sensitivity of LNCaP-AI cells to apoptotic stimuli and their reliance on androgens for growth.

### 3.3.2 The SK1 inhibitor SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) induces the proteasomal degradation of SK1 in LNCaP and LNCaP-AI cells by activating the proteasome

The data presented in the current Chapter demonstrate that the chronic treatment of LNCaP and LNCaP-AI cells with SKi results in the proteasomal degradation of SK1. Similarly, proteasome-mediated down-regulation of SK1 expression in response to SKi was observed in other cell lines, including cancerous (MCF-7 breast cancer cells) and non-cancerous (hPASMC; HEK 293) cells (Loveridge et al., 2010; Lim et al., 2011b).

The treatment of LNCaP and LNCaP-AI cells with SKi increases the flux of cellular proteins through the proteasomal degradative pathway. This was demonstrated by the measurement of proteasomal activity and by the finding that the levels of proteins which are subject to proteasomal degradation (cyclin D1, c-Myc) are markedly

reduced in cells treated with SKi, an effect that can be reversed by the proteasome inhibitor, MG132.

SKi has also been shown to stimulate proteasomal activity in MCF-7 cells (Loveridge et al., 2010) and to reduce cyclin D1 protein levels in these cells.

The activation of the proteasome by SKi is in line with results indicating that SKi can partially counter the inhibition of the proteasome by MG132. This is suggested by two lines of evidence. First, SK1a levels are not fully recovered in LNCaP and LNCaP-AI cells treated with SKi and MG132 compared with cells treated with MG132 alone. Second, SKi reduces MG132-induced PARP cleavage in LNCaP and LNCaP-AI cells. This finding suggests that activation of the proteasome by SKi can partially reverse the induction of apoptosis that occurs when the proteasome is inhibited by MG132. Indeed, inhibition of the proteasome by MG132 causes the accumulation of unfolded/misfolded proteins within the endoplasmic reticulum ("ER stress"), resulting in the activation of the so-called "unfolded protein response" which culminates in the induction of apoptosis (Obeng et al., 2006). By accelerating the flux of cellular proteins through the proteasome, SKi is likely to favour the removal of unfolded/misfolded proteins thereby relieving cells from MG132-induced ER stress to reduce apoptosis.

It is well established that proteins have to be modified by covalent attachment of a lysine-48-linked polyubiquitin chain to be targeted for degradation by the proteasome. Kihara and colleagues reported that SK1 is polyubiquitinated and degraded by the proteasome under basal conditions (Kihara et al., 2006). Indeed, SK1a and SK1b accumulate in LNCaP-AI cells when treated with MG132, suggesting an active turnover of these proteins through the ubiquitin-proteasomal pathway. Loveridge et al. also showed that SK1 is polyubiquitinated under basal conditions and that SKi promotes the proteasomal degradation of polyubiquitinated SK1 by activating the proteasome (Loveridge et al., 2010).
Taken together, these findings are consistent with a model where SKi activates the proteasome to increase removal of polyubiquitinated SK1.

Others have shown that SKi induces the cathepsin B-dependent lysosomal degradation of SK1 in various human cell lines (Ren et al. 2010). However, we can exclude a role for this pathway in the SKi-induced degradation of SK1 as the specific cathepsin B inhibitor CA074Me did not reverse the effect of SKi in LNCaP cells. In addition, an alternative proteasomal inhibitor, lactacystin, which lacks inhibition of cathepsin B also blocked the SKi-induced down-regulation of SK1a in hPASMC (Loveridge et al., 2010). The difference with the study by Ren et al. might be related to the fact that the relative contribution of lysosomal and proteasomal pathways to protein degradation may vary between cell types (Clague and Urbè, 2010).

Obeid and colleagues demonstrated that DNA damaging agents reduce SK1 expression via a post-transcriptional mechanism that involves multiple proteases, including cathepsin B and effector caspase-3 and -7 (Taha et al., 2004). The data presented in the current study provide evidence that caspase-3/7 do not participate in the down-regulation of SK1 in prostate cancer cells treated with SKi, as the caspase-3/7 inhibitor, Ac-DEVD-CHO had no effect on the degradation of SK1 in response to SKi. Similarly, Ac-DEVD-CHO failed to rescue SKi-induced down-regulation of SK1 in hPASMC and MCF-7 cells (Loveridge et al., 2010). Moreover, SKi reduces SK1a expression in LNCaP-AI cells that do not undergo apoptosis upon treatment with SKi.

Obeid and colleagues have also demonstrated that SK1 down-regulation in response to genotoxic agents is dependent on p53 accumulation (Taha et al., 2004; Heffernan-Stroud et al., 2012). SKi treatment did not result in p53 up-regulation in LNCaP cells (Chapter 6), indicating that p53 is not required for SKi-induced degradation of SK1, although this needs to be confirmed using a p53-specific inhibitor.

### 3.3.3 SKi activates the proteasome by tilting the sphingolipid rheostat toward ceramide

The data presented in this Chapter provide evidence that the stimulation of proteasome activity in LNCaP cells treated with SKi and the subsequent down-

regulation of SK1 expression are due to acute SK1 catalytic inhibition by SKi, which results in the accumulation of ceramide formed by the salvage pathway. This is based on the finding that SKi-induced proteasomal degradation of SK1 and cyclin D1 is partially reversed by the ceramide synthase inhibitor fumonisin B1 while is not affected by myriocin (which by inhibiting serine palmitoyltransferase reduces the *de novo* ceramide formation). In addition, the cell-permeable analogue of ceramide, C2-ceramide mimics the effect of SKi, promoting the proteasomal degradation of SK1 in LNCaP and LNCaP-AI cells, while C2-dihydroceramide fails to do so. Indeed, C2-ceramide also induced the MG132-sensitive down-regulation of SK1 in hPASMC (Loveridge et al., 2010).

Furthermore, C2-ceramide activates the proteasome in astrocytes (Calatayud et al., 2005) and MCF-7 breast cancer cells (Loveridge et al., 2010). In line with these findings, the treatment of LNCaP-AI cells with C2-ceramide resulted in increased proteasomal activity, as assessed using a proteasomal luminogenic activity assay. In addition, C2-ceramide reduces cyclin D1 expression and prevents MG132-induced apoptosis in LNCaP cells, which is also consistent with the ability of this lipid to activate the proteasome.

The mechanism by which ceramide activates the proteasome remains to be elucidated. In this regard, preliminary data showed that PP2A might mediate the effect of ceramide, as the inhibition of this enzyme with okadaic acid partially reverses SKi-induced proteasomal degradation of cyclin D1.

The finding that S1P inhibits the proteasome in MCF-7 breast cancer cells (Loveridge et al., 2010) suggests that the flux of proteins through the proteasomal degradative pathway might be modulated by the intracellular balance between ceramide and S1P. Interestingly, S1P has been recently shown to inhibit PP2A activity via $S1P_2$ in chronic myeloid leukemia cells (Salas et al., 2011).

The partial reversal of SKi effect by fumonisin B1 is in line with previous studies by Garzotto et al., reporting that the same concentration of fumonisin B1 could not

completely abrogate ceramide accumulation and apoptosis in LNCaP cells challenged with phorbol ester (PMA) (Garzotto et al., 1998; Garzotto et al., 1999). Thus, the accumulation of lower levels of ceramide in LNCaP cells co-treated with SKi and fumonisin B1 might be sufficient to activate the proteasome, although to a lesser extent. In order to test this hypothesis, it would be necessary to measure ceramide levels in LNCaP cells challenged with SKi in the presence or absence of fumonisin B1.

Alternatively, the inability of fumonisin B1 to fully reverse SKi effect might indicate that the activation of the proteasome by SKi is only partly dependent on the inhibition of SK1 activity (i.e. on ceramide accumulation), while additional mechanism(s) might be involved.

Consistent with the possibility that the SKi-induced proteasomal degradation of SK1 is partly due to acute inhibition of SK1 catalytic activity and subsequent ceramide accumulation, SKi (10 μM), which induces the proteasomal degradation of endogenous SK1 in LNCaP cells, failed to down-regulate over-expressed SK1a and SK1b, suggesting that the proteasome is not activated in these cells. Indeed, the ceramide/S1P balance is predicted to be more resistant to perturbation by SKi in cells over-expressing SK1. In contrast, a higher concentration of SKi (50 μM) induced the degradation of ectopically expressed recombinant SK1a and SK1b, suggesting that the proteasome is activated under these conditions due to a higher fractional inhibition of SK1 activity.

The results of SK1 siRNA studies provide evidence that SKi acutely inhibits the activity of *both* SK1a *and* SK1b to activate the proteasome in LNCaP cells. Indeed, treatment of these cells with SK1 siRNA, which reduces the expression of SK1a but not SK1b, does not result in proteasome activation, as indicated by the failure to reduce cyclin D1 and c-Myc expression. This possibility is consistent with the finding that SKi inhibits both SK1a and SK1b *in vitro*.

SKi activates the proteasome to a similar extent in LNCaP and LNCaP-AI cells, as assessed by the measurement of proteasomal activity. In line with this, the expression of proteins targeted to the proteasome (SK1a, cyclin D1) was reduced in LNCaP-AI cells as efficiently as in LNCaP cells. In light of the data discussed above, this finding indicates that SKi acutely inhibits both SK1a and SK1b in LNCaP-AI cells.
Interestingly, although SKi stimulates proteasomal activity to a similar extent in LNCaP and LNCaP-AI cells, SK1b evades the proteasome in LNCaP-AI cells treated with SKi. This finding prompted the investigation of the molecular basis underlying the "protection" of SK1b from proteasomal degradation induced by SKi (see below). Significantly, the resistance of SK1b to proteasomal degradation is associated with a different effect of SKi on sphingolipid levels in LNCaP cells *versus* LNCaP-AI cells and consequently with a distinct cell fate outcome in response to this inhibitor, as will be discussed below.

Finally, the inhibitor kinetic analysis revealed that SKi has a poor inhibitory effect on SK1 activity *in vitro*, with an $IC_{50}$ of approximately 100 µM, which is in good agreement with a previous study (Ren et al., 2010). However, SKi effectively inhibits endogenous SK1a and SK1b in intact LNCaP and LNCaP-AI cells, as demonstrated by the ceramide-dependent activation of the proteasome in these cells in response to SKi treatment. A possible explanation is that SKi applied to the outside of cells might accumulate into cells thereby reaching intracellular concentrations that are significantly higher than the extracellular concentration and efficiently inhibit SK1 activity, resulting in ceramide accumulation. This possibility is supported by the finding that FTY720 is phosphorylated by SK2 when applied to cells at nanomolar concentrations although the $K_M$ of SK2 for FTY720 is in the micromolar range (Billich et al., 2003), indicating that the cells concentrate the inhibitor to concentrations that are in the $K_M$ of SK2.

### 3.3.4 Effect of SKi on sphingolipid levels in LNCaP and LNCaP-AI cells

SK1 is a key modulator of the ceramide/S1P ratio and its inhibition has profound effects on sphingolipid metabolism (Ogretmen and Hannun, 2004). We therefore

assessed the effect of SK1 down-regulation in response to SKi on sphingolipid intracellular levels, measured by LC-MS (in collaboration with Dr E. Berdyshev, University of Illinois at Chicago) (Loveridge et al., 2010; Lim et al., 2012b) (Supplementary data, Figure S6).

Treatment of LNCaP cells with SKi (10 μM, 24 hours) decreased S1P levels and markedly increased C22:0 ceramide levels, while it had no significant effect on the other ceramide species that could be detected in these cells (C14:0, C16:0, C18:1, C18:0, C20:0, C24:1, C24:0, C26:1, C26:0). Sphingosine levels were also increased. These data are indicative of a perturbation of the ceramide/S1P balance due to SKi-induced inhibition/down-regulation of SK1a and SK1b in LNCaP cells.

SKi also substantially increased the levels of various dihydroceramide species in LNCaP cells. The same effect was observed in SKi-treated LNCaP-AI cells. Similarly, the accumulation of dihydroceramide in response to SKi treatment was reported by others (Illuzzi et al., 2010). Spiegel and colleagues have suggested that S1P might inhibit dihydroceramide synthase (CerS), based on the finding that SK1 over-expression markedly increases dihydrosphingosine levels while decreasing dihydroceramide levels in HEK 293 cells (Maceyka et al., 2005). According to this model, down-regulation of SK1 by SKi would increase dihydroceramide levels in LNCaP cells by relieving dihydroceramide synthase from S1P inhibitory effect. It should be noted, however, that S1P inhibits CerS2 only (Laviad et al., 2008), which utilizes long-chain acyl-CoA (C20-C26) for dihydroceramide synthesis, while it shows low or no activity on C16- or C18-CoA (Laviad et al., 2008). Since SKi elevates all the dihydroceramide species (including C16- and C18-dihydroceramide), it is more likely that SKi effect is due to the inhibition of dihydroceramide desaturase activity by SKi. Interestingly, it has been shown that resveratrol which is structurally similar to SKi, inhibits dihydroceramide desaturase and increases dihydroceramide levels (Signorelli et al., 2009).

An alternative explanation for the accumulation of dihydroceramide in SKi-treated cells is provided by a study from Hannun and colleagues, showing that oxidative stress induces a marked inhibition of dihydroceramide desaturase activity thereby resulting in increased dihydroceramide levels in various human cell lines (Idkowiak-

Baldys et al., 2010). Since SKi activates oxidative stress in both LNCaP and LNCaP-AI cells (Chapter 6), the mechanism described by Hannun and colleagues might account for the accumulation of dihydroceramide in cells treated with this inhibitor.

The finding that SKi treatment substantially elevates the levels of various dihydroceramide species while it does not induce the accumulation of the corresponding ceramides suggests that all these ceramide species are predominantly formed through the *de novo* pathway in LNCaP cells. In contrast, C22:0 ceramide might be predominantly synthesized from the salvage pathway, as SKi causes the accumulation of this ceramide species. Indeed, Berdyshev and colleagues have previously shown that ceramide formed from the salvage pathway is a major contributor to the intracellular pool of S1P (Berdyshev et al., 2006). These data also suggest that fumonisin B1 might inhibit SKi effect on the proteasome by targeting the back-conversion of sphingosine to C22:0 ceramide, i.e. C22:0 ceramide activates the proteasome.

Interestingly, the increase in C22:0 ceramide levels in SKi-treated LNCaP cells is 100-fold higher than S1P levels. This finding suggests that SK1-derived S1P is actively removed from cells by the action of S1P lyase. Thus, SK1 and S1P lyase might be functionally coupled in LNCaP cells to remove ceramide and thereby protect cells from apoptosis (see below).

Unlike LNCaP cells, treatment of LNCaP-AI cells with SKi failed to increase ceramide and sphingosine levels. This is consistent with the failure of SKi to down-regulate SK1b expression in LNCaP-AI cells and indicates that SK1b activity is sufficient to maintain the flow through the ceramide-sphingosine-S1P pathway. Interestingly, however, SKi reduced S1P mass in LNCaP-AI cells. A possible explanation is that S1P might be removed at a faster rate by S1P lyase in LNCaP-AI cells treated with SKi (i.e. SKi might induce the up-regulation of S1P lyase activity/expression).

The data from the analysis of sphingolipid levels in LNCaP and LNCaP-AI cells support a model in which the modulation of ceramide levels might depend on the

concerted action of SK1 and S1P lyase via the phosphorylation of sphingosine to S1P by SK1 and the irreversible removal of SK1-derived S1P by S1P lyase. Consistent with this model, ceramide levels are lower in LNCaP-AI cells compared with LNCaP cells and this is associated with the up-regulation of SK1 and, possibly, S1P lyase in LNCaP-AI cells *versus* LNCaP cells.

Taken together, the data presented so far provide evidence that SKi has a biphasic effect on ceramide levels in LNCaP cells. The first phase is due to acute inhibition of the activity of SK1a and SK1b to increase ceramide levels and activate the proteasome. The second phase is due to SK1a and SK1b proteasomal degradation which results in sustained accumulation of ceramide to induce apoptosis (see below) (Figure 3.28).

In LNCaP-AI cells, SKi acutely inhibits the activity of both SK1a and SK1b, as evidenced by the activation of the proteasome in these cells in response to SKi. However, the resistance of SK1b to proteasomal degradation prevents the delayed accumulation of ceramide in LNCaP-AI cells.

### 3.3.5 Mechanisms underlying the different sensitivity of SK1a and SK1b to SKi-induced proteasomal degradation

A major finding of the current study is that SK1 isoforms SK1a and SK1b exhibit distinct properties. In particular, SK1b is resistant to SKi-induced proteasomal degradation in LNCaP-AI cells. Similarly, SK1a and SK1b show a different sensitivity to SKi when over-expressed in LNCaP cells, where SK1a is down-regulated by SKi while SK1b is not. These data suggest that the sensitivity of SK1b to SKi-induced degradation is dependent on its expression level. Indeed, the expression of SK1b is higher in LNCaP-AI cells compared with LNCaP cells. Consistent with this possibility, the resistance of SK1b to SKi-induced proteasomal degradation in LNCaP-AI cells can be overcome by the combined treatment with SKi and SK1 siRNA.

It is reasonable to suppose that the expression level of SK1b might contribute to its resistance to proteasomal degradation by promoting the interaction with a partner protein which might physically "shield" SK1b from proteasomal degradation. Thus, the up-regulation of SK1b levels might bring SK1b concentration closer to the binding affinity of the partner protein, thereby resulting in enhanced association. However, it was not possible to detect any protein associated with SK1b in response to treatment with SKi in LNCaP cells over-expressing recombinant SK1b. This finding suggests that SK1b protection from proteasomal degradation might not be due to its binding to a "shielding protein".

An alternative possibility is that SK1b resistance to SKi-induced degradation might be due to post-translational modifications of this protein which might reduce the availability of SK1b for proteasomal degradation. In this regard, evidence exists for a cross-talk between protein lysine acetylation and ubiquitination that modulates protein stability (Caron et al., 2005). In particular, lysine acetylation can inhibit ubiquitination, thereby preventing proteasomal degradation of the target protein. This can be either via a direct mechanism (competition for the same lysine residues) or via a more complex mechanism (the acetylation of a protein interferes with its ability to interact with the ubiquitin-proteasomal machinery) (Caron et al., 2005).

An online tool for the prediction of protein acetylation sites (PAIL, Prediction of Acetylation on Internal Lysines, available at http://bdmpail.biocuckoo.org/) identified seven potential acetylation sites in SK1b, two of which (Lysine 58 and Lysine 61) are unique to SK1b as they are in the N-terminal extension that is not present in SK1a. Interestingly, a study by Zhao and coworkers demonstrated that SK1b is actually acetylated *in vivo* at Lysine 58 (Zhao et al., 2010). Thus, SK1b protection from SKi-induced proteasomal degradation might result from its acetylation at Lysine 58, which would prevent SK1b ubiquitination and therefore its targeting to the proteasome. According to this model, the up-regulation of SK1b expression would contribute to SK1b resistance by increasing the concentration of this protein closer to the $K_M$ of the enzyme that catalyses its acetylation. In addition, it cannot be excluded that the differential susceptibility of SK1b to SKi-induced

proteasomal degradation in LNCaP cells *versus* LNCaP-AI cells might be due to differences in the expression/activity levels of lysine acetyltransferases (KAT) and/or lysine deacetylases (KDAC) between the two cell lines (i.e. lower KAT activity and/or higher KDAC activity in LNCaP cells compared with LNCaP-AI cells).
This model would also account for the different sensitivity of endogenous SK1a and SK1b to proteasomal degradation in response to SKi in LNCaP cells (i.e. while SK1a is completely removed by SKi, a fraction of SK1b is retained), as the Lysine 58 acetylation site is unique to SK1b.

Future experiments will be aimed at testing a role for Lysine 58 acetylation in protecting SK1b. For this purpose, plasmids encoding SK1b variants mutated at Lysine 58 (acetylation-mimicking mutant and non-acetylable mutant) were generated to test the sensitivity of these mutants to SKi-induced proteasomal degradation (in collaboration with Dr Stuart Pitson, University of Adelaide). In addition, proteomic analysis of cell extracts from LNCaP and LNCaP-AI cells treated with SKi would assist in assessing any changes in Lysine 58 acetylation of SK1b in response to SKi.

Interestingly, C2-ceramide was able to "by-pass" the resistance of SK1b in LNCaP-AI cells. This finding suggests that the modifications that protect SK1b from proteasomal degradation in response to SKi might be induced by SKi itself. According to the proposed model, SKi might activate KAT and/or inhibit KDAC, thereby promoting SK1b acetylation and increasing its stability.

Significantly, the resistance of SK1b in LNCaP-AI cells can be overcome by two different SK1 inhibitors, FTY720 and (*S*)-FTY720 vinylphosphonate (Chapter 4). This finding suggests that the inhibitor-induced conformation adopted by SK1a/SK1b might be an additional factor that contributes to determine the susceptibility of these enzymes to degradation by the proteasome (i.e. the binding to the inhibitor might induce conformational changes in the enzyme to prime it to the proteasome). In addition, (*S*)-FTY720 vinylphosphonate might overcome the resistance of SK1b to proteasomal degradation by activating the E3 ubiquitin ligase specific for SK1b. Indeed, (*S*)-FTY720-vinylphosphonate is structurally similar to

S1P, which binds to the RING domain of TRAF2 thereby stimulating its E3 ubiquitin ligase activity (Alvarez et al., 2010). In contrast, by lowering S1P levels SKi might inhibit the E3 ubiquitin ligase specific for SK1b. This would explain why SK1b is resistant to SKi in LNCaP-AI cells while it is sensitive to ceramide-induced proteasomal degradation.
All these issues are further discussed in Chapter 4.

Importantly, a different sensitivity of SK1a and SK1b to SKi-induced proteasomal degradation was also observed in a HEK 293 cell system with inducible SK1a or SK1b expression, where SK1a was removed in response to SKi while SK1b was not (Lim et al., 2012b). This finding provides evidence that the regulatory mechanisms that confer on SK1b the ability to evade the ubiquitin-proteasomal machinery are not unique to prostate cancer cells.

### 3.3.6 SKi induces apoptosis in LNCaP cells by removing SK1a and SK1b

Chronic treatment with SKi induced cell death by apoptosis in LNCaP cells, as indicated by cell shrinkage, activation of a pro-survival pathway (ERK-1/2), activation of caspase-3 (a well established initiator of apoptosis) and PARP cleavage. In addition, the metabolomic analysis of LNCaP cells revealed the accumulation of diadenosine triphosphate in response to treatment with SKi (unpublished data), a metabolite whose intracellular levels are increased in cells challenged with various apoptotic stimuli and correlate with the degree of apoptosis induced by these treatments (Fisher et al., 2008). In contrast, the chronic treatment of LNCaP-AI cells with SKi did not result in apoptosis, as evidenced by the lack of apoptotic morphological features, caspase-3 activation and PARP cleavage. Also, diadenosine triphosphate intracellular levels did not change in SKi-treated LNCaP-AI cells (unpublished data).

Significantly, the differential effect of SKi on LNCaP and LNCaP-AI cell survival is associated with the different sensitivity of SK1b to SKi-induced proteasomal degradation in the two cell lines. Thus, the inability of SKi to reduce SK1b

expression in LNCaP-AI cells might explain the resistance of these cells to SKi-induced apoptosis. This is supported by the finding that treatment of LNCaP and LNCaP-AI cells with SK1 siRNA, which removes SK1a but not SK1b, does not result in apoptosis, whereas LNCaP-AI cells undergo apoptosis when treated with a combination of SK1 siRNA and SKi, which results in the down-regulation of both SK1a and SK1b.

It is well established that the balance between pro-apoptotic ceramide and pro-survival S1P, which is critically regulated by SK1, controls cell fate (Cuvillier et al., 1996). MS analysis of sphingolipid levels provides evidence that SKi induces apoptosis in LNCaP cells by increasing the intracellular levels of pro-apoptotic ceramide. Indeed, SKi treatment does not result in ceramide accumulation in LNCaP-AI cells (where SK1b is retained), and these cells are resistant to SKi-induced apoptosis. Interestingly, SKi reduces S1P levels in both LNCaP and LNCaP-AI cells. Taken together, these findings suggest that SK1 might maintain prostate cancer cell survival by preventing the accumulation of ceramide rather than promoting the formation of S1P.

The mechanism by which ceramide accumulation activates apoptosis in SKi-treated LNCaP cells has not been investigated. In this regard, it is well established that ceramide promotes apoptotic cell death via different mechanisms. These include the activation of the intrinsic and extrinsic apoptotic cascades and the modulation of intracellular signalling pathways that regulate cell survival (JNK/p38, ERK-1/2, Akt) (Oskouian and Saba, 2010).
The finding that SKi treatment results in caspase-3 activation in LNCaP cells is consistent with a role for ceramide in mediating SKi-induced apoptosis. Indeed, ceramide has been shown to activate caspase-3-mediated apoptosis (Cuvillier et al., 1998). It cannot be excluded, however, that additional ceramide-activated pathways (caspase-independent) might contribute to SKi-induced apoptosis in LNCaP cells.

The finding that SKi promotes apoptosis in LNCaP cells by elevating intracellular ceramide is in line with previous reports showing that siRNA knock down of SK1 or

treatment with SK1 inhibitors reduce prostate cancer cell viability, an effect that is associated with increased ceramide/S1P ratio (Pchejetski et al., 2005; Akao et al., 2006; Brizuela at al., 2010; Pchejetski et al., 2010). The data presented in this Chapter extend these studies by demonstrating that SK1 inhibitors might promote apoptosis by inducing the removal of SK1 via the ubiquitin-proteasomal degradation pathway (which is translated in a sustained accumulation of ceramide) rather than by simply reversibly inhibiting SK1 activity. In addition, the present study uncovered a crucial role for SK1b in modulating the ceramide/S1P rheostat and therefore in maintaining prostate cancer cell survival.

As discussed in Chapter 1, a number of studies have shown that the resistance of prostate cancer cells to chemotherapy and irradiation is associated with the failure of these treatments to down-regulate SK1 and to shift the ceramide/S1P balance toward pro-apoptotic ceramide (Nava et al., 2000b; Pchejetski et al., 2005; Pchejetski et al., 2008; Sauer et al., 2009; Pchejetski et al., 2010). In light of the findings presented in the current study, it is tempting to speculate that the altered regulation of SK1b might control the resistance of prostate cancer cells to therapeutic agents. If this was the case, irradiation and chemotherapeutics might not be able to elevate the ceramide/S1P ratio in therapy-resistant prostate cancer cells because they might fail to reduce SK1b expression. To test this possibility, it will be necessary to assess whether the modifications that render SK1b protein less sensitive to SKi-induced degradation are also protective toward the down-regulation promoted by therapeutic agents.

It is important to appreciate that the regulation of the sensitivity of prostate cancer cells to therapeutic agents is likely to be complex and to involve multiple mechanisms. For example, docetaxel induces apoptosis in prostate cancer cells via both SK1-dependent and -independent mechanisms (Sauer et al., 2009). Nonetheless, *in vivo* studies demonstrated the efficacy of down-regulating SK1 to sensitize prostate cancer cells to anti-cancer treatments (Nava et al., 2000b; Pchejetski et al., 2008; Sauer et al., 2009; Pchejetski et al., 2010). The data presented in this study provide evidence that removing both SK1a and SK1b is necessary to induce apoptosis of therapeutic resistant prostate cancer cells.

It is important to appreciate that although SKi inhibits the activity of SK2 *in vitro* (Ren et al., 2010; Lim et al., 2011a), its effects on ceramide levels and therefore on apoptosis in LNCaP cells are likely independent of SK2. Indeed, the SK2-specific inhibitor, (*R*)-FTY720-OMe does not affect ceramide mass nor activates apoptosis in LNCaP cells (Chapter 5). These findings indicate that SKi acts predominantly on SK1 in LNCaP cells. This is further supported by the finding that SKi and (*R*)-FTY720-OMe have opposing effects in regulating autophagy in these cells (see below).

### 3.3.7 SK1 and SK2 have opposing roles in the regulation of autophagy in LNCaP and LNCaP-AI cells

Autophagy is a highly regulated process that takes place in all eukaryotic cells, by which cellular components (proteins and organelles) are degraded by lysosomal hydrolases (Klionsky and Emr, 2000). Under basal conditions, this process prevents the accumulation of abnormal protein aggregates and damaged organelles to maintain intracellular homeostasis, whereas under nutrient starvation or stress conditions it enables recycling of intracellular components to sustain cellular metabolism (Klionsky and Emr, 2000).

The current study provides evidence that SK1 and SK2 regulate autophagy in LNCaP and LNCaP-AI cells. This is based on the finding that the pharmacological inhibition of these enzymes affects the autophagic pathway in these cells, as assessed by monitoring LC3 expression by immunoblotting analysis. In particular, treatment of LNCaP and LNCaP-AI cells with SKi induces a marked accumulation of LC3-I which is not accompanied by an increase in LC3-II levels, suggesting that this inhibitor impairs the conversion of LC3-I to LC3-II, i.e. SKi inhibits autophagy. In contrast, the SK2-specific inhibitor, (*R*)-FTY720-OMe stimulates autophagy in these cells, as evidenced by the accumulation of LC3-II. Thus, these data suggest that SK1 promotes, while SK2 inhibits autophagy in LNCaP and LNCaP-AI cells.

These findings are in line with previous studies reporting that SK1 over-expression stimulates autophagy in MCF-7 breast cancer cells (Lavieu et al., 2006) and that the SK2-specific inhibitor, ABC292640 induces autophagy in various human cancer cell lines, including PC-3 prostate cancer cells (Beljanski et al., 2010).

The finding that SK1 and SK2 have opposing effects on autophagy is consistent with the notion that these enzymes might regulate distinct functional pools of sphingolipids, as will be discussed in Chapter 7. The mechanisms by which SK1 and SK2 modulate autophagy remain to be elucidated. Since SKi reduces S1P levels in both LNCaP and LNCaP-AI cells, it might be speculated that the regulation of autophagy by SK1 might be mediated by S1P. Consistent with this possibility, S1P induces autophagy in MCF-7 cells (Lavieu et al., 2006) and PC-3 cells (Chang et al., 2009). (*R*)-FTY720-OMe also reduces S1P levels in LNCaP cells (Chapter 5), but in contrast to SKi, promotes autophagy in these cells. A model that might explain these findings is one in which SK1-derived S1P might promote autophagy while SK2-derived S1P might inhibit this cellular process. This difference might be related to the sub-cellular compartment where S1P is generated, which might determine the interaction of S1P with distinct downstream effectors to elicit different cellular responses. This hypothesis is entirely consistent with previous studies. For instance, it has been shown that SK1 promotes cell proliferation when targeted to the plasma membrane (Pitson et al., 2005) whereas it inhibits DNA synthesis when artificially targeted to the nucleus (Igarashi et al., 2003).

To obtain further insight into the role of SK1 and SK2 in regulating autophagy, it would be useful to assess the effect of genetic manipulation of SK1/SK2 expression (over-expression and siRNA-mediated knock-down) on the autophagic pathway.

Beljanski and colleagues hypothesised that ABC292640 might induce autophagy by increasing the intracellular levels of ceramide (Beljanski et al., 2010), based on a previous report that ceramide stimulates autophagy in cancer cells (Scarlatti et al., 2004). However, treatment of LNCaP cells with (*R*)-FTY720-OMe did not increase ceramide levels (Chapter 5).

A recent study reported that FTY720 induces autophagy in human cancer cell lines by down-regulating the expression of nutrient transporter proteins, thereby limiting the nutrient uptake by the cells (Romero Rosales et al., 2011). The loss of transporters is mediated by PP2A, which is activated by FTY720 either directly or indirectly (via ceramide accumulation) (Romero Rosales et al., 2011). Thus, although (*R*)-FTY720-OMe does not induce ceramide accumulation in LNCaP cells, it would be interesting to assess whether this inhibitor, which is structurally similar to FTY720, can activate PP2A and down-regulate nutrient transporters.

Interestingly, the finding that SKi inhibits autophagy in LNCaP and LNCaP-AI cells might provide an explanation as to why treatment of these cells with SKi induces the activation of an oxidative stress response in these cells (Chapter 6). In fact, blocking the autophagic pathway would impair the removal of damaged mitochondria from cells (which indeed occurs via the autophagic degradative pathway) thereby leading to abnormal reactive oxygen species generation (Jin and White, 2008). To test this hypothesis it will be necessary to assess whether preventing the sequestration of mitochondria into autophagosome results in oxidative stress in LNCaP and LNCaP-AI cells.

Finally, the stimulation of autophagy might represent one of the mechanisms by which SK1 promotes the resistance of prostate cancer cells to therapeutic agents. Indeed, it has been shown that autophagy represents a protective mechanism that allows cancer cells to survive under stress conditions, including cytotoxic damage induced by chemotherapeutic drugs and radiation (Chen et al., 2010). Thus, SK1 inhibitors might sensitise prostate cancer cells to chemotherapy and radiotherapy by blocking autophagy-mediated survival.

## 3.4 Summary

A major finding of the current study is that the SK inhibitor SKi induces the proteasomal degradation of SK1 in LNCaP and LNCaP-AI cells. The data presented in this Chapter provide evidence that this is due to the acute inhibition of SK1 activity, which disrupts the ceramide/S1P balance and leads to ceramide-dependent activation of the proteasome.

Significantly, the present study demonstrated that SK1 isoforms SK1a and SK1b exhibit different properties. In particular, SKi induces the proteasomal degradation of both SK1a and SK1b in LNCaP cells. In contrast, SKi promotes the proteasomal degradation of SK1a in LNCaP-AI cells, while it fails to reduce the expression of SK1b. This is translated into specific changes in sphingolipid levels and a different cell fate outcome. Indeed, SKi-induced removal of SK1a and SK1b from LNCaP cells results in sustained accumulation of ceramide and subsequent ceramide-dependent apoptosis. In contrast, SKi fails to increase ceramide levels in LNCaP-AI cells, where SK1b is retained, and these cells do not undergo apoptosis. However, LNCaP-AI cells are forced to undergo apoptosis upon removal of both SK1a and SK1b.

Thus, the present study extend the current knowledge on the function of SK1 in regulating prostate cancer cell survival, providing compelling evidence that eliminating both SK1a and SK1b is necessary to effectively disrupt the ceramide/S1P balance and promote apoptosis in prostate cancer cells. Therefore, elucidating the mechanisms underlying the resistance of SK1b is a critical issue for designing more effective SK1 inhibitors for prostate cancer treatment. In this regard, the data obtained so far provide evidence that the sensitivity of SK1b to SKi-induced proteasomal degradation is dependent on its expression level and on modifications that are unique to SK1b and might occur in conjunction with the progression to androgen independence. Selective knock-down of SK1b would assist in defining whether this isoform is solely responsible for maintaining prostate cancer cell survival or whether this is controlled by both SK1a and SK1b.

In addition, pharmacological studies provided evidence that SK1 and SK2 might oppositely regulate autophagy in prostate cancer cells. Future experiments will be aimed at understanding the mechanisms involved and the functional significance.

Finally, further work is required to elucidate the molecular basis of SK1 up-regulation in LNCaP-AI cells compared to LNCaP cells and to assess how this contributes to androgen escape.

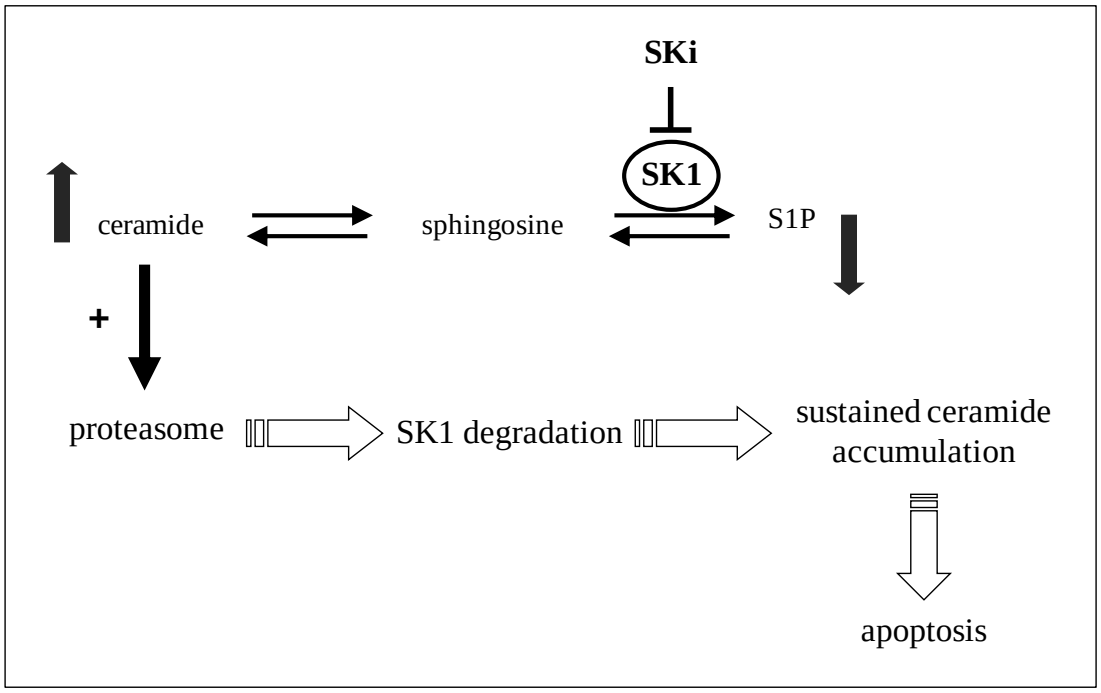



**Figure 3.28** ***Schematic representation of the mechanism by which the SK1 inhibitor, SKi induces the proteasomal degradation of SK1 and apoptosis in LNCaP cells.***

# CHAPTER 4:

# EFFECT OF FTY720 AND (*S*)-FTY720 VINYLPHOSPHONATE ON SK1 EXPRESSION AND SURVIVAL OF LNCaP-AI CELLS

# 4. EFFECT OF FTY720 AND (*S*)-FTY720 VINYLPHOSPHONATE ON SK1 EXPRESSION AND SURVIVAL OF LNCaP-AI CELLS

## 4.1 Introduction

The findings reported in the previous Chapter support a model in which SKi inhibits SK1 activity, and the ceramide that accumulates induces SK1 proteasomal degradation by activating the proteasome. We therefore tested whether other SK1 inhibitors induce the proteasomal degradation of SK1. For this purpose we used two novel SK1 inhibitors, structurally dissimilar from SKi: FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) and its analogue (*S*)-FTY720 vinylphosphonate (Tonelli et al., 2010; Lim et al., 2011b). The structures of these inhibitors are presented in Figure 1.5.

FTY720 is a sphingosine analogue which is taken up by cells and converted to (*S*)-FTY720 phosphate by SK2. The phosphorylated (*S*)-FTY720 is then released by cells and acts as an $S1P_1$ "functional antagonist" (Brinkmann et al., 2002; Matloubian et al., 2004; Gräler and Goetzl, 2004) (Chapter 1). We have recently shown that FTY720 also inhibits the activity of purified SK1a (Tonelli et al., 2010). Although FTY720 had been previously reported to inhibit SK1 activity in a chromatographic fraction containing an anti-SK1 immunoreactive protein (Vessey et al., 2007), the study by Tonelli and colleagues is the first to demonstrate that FTY720 inhibits SK1 activity using purified SK1.

Further kinetic studies revealed that FTY720 is a competitive inhibitor with sphingosine ($K_{ic}$= 2 μM) (Lim et al., 2011b) and an uncompetitive inhibitor against ATP ($K_{iu}$= 13 μM) (Lim et al., 2012b). FTY720 also competes with sphingosine for the catalytic site of SK2, thereby inhibiting SK2 activity, when sphingosine is used as the substrate (Paugh et al., 2003).

An unsaturated phosphonate enantiomer of FTY720, (*S*)-FTY720 vinylphosphonate, has been synthesized (Lu et al., 2009), which also inhibits purified SK1a activity (Tonelli et al., 2010). Remarkably, minor modifications in FTY720 chemical

structure confer to (*S*)-FTY720 vinylphosphonate distinct inhibition kinetic properties. Thus, (*S*)-FTY720 vinylphosphonate is an uncompetitive inhibitor with respect to sphingosine ($K_{iu}$ = 14.5 μM) (Lim et al., 2011b) and a mixed inhibitor against ATP ($K_{ic}$= 10 μM; $K_{iu}$= 65 μM) (Lim et al., 2012b). Additionally, (*S*)-FTY720 vinylphosphonate inhibits SK2 activity (Lim et al., 2011a).

Both (*S*)-FTY720 phosphate and (*S*)-FTY720 vinylphosphonate bind to S1P receptors with nanomolar potency (Mandala et al., 2002; Brinkmann et al., 2002; Valentine et al., 2010). However, although (*S*)-FTY720 phosphate binds to and activates $S1P_{1,3,4,5}$ (Mandala et al., 2002; Brinkmann et al., 2002), (*S*)-FTY720 vinylphosphonate is a pan-antagonist of S1P receptors (Valentine et al., 2010).

In addition to its action at S1P receptors and SK, FTY720 inhibits phospholipase A2 (Payne et al., 2007), S1P lyase (Bandhuvula et al., 2005) and ceramide synthase (Lahiri et al., 2009; Berdyshev et al., 2009) and activates PP2A and PP2A-like phosphatases (Neviani et al., 2007; Matsuoka et al., 2003). The effects on these molecular targets might underlie the anti-cancer activity of FTY720 that has been reported in *in vitro* and *in vivo* tumour models. In this regard, FTY720 has been shown to decrease cell proliferation and promote apoptosis in various cancer cell lines, including prostate cancer cells (Wang et al., 1999; Azuma et al., 2003; Ho et al., 2005; Hung et al., 2008; Nagaoka et al., 2008) and to inhibit prostate cancer cell invasive ability (Zhou et al., 2006). Also, pre-clinical studies demonstrated that FTY720 reduces tumour growth and metastasis in prostate cancer (Pchejetski et al., 2010), melanoma (LaMontagne et al., 2006), hepatocellular carcinoma (Ho et al., 2005) and breast cancer (Azuma et al., 2002) murine models and exhibits additional anti-cancer properties due to the inhibition of tumour angiogenesis and vascular permeability (Ho et al., 2005; LaMontagne et al., 2006).

A novel mechanism of action has been recently described for FTY720, which might also contribute to its anti-tumoral activity. In particular, FTY720 down-regulates the expression of nutrient transporter proteins in *in vitro* and *in vivo* cancer models in a S1P receptors-independent manner, thereby inducing cell death by starvation (Romero Rosales et al., 2011).

Importantly, FTY720 has been approved by the FDA/EMA for the treatment of multiple sclerosis (Brinkmann et al., 2010), representing the first clinically useful SK1 inhibitor.

## 4.2 Results

### 4.2.1 Effect of FTY720 and (*S*)-FTY720 vinylphosphonate on SK1 expression and proteasome activity in LNCaP-AI cells

Chronic treatment of LNCaP-AI cells with FTY720 or (*S*)-FTY720 vinylphosphonate reduced SK1a expression, an effect that was reversed by MG132 (Figure 4.1; supplementary data, Figure S2). Remarkably, while SKi failed to induce SK1b proteasomal degradation in LNCaP-AI cells (Chapter 3), an MG132-sensitive down-regulation of SK1b protein levels was observed in these cells in response to both inhibitors (Figure 4.1; supplementary data, Figure S3). However, FTY720 appears less effective than an equal concentration of (*S*)-FTY720 vinylphosphonate in inducing the proteasomal degradation of SK1b (Figure 4.1; supplementary data, Figure S3).

FTY720 appears to activate the proteasome as it induced down-regulation of cyclin D1 and c-Myc expression, which are targeted to the proteasome (Chapter 3). In contrast, (*S*)-FTY720 vinylphosphonate failed to reduce c-Myc protein levels in LNCaP and LNCaP-AI cells even when used at high micromolar concentrations (Figure 4.2c), suggesting that the treatment with this inhibitor does not result in the activation of the proteasomal machinery in these cell lines.

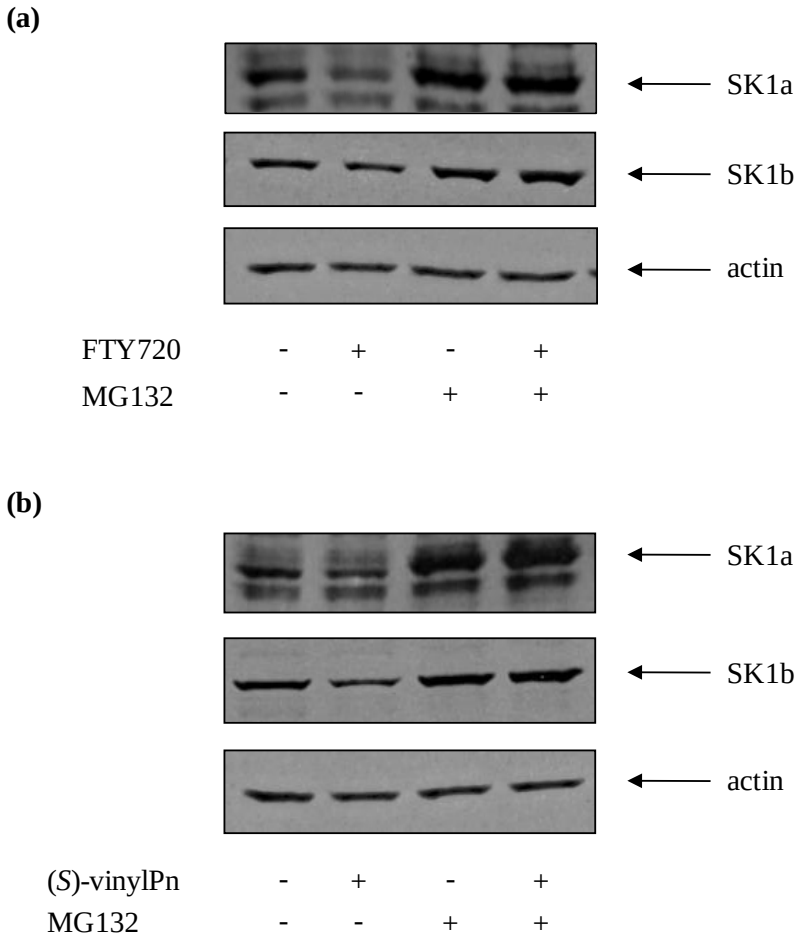



**Figure 4.1** ***Effect of FTY720 and (S)-FTY720 vinylphosphonate on SK1 expression in LNCaP-AI cells.*** *Cells were treated for 48 hours with 10 μM of (a) FTY720 or (b) (S)-FTY720 vinylphosphonate ((S)-vinylPn), in the absence or presence of MG132 (10 μM, 30 minutes pre-treatment). SK1a and SK1b protein expression was then measured by western blotting analysis. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of SK1a and SK1b immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SK1a: FTY720 vs control, <0.01; (S)-FTY720-vinylphosphonate vs control, <0.001; MG132 vs control, <0.001; MG132+FTY720 vs FTY720, <0.05; MG132+(S)-FTY720-vinylphosphonate vs (S)-FTY720-vinylphosphonate, <0.01; SK1b: FTY720 vs control, <0.001; (S)-FTY720-vinylphosphonate vs control, <0.001; MG132 vs control, <0.01; MG132+FTY720 vs FTY720, <0.05; MG132+(S)-FTY720-vinylphosphonate vs (S)-FTY720-vinylphosphonate, <0.05.*

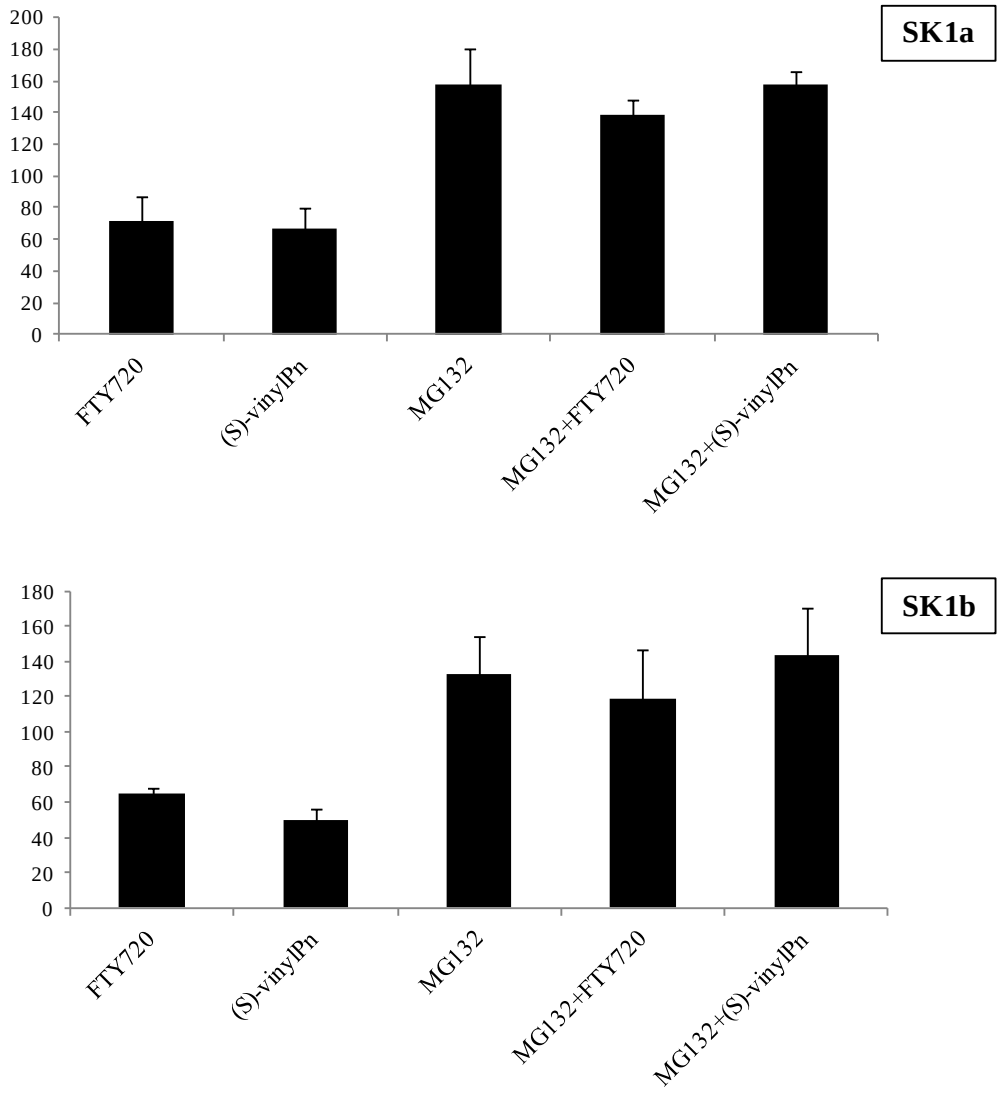



**Figure 4.1** ***Effect of FTY720 and (S)-FTY720 vinylphosphonate on SK1 expression in LNCaP-AI cells (continued).***

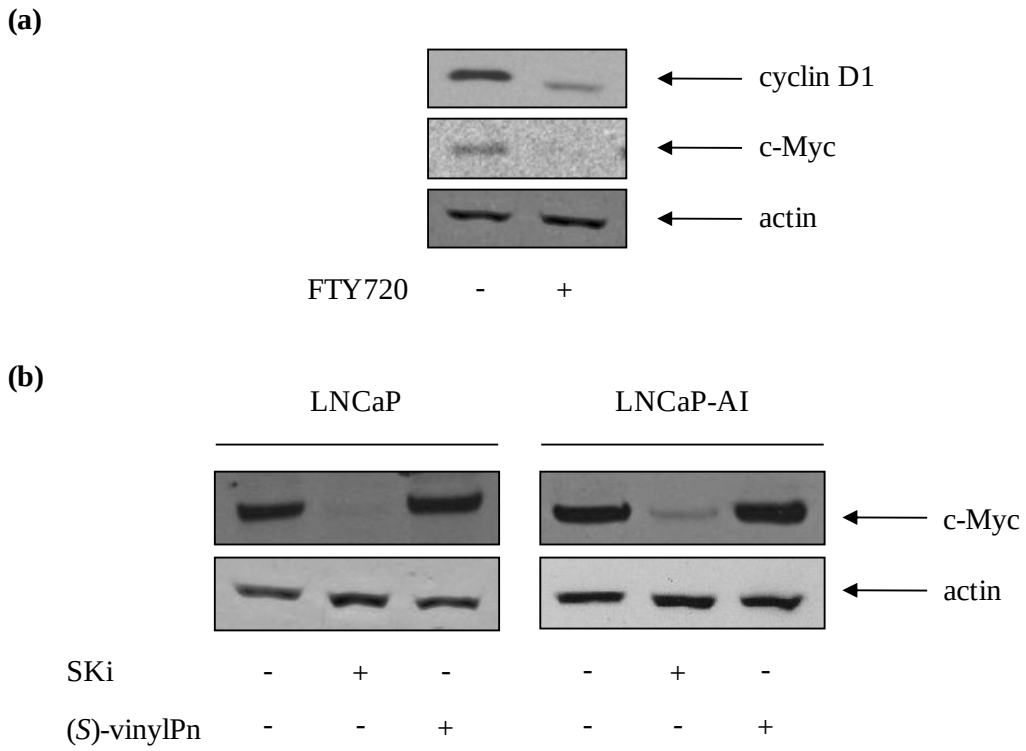



**Figure 4.2** ***Effect of FTY720 and (S)-FTY720 vinylphosphonate on proteasome activity in LNCaP and LNCaP-AI cells.*** *(a) Western blots showing the effect of FTY720 (25 μM, 24 hours) on cyclin D1 and c-Myc expression in LNCaP cells. The blot was then stripped and re-probed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments. (b) Western blots showing the lack of effect of (S)-FTY720 vinylphosphonate ((S)-vinylPn) (50 μM, 24 hours) on c-Myc expression in LNCaP and LNCaP-AI cells. SKi (50 μM, 24 hours) was used as a positive control. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments.*

### 4.2.2 Effect of FTY720 and (*S*)-FTY720 vinylphosphonate on LNCaP-AI cell survival

Since (*S*)-FTY720 vinylphosphonate reduces the expression of both SK1a and SK1b in LNCaP-AI cells, we established whether this inhibitor induces apoptosis, and comparison was made with FTY720.

Chronic treatment of LNCaP-AI cells with (*S*)-FTY720 vinylphosphonate induced the onset of apoptosis, as assessed by detection of 89 kDa cleaved PARP, while FTY720 failed to do so (Figure 4.3a). Similarly, treatment of androgen-independent PC-3 prostate cancer cells with (*S*)-FTY720 vinylphosphonate resulted in increased levels of cleaved PARP (Figure 4.3b).

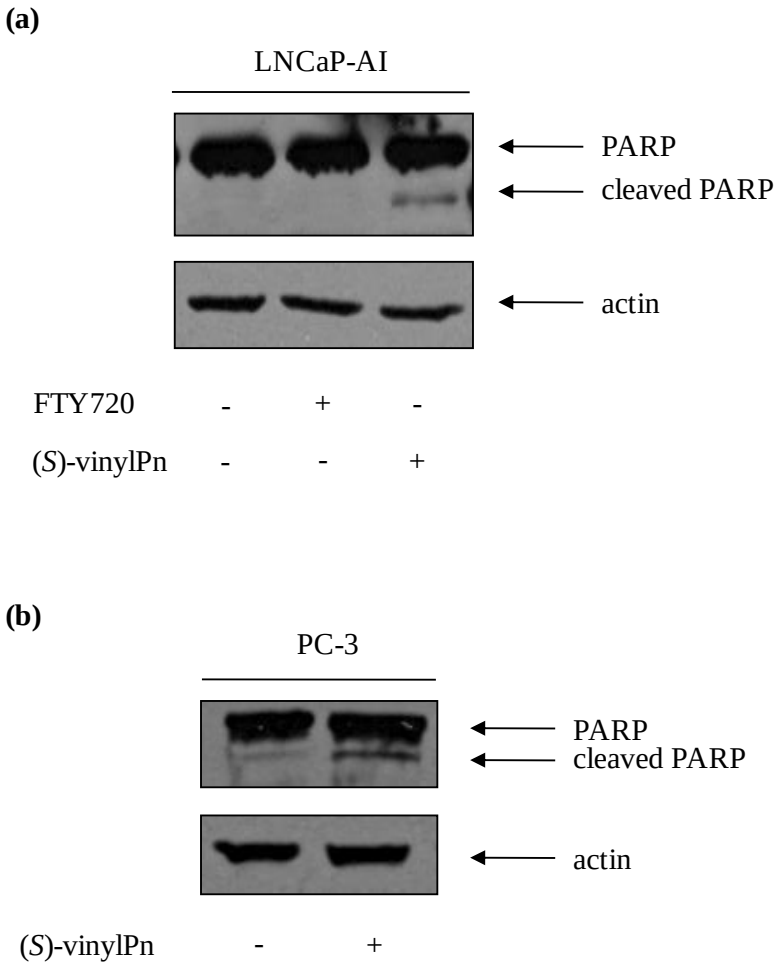



**Figure 4.3** ***Effect of FTY720 and (S)-FTY720 vinylphosphonate on PARP cleavage in LNCaP-AI and PC-3 cells.*** *(a) LNCaP-AI cells were treated for 48 hours with FTY720 or (S)-FTY720 vinylphosphonate ((S)-vinylPn) (either compound at 10 μM), and (b) PC-3 cells were treated with (S)-FTY720 vinylphosphonate ((S)-vinylPn) (10 μM, 24 hours), before measuring cleaved PARP levels by western blotting analysis using an antibody that recognizes both full-length (116 kDa) and cleaved (89 kDa) PARP. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

## 4.3 Discussion

### 4.3.1 SK inhibitors FTY720 and (*S*)-FTY720 vinylphosphonate induce the proteasomal degradation of SK1a and SK1b in LNCaP-AI cells

This study demonstrates that the chronic treatment of LNCaP-AI cells with FTY720 or (*S*)-FTY720 vinylphosphonate, two novel SK1 inhibitors (Tonelli et al., 2010; Lim et al., 2011b), results in SK1a and SK1b proteasomal degradation, although sensitivity of SK1b to FTY720 is reduced compared with (*S*)-FTY720 vinylphosphonate. Similarly, an MG132-sensitive down-regulation of SK1a expression was reported to occur in primary hPASMC and MCF-7 breast cancer cells treated with FTY720 or (*S*)-FTY720 vinylphosphonate (Tonelli et al., 2010; Lim et al., 2011b).

In contrast with SKi, (*S*)-FTY720 vinylphosphonate does not appear to activate the proteasome, as evidenced by the lack of effect on c-Myc expression. The data presented in the previous Chapter provide evidence that the acute inhibition of SK1 activity by SKi results in proteasome activation. *In vitro* assays demonstrated that (*S*)-FTY720 vinylphosphonate is more effective than SKi in inhibiting purified SK1 catalytic activity (Tonelli et al., 2010; Lim et al., 2011b). Thus, the finding that (*S*)-FTY720 vinylphosphonate does not stimulate proteasomal activity in LNCaP and LNCaP-AI cells was unexpected. A possible explanation is that (*S*)-FTY720 vinylphosphonate might be less effective than SKi in inhibiting SK1 activity in intact cells. This might be related to the nature of the inhibition. In fact, SKi is a competitive inhibitor with sphingosine (Lim et al., 2011b) whereas (*S*)-FTY720 vinylphosphonate is competitive with ATP, but like SKi also has an allosteric effect (Lim et al., 2012b). Thus, it might be speculated that in the presence of low cellular concentrations of sphingosine and high levels of ATP, SKi might be more effective than (*S*)-FTY720 vinylphosphonate in inhibiting SK1 activity (because competitive inhibition might be favoured) in these cells, thereby providing an explanation as to why only SKi activates the proteasome.

An additional explanation for the lack of effect of (*S*)-FTY720 vinylphosphonate on proteasome activity is that, being structurally similar to S1P, (*S*)-FTY720 vinylphosphonate itself might inhibit the proteasome. Indeed, S1P has been shown to exert an inhibitory effect on proteasome activity in MCF-7 cells (Loveridge et al., 2010). This effect might counter the activation of the proteasome due to ceramide accumulation.

Interestingly, treatment with (*S*)-FTY720 vinylphosphonate results in a substantial increase in S1P content in LNCaP and LNCaP-AI cells (see below) and this might also contribute to neutralise the effect of ceramide on the proteasome, although the temporal relationship between the (*S*)-FTY720 vinylphosphonate-induced S1P accumulation and the effect on the proteasome remains to be determined.

There are at least two possible mechanisms by which (*S*)-FTY720 vinylphosphonate might promote SK1 proteasomal degradation without activating the proteasome. These two models are not necessarily mutually exclusive and might operate together. First, the binding of this inhibitor to SK1 might induce conformational changes in the enzyme, which might result in an increased susceptibility of SK1 to proteasomal degradation. Indeed, it is well established that protein unfolding represents a signal for degradation by the ubiquitin-proteasome system. Second, (*S*)-FTY720 vinylphosphonate might stimulate the activity of the E3 ubiquitin ligase specific for SK1, thereby promoting the polyubiquitination and, consequently, the degradation of this protein via the proteasome. In this regard, it has been recently reported that S1P binds to the RING domain of TRAF2, thereby stimulating its E3 ubiquitin ligase activity (Alvarez et al., 2010). Since (*S*)-FTY720 vinylphosphonate is structurally similar to S1P, it is possible that these compounds might also bind to and activate E3 ubiquitin ligase(s).

In order to test this hypothesis, it would be necessary to assess whether the treatment with (*S*)-FTY720 vinylphosphonate results in an increased ubiquitination of SK1 in LNCaP-AI cells.

Significantly, (*S*)-FTY720 vinylphosphonate induced the proteasomal degradation of SK1b in LNCaP-AI cells while FTY720 and SKi were less effective (Chapter 3).

There are at least two possibilities that might explain this, based on the two different models proposed above. First, SK1b might adopt a conformation or be protected by an accessory protein, which confers resistance to proteasomal degradation when bound to SKi (and to some degree, to FTY720) and specifically in LNCaP-AI cells. In contrast, the binding to (*S*)-FTY720 vinylphosphonate might force SK1b to adopt a conformation (or induce dissociation of the accessory protective protein) which increases its susceptibility to proteasomal degradation. Second, the process of ubiquitination might represent the rate-limiting step in SK1b processing through the proteasomal degradative machinery in LNCaP-AI cells. Thus, (*S*)-FTY720 vinylphosphonate might overcome the "resistance" of SK1b to proteasomal degradation by activating the E3 ubiquitin ligase specific for this protein. In contrast, SKi might not be able to efficiently remove SK1b from LNCaP-AI cells because although stimulating the proteasome, the basal polyubiquitination of SK1b might be impaired in LNCaP-AI cells through an unidentified mechanism.

This is consistent with the finding by Loveridge et al. that SKi does not induce the polyubiquitination of SK1a (Loveridge et al., 2010). As discussed in Chapter 3, the reduced sensitivity of SK1b to SKi-induced proteasomal degradation might be contingent on acetylation of SK1b that prevents its polyubiquitination (and subsequent removal by the proteasome) due to competition between the two protein modifications. By activating the E3 ligase, (*S*)-FTY720 vinylphosphonate might favour SK1b polyubiquitination over acetylation to promote the proteasomal degradation of SK1b.

Whether (*S*)-FTY720 vinylphosphonate induces SK1b unfolding and/or activates the SK1b-specific E3 ubiquitin ligase remains to be determined. Significantly, the increased efficacy of (*S*)-FTY720 vinylphosphonate compared with FTY720 in down-regulating SK1b expression in LNCaP-AI cells might determine the sensitivity of these cells to the induction of apoptosis (see below).

Consistent with the ability of (*S*)-FTY720 vinylphosphonate to induce the proteasomal degradation of both SK1a and SK1b, the treatment of LNCaP-AI cells

with this inhibitor increased ceramide (C16:0 and C24:0) and sphingosine levels, as assessed by MS analysis of sphingolipid levels in these cells (in collaboration with Dr E. Berdyshev, University of Illinois at Chicago; unpublished data) (Supplementary data, Figure S7). In contrast, SKi did not induce the accumulation of ceramide nor sphingosine in LNCaP-AI cells, where SK1b is resistant to SKi-induced degradation (Chapter 3), suggesting a key role for SK1b in regulating ceramide levels.

Unexpectedly, (*S*)-FTY720 vinylphosphonate induced a substantial increase in S1P intracellular levels in LNCaP-AI cells (Supplementary data, Figure S7). Similarly, treatment of LNCaP cells with (*S*)-FTY720 vinylphosphonate resulted in the accumulation of S1P, an effect that was far more pronounced than in LNCaP-AI cells (unpublished data). This might be due to the inhibition of S1P lyase activity by (*S*)-FTY720 vinylphosphonate, and future experiments will be aimed at testing this possibility. Indeed, it has been reported that FTY720 inhibits S1P lyase activity *in vitro* and *in vivo* (Bandhuvula et al., 2005). As discussed in Chapter 3, the finding that S1P levels are lower in LNCaP-AI cells than in LNCaP cells despite a higher SK1 expression suggests that S1P lyase activity might be up-regulated in LNCaP-AI cells. If (*S*)-FTY720 vinylphosphonate increased S1P levels by inhibiting S1P lyase activity, it is expected that a higher expression of this enzyme would result in a less marked accumulation of S1P in LNCaP-AI cells compared with LNCaP cells in response to (*S*)-FTY720 vinylphosphonate. Alternatively, the net effect on S1P levels might be less pronounced in LNCaP-AI cells because of the down-regulation of SK1 expression by (*S*)-FTY720 vinylphosphonate.

Interestingly, (*S*)-FTY720 vinylphosphonate induced the up-regulation of ectopically expressed SK2 in LNCaP cells (Chapter 5), which, if recapitulated for the endogenous SK2 might also account for the elevation in S1P intracellular levels. Indeed, SK2 over-expression has been previously shown to increase S1P levels (Liu et al., 2000; Liu et al., 2003; Maceyka et al., 2005).

### 4.3.2 (*S*)-FTY720 vinylphosphonate, but not FTY720 induces apoptosis of LNCaP-AI cells

This study revealed that (*S*)-FTY720 vinylphosphonate induces the onset of apoptosis in LNCaP-AI cells, as assessed by detection of PARP cleavage, while FTY720 was not effective in activating apoptosis in LNCaP-AI cells. Significantly, the differential effect on survival can be correlated with the extent to which the two inhibitors down-regulate SK1b expression in LNCaP-AI cells, (*S*)-FTY720 vinylphosphonate being more effective than FTY720 in removing SK1b from these cells. This suggests that (*S*)-FTY720 vinylphosphonate might reduce SK1 levels below a threshold which is required for survival, thereby promoting apoptosis, while FTY720 fails to do so. This hypothesis is in line with the finding that LNCaP-AI cells undergo apoptosis when both SK1a and SK1b are removed (Chapter 3).

MS analysis of sphingolipid levels provides evidence that (*S*)-FTY720 vinylphosphonate induces a loss of LNCaP-AI cell viability by increasing the intracellular levels of the pro-apoptotic sphingolipids ceramide and sphingosine. Indeed, SKi treatment leads to ceramide accumulation and apoptosis in LNCaP cells, while an elevation in ceramide mass is not observed in LNCaP-AI cells which are resistant to SKi-induced apoptosis (Chapter 3). Thus, it might be speculated that the modest effect of FTY720 on SK1 expression in LNCaP-AI cells might not induce ceramide accumulation and these cells therefore do not undergo apoptosis.

The substantial increase in S1P intracellular levels induced by (*S*)-FTY720 vinylphosphonate might also contribute to the pro-apoptotic action of this inhibitor on LNCaP-AI cells. Indeed, a study by Mulders and coworkers reported that high micromolar concentrations of S1P promote apoptosis in PC-3 and DU145 prostate cancer cell lines, associated with increased intracellular $Ca^{2+}$ levels (Mulders et al., 2007). Moreover, previous studies reported that SK2 over-expression results in S1P accumulation and apoptotic cell death (Liu et al., 2003; Maceyka et al., 2005), providing evidence that SK2-derived S1P is linked to apoptosis.

Taken together, the findings presented in this Chapter and Chapter 3 support a critical role for SK1 in promoting LNCaP and LNCaP-AI cell survival and suggest that this might be achieved by the ability of this enzyme to remove ceramide rather than to generate S1P.

These findings are in line with previous studies demonstrating that inhibition of SK1 activates apoptosis in prostate cancer cells by shifting the ceramide/S1P balance toward ceramide (Pchejetski et al., 2005, 2008, 2010; Brizuela et al., 2010), as it has been extensively discussed in Chapter 1. However, this study is the first to demonstrate that the induction of apoptosis by SK1 inhibitors in prostate cancer cells is associated with the proteasomal degradation of SK1 (resulting in a sustained deregulation of the ceramide/S1P balance) and to highlight a crucial role for both SK1a and SK1b in promoting prostate cancer cell viability. Additionally, the finding that (*S*)-FTY720 vinylphosphonate reduces cell viability in LNCaP-AI cells is particularly significant, as the therapeutic options for the treatment of hormone refractory prostate cancer are currently limited.

### 4.3.3 SK1 as a novel target of FTY720

The finding that FTY720 modulates the activity (Tonelli et al., 2010) and expression of SK1 extends the number of intracellular targets of this agent. In light of this newly uncovered mechanism of action of FTY720, it is tempting to speculate that the previously reported anti-cancer properties of this agent (i.e. inhibition of cell proliferation, survival and migration *in vitro* and suppression of tumour growth, metastasis and angiogenesis *in vivo*) might be at least in part due to its ability to inhibit SK1 catalytic activity and/or induce proteasomal degradation of SK1.

Indeed, other studies provide evidence that FTY720 behaves as a typical SK1 inhibitor in cancer cells. In particular, a study by Pchejetski and colleagues reported that treatment of PC-3 prostate cancer cells with FTY720 increases the intracellular levels of ceramide and sphingosine while reducing intracellular S1P to induce apoptosis, an effect that was reversed by SK1 over-expression (Pchejetski et al., 2010). The findings reported in this Chapter indicate that the down-regulation of SK1

expression rather than the reversible inhibition of SK1 activity might be a crucial step in FTY720-induced apoptosis of PC-3 cells.
In this context, the different sensitivity of PC-3 and LNCaP-AI cells to FTY720-induced apoptosis might be ascribed to differences in the expression and/or regulation of SK1a/b between the two cell lines. As a consequence, FTY720 might lower SK1 expression to below a threshold at which cells undergo apoptosis in PC-3 cells but not in LNCaP-AI cells. This hypothesis is supported by the finding that SK1 siRNA promotes the onset of apoptosis in PC-3 cells (Pchejetski et al., 2005) but not in LNCaP-AI cells, where SK1b is not knocked-down (Chapter 3).

The study by Pchejetski and colleagues also demonstrated that FTY720 mimics the effect of SK1 siRNA and well-established pharmacological inhibitors of SK1 in sensitising prostate cancer cells to γ-irradiation both *in vitro* and *in vivo* (Pchejetski et al., 2010; Nava et al., 2000b), an effect that was associated with the inhibition of SK1 activity and was reversed by SK1 over-expression (Pchejetski et al., 2010).

Since the effects of FTY720 in prostate cancer cells appear to be due to SK1 inhibition, it is likely that (*S*)-FTY720 vinylphosphonate, which is more effective than FTY720 in reducing SK1 expression in LNCaP-AI cells, might prove to be even more effective than FTY720 in suppressing prostate cancer cell growth *in vivo* and sensitizing these cells to γ-irradiation.

Finally, additional evidence of the efficacy of FTY720 as an SK1 inhibitor in cancer cells is provided by the finding that this compound suppresses the S1P-induced rearrangement of actin into membrane ruffles/lamellipodia (indicative of a migratory phenotype) in MCF-7 breast cancer cells (Lim et al., 2011b), recapitulating the effect of siRNA knock-down of SK1 (Long et al., 2010a). A similar effect was observed in response to (*S*)-FTY720 vinylphosphonate (Lim et al., 2011b).

## 4.4 Summary

In common with SKi, two novel SK inhibitors, FTY720 and (*S*)-FTY720 vinylphosphonate, modulate the expression of SK1 in LNCaP-AI cells. In contrast with SKi and FTY720, (*S*)-FTY720 vinylphosphonate promotes SK1 proteasomal degradation without stimulating proteasome activity. Moreover, (*S*)-FTY720 vinylphosphonate overcomes the resistance of SK1b to proteasomal degradation. The current study can be progressed with further investigation of the mechanisms by which (*S*)-FTY720 vinylphosphonate modulates SK1 expression in LNCaP-AI cells.

Taken together, the data presented in this Chapter support a model where acute inhibition of SK1 activity by (*S*)-FTY720 vinylphosphonate in LNCaP-AI cells does not substantially acutely tilt the ceramide/S1P balance toward ceramide, as suggested by the lack of effect of this agent on proteasome activity. However, chronic treatment with this inhibitor promotes accumulation of ceramide and sphingosine in LNCaP-AI cells, resulting from the proteasomal degradation of both SK1a and SK1b. This, in turn, is associated with the activation of apoptosis. In contrast with (*S*)-FTY720 vinylphosphonate, FTY720 does not induce the onset of apoptosis in LNCaP-AI cells, and this can be correlated with a less marked effect of this inhibitor on SK1b expression. Finally, chronic treatment of LNCaP cells with SKi promotes accumulation of C22:0 ceramide, while (*S*)-FTY720 vinylphosphonate promotes accumulation of C16:0 ceramide in LNCaP-AI cells, suggesting that androgen-independence might also be associated with changes in the substrate utilisation by the ceramide synthase that is functionally coupled with SK1a/b in the ceramide-sphingosine-S1P rheostat.

For its ability to overcome SK1b resistance and activate apoptosis in LNCaP-AI cells, (*S*)-FTY720 vinylphosphonate might prove useful in the clinical management of castration-resistant prostate cancer.

# CHAPTER 5:

# MODULATION OF SK2 EXPRESSION BY SK INHIBITORS AND EFFECT OF SK2-SELECTIVE ABLATION IN LNCaP CELLS

# 5. MODULATION OF SK2 EXPRESSION BY SK INHIBITORS AND EFFECT OF SK2-SELECTIVE ABLATION IN LNCaP CELLS

## 5.1 Introduction

In contrast with SK1, there are no published reports showing a correlation between SK2 over-expression and cancer. In addition, SK2 over-expression was reported to exert an anti-proliferative and pro-apoptotic effect (Liu et al., 2003; Maceyka et al., 2005). Thus, most of the research on SK and cancer has focused on SK1. As discussed in Chapter 1, however, emerging evidence suggests that similar to SK1, SK2 also plays an important role in cancer, promoting tumour cell survival, proliferation and migration (Hait et al., 2005; Van Brocklyn et al., 2005; Gao and Smith, 2011). Significantly, a recent report demonstrated that SK2 knock-down has stronger anti-tumour activities than SK1 knock-down in various human cancer cell lines (Gao and Smith, 2011). In addition, SK2 ablation inhibits tumour growth in a murine model of breast cancer (Weigert et al., 2009) and sensitizes cancer cells to chemotherapeutic agents (Sankala et al., 2007; Beljanski et al., 2011). These studies provide strong evidence that SK2 might represent a promising target for cancer therapy and prompt further investigation of the function of SK2 in tumour cells.

Two selective inhibitors of SK2 have been recently described in the literature, namely ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide] (French et al., 2010) and (*R*)-FTY720 methyl ether [referred to as (*R*)-FTY720-OMe in the text] (Lim et al., 2011a) (Figure 1.5). ABC294640 has been synthesised from SKi (Smith et al., 2008), while (*R*)-FTY720-OMe is a non-phosphorylable analogue of FTY720, which has been synthesised by replacing one of the prochiral hydroxyl groups of FTY720 with a methoxy group (Lim et al., 2011a).

ABC294640 and (*R*)-FTY720-OMe inhibit SK2 *in vitro*, without affecting SK1 activity. Both compounds act as competitive inhibitors with respect to sphingosine

and exhibit similar potency ($K_{ic}$= 10 µM and 16.5 µM, respectively) (French et al., 2010; Lim et al., 2011a). When tested against a panel of protein kinases, ABC294640 was found to be highly selective for SK2 (French et al., 2010). Interestingly, (*R*)-FTY720-OMe reduces SK2 expression in HEK 293 cells (Lim et al., 2011a), suggesting that the efficacy of this inhibitor might be superior to that predicted from the reversible inhibition of SK2 activity. Importantly, (*R*)-FTY720-OMe does not affect SK1 expression in HEK 293 cells (Lim et al., 2011a), demonstrating the selectivity of this compound not only in inhibiting the catalytic activity, but also in modulating the expression of SK2 over SK1. Hence, (*R*)-FTY720-OMe represents a useful tool to interrogate SK2 function in cell systems.

Besides inhibiting SK2 activity *in vitro*, ABC294640 and (*R*)-FTY720-OMe are also active in cells. Significantly, ABC294640 has anti-tumour effects *in vitro* and *in vivo*, reinforcing the hypothesis that targeting SK2 might represent a successful strategy for cancer treatment (French et al., 2010). Similarly, (*R*)-FTY720-OMe reduces cell viability and inhibits S1P-stimulated migration (Lim et al., 2011).

Having established that the chronic treatment with SKi and (*S*)-FTY720-vinylphosphonate modulates SK1 expression in LNCaP and LNCaP-AI cells (Chapter 3; Chapter 4), we also investigated the effect of these inhibitors on SK2 expression. In addition, the SK2-selective inhibitor, (*R*)-FTY720-OMe was used to probe the role of SK2 in regulating prostate cancer cell survival. Indeed, the knowledge about the function of SK2 in prostate cancer is very limited.

## 5.2 Results

### 5.2.1 Effect of SKi and (*S*)-FTY720-vinylphosphonate on ectopically expressed SK2 in LNCaP cells

LNCaP and LNCaP-AI cells express *SK2*, as assessed by RT-PCR using SK2 gene-specific primers that amplified a single product of 315 bp (Figure 5.1). RT-PCR analysis also revealed that SK2 mRNA levels are not significantly different in LNCaP cells compared with LNCaP-AI cells (LNCaP cells *vs* LNCaP-AI cells, p value >0.05) (Figure 5.1).

The commercially available anti-SK2 antibodies perform poorly in immunoblotting experiments, as they lack of selectivity. Therefore, to investigate the effect of SK inhibitors on SK2 protein expression, it was necessary to over-express recombinant SK2 in LNCaP and LNCaP-AI cells by transient transfection with a plasmid construct encoding Myc-tagged SK2.

Western blotting analysis of lysates from these cells using an anti-Myc antibody detected a band of the predicted size (molecular weight of 63 kDa), confirming that the recombinant protein was expressed (Figure 5.2). Recombinant SK2 was not detected in lysates from LNCaP cells transfected with the vector plasmid, confirming the specificity of the anti-Myc antibody used to detect recombinant SK2 (Figure 5.2).

Treatment with SKi (10 μM) did not down-regulate recombinant SK2 in LNCaP cells, while it was effective in reducing the expression of endogenous c-Myc (Figure 5.3a). In contrast, a higher concentration of SKi (50 μM) down-regulated the expression of recombinant SK2 in LNCaP and LNCaP-AI cells (Figure 5.3).

Surprisingly, treatment of LNCaP cells with (*S*)-FTY720-vinylphosphonate resulted in elevated expression of recombinant SK2 protein levels (66±16% increase in treated cells *vs* control, p value <0.01) (Figure 5.4).

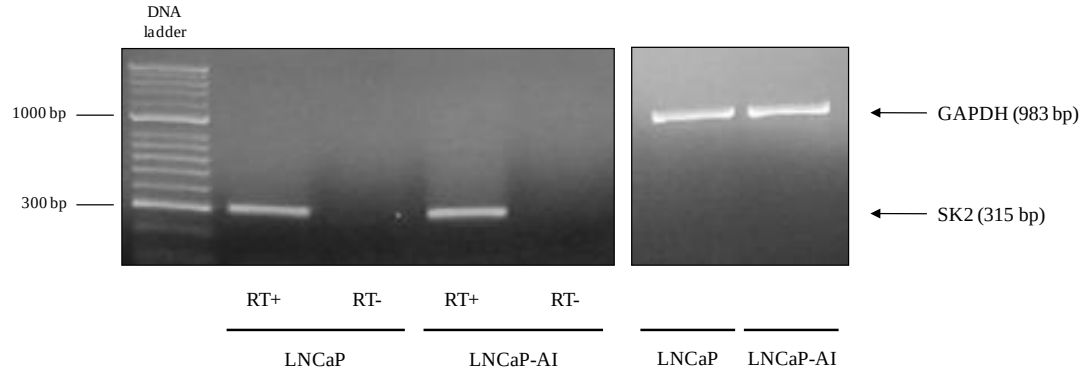



***Figure 5.1 Expression of SK2 in LNCaP and LNCaP-AI cells.*** *SK2 mRNA levels were examined by RT-PCR using gene-specific primers, followed by agarose gel electrophoresis of the amplification products. A negative control with omission of reverse transcriptase (RT-) was included to establish a lack of genomic DNA contamination. The expression of the housekeeping gene GAPDH was analysed using gene-specific primers to ensure that comparable amounts of cDNA were used for the two cell lines. The results shown are representative of three independent experiments.*

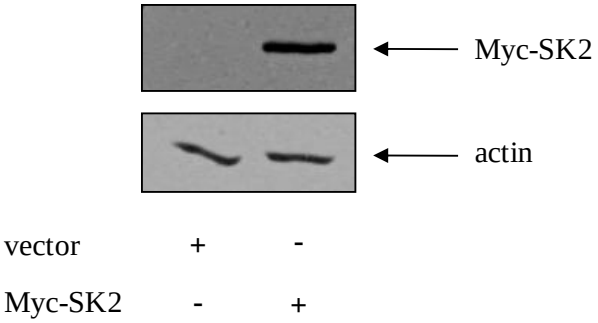



***Figure 5.2 Expression of recombinant SK2 in LNCaP cells.*** *Cells were transiently transfected with Myc-tagged SK2 plasmid construct (Myc-SK2) or vector plasmid construct (vector), as described under Methods section. Ectopic expression of recombinant SK2 was measured by western blotting analysis using an anti-Myc antibody. Blot was then stripped and re-probed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of more than three separate experiments.*

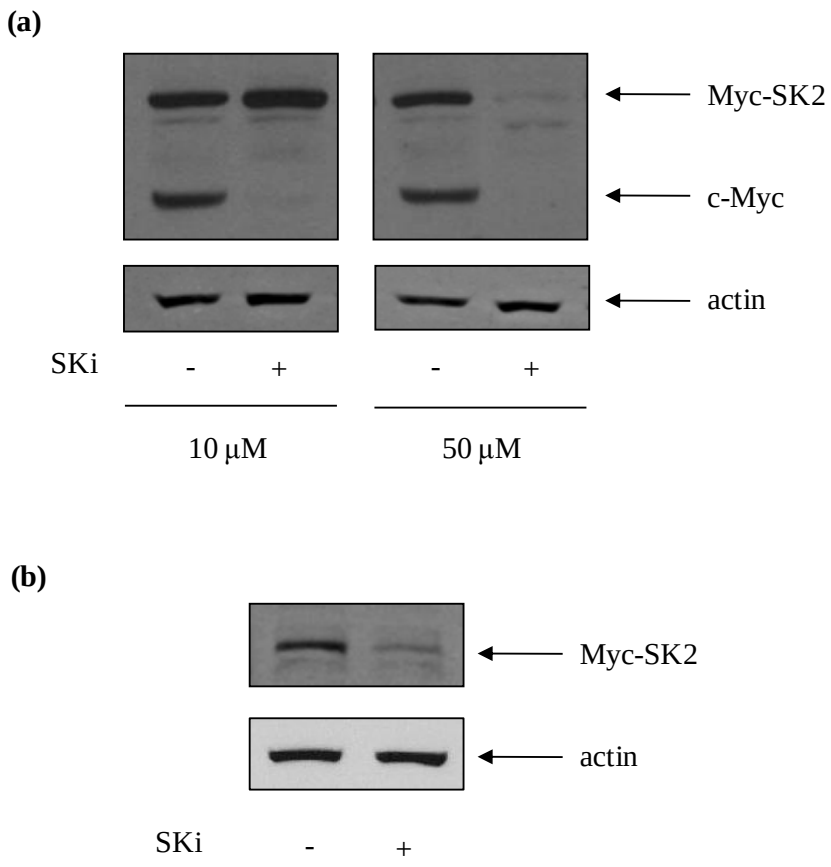



**Figure 5.3** ***Effect of SKi on recombinant SK2 expression in LNCaP and LNCaP-AI cells.*** *Cells transiently over-expressing Myc-tagged SK2 were treated with the indicated concentrations of SKi or with the vehicle alone (DMSO, 0.1% v/v) for 24 hours. The effect of SKi on recombinant SK2 expression in (a) LNCaP and (b) LNCaP-AI cells was then measured by western blotting analysis using an anti-Myc antibody. Also shown in (a) is the effect of SKi on endogenous c-Myc expression in LNCaP cells. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of at least three separate experiments.*

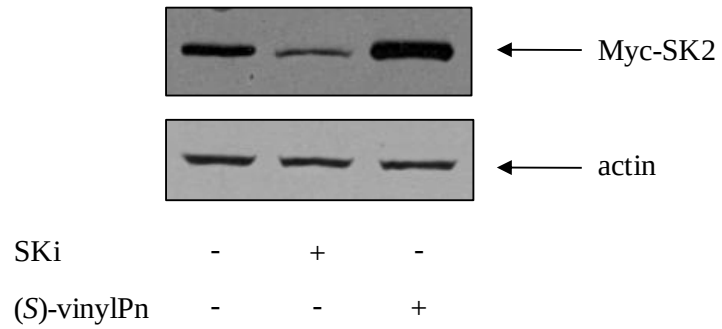



**Figure 5.4** ***Effect of (S)-FTY720-vinylphosphonate on recombinant SK2 expression in LNCaP cells.*** *Cells transiently over-expressing Myc-tagged SK2 were treated with (S)-FTY720-vinylphosphonate ((S)-vinylPn) or with the vehicle alone (DMSO, 0.1% v/v) for 24 hours. SKi (50 μM, 24 hours) was used as a control. Recombinant SK2 levels were measured by western blotting analysis using an anti-Myc antibody. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

The mechanism by which high concentrations of SKi induce the down-regulation of SK2 in LNCaP cells was then investigated. The proteasome inhibitor MG132 did not prevent SKi effect on recombinant SK2 expression in LNCaP cells, while c-Myc protein levels were restored under the same conditions (Figure 5.5a). The treatment with MG132 alone induced a substantial accumulation of recombinant SK2, suggesting that this protein is actively processed through the ubiquitin-proteasome pathway (Figure 5.5a).

Pre-treatment with CA074Me, which inhibits the lysosomal protease cathepsin B also failed to reverse the effect of SKi on recombinant SK2 protein levels in LNCaP cells (Figure 5.5b), excluding a role for the lysosomal degradation pathway in mediating SK2 down-regulation in response to SKi.

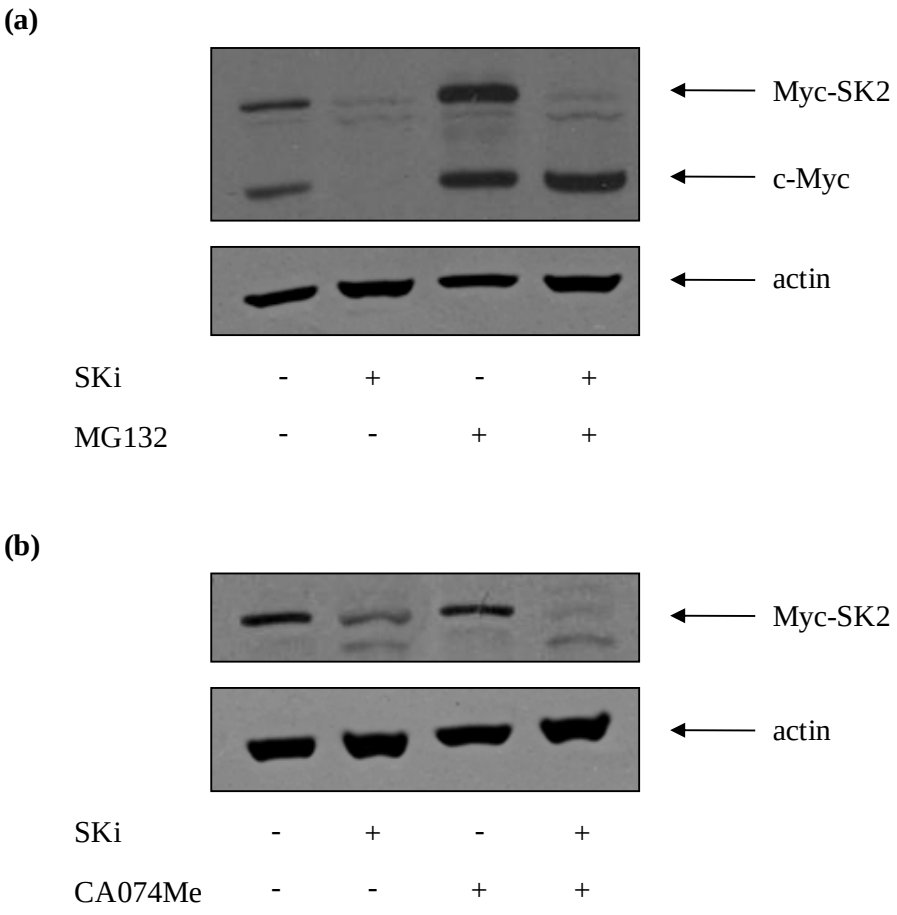



**Figure 5.5** ***Effect of proteasomal or lysosomal degradative pathway inhibition on SKi-induced down-regulation of recombinant SK2 expression in LNCaP cells.*** *Cells transiently over-expressing Myc-tagged SK2 were pre-treated with (a) the proteasome inhibitor MG132 (10 μM, 30 minutes) or (b) the cathepsin B inhibitor CA074Me (10 μM, 30 minutes) before being treated with SKi (50 μM, 24 hours) or with the vehicle (DMSO, 0.1% v/v). The levels of recombinant SK2 were measured by western blotting analysis using an anti-Myc antibody. Also shown in (a) is the reversal of SKi effect on endogenous c-Myc expression by MG132. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

### 5.2.2 Effect of (*R*)-FTY720-OMe on ectopically expressed SK2 and proteasome activity in LNCaP cells

We next investigated the effect of the SK2-specific inhibitor (*R*)-FTY720-OMe on recombinant SK2 expression in LNCaP cells.

Treatment with (*R*)-FTY720-OMe (10 μM) induced the down-regulation of SK2 protein levels in these cells (Figure 5.6). Higher concentrations of the inhibitor did not further reduce SK2 expression (Figure 5.6a).

The effect of (*R*)-FTY720-OMe persisted in the presence of the proteasome inhibitor, MG132. Indeed, in cells pre-treated with MG132, (*R*)-FTY720-OMe reduced SK2 expression to the same extent as in control cells not pre-treated with MG132 (Figure 5.6b). This finding suggests that the proteasomal degradation pathway does not mediate SK2 down-regulation in response to (*R*)-FTY720-OMe treatment.

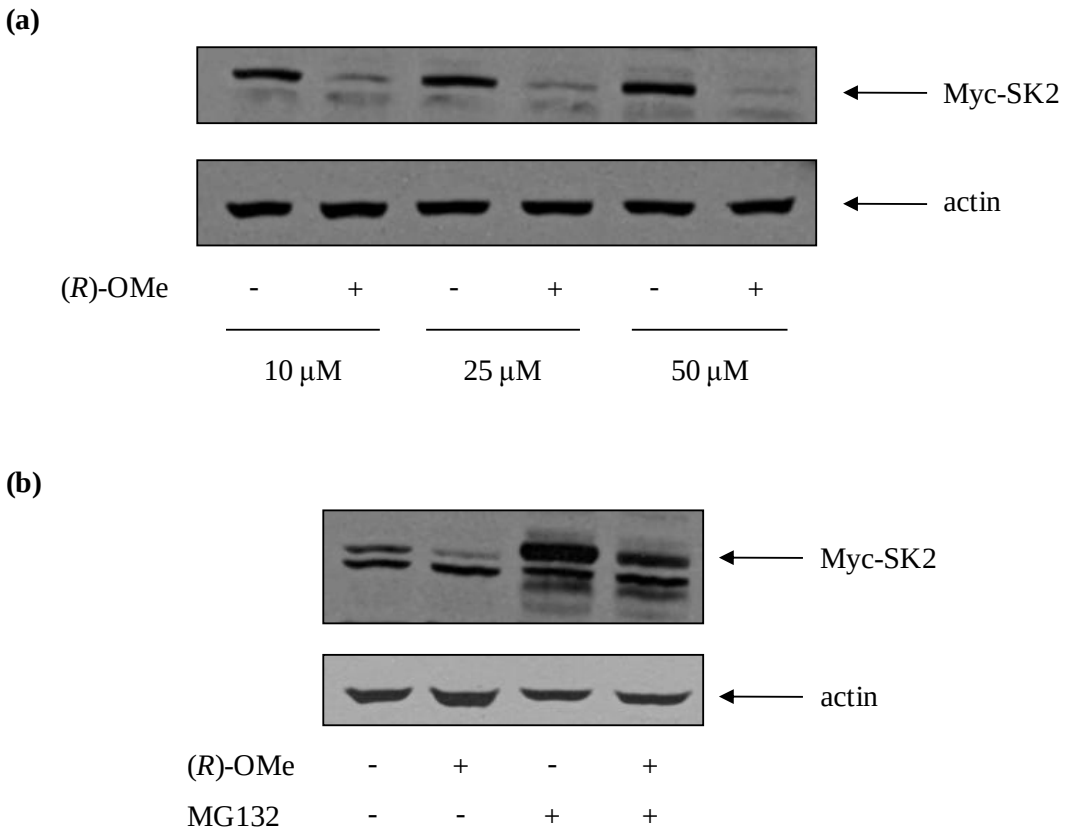



**Figure 5.6** ***Effect of (R)-FTY720-OMe on SK2 expression in LNCaP cells.*** *(a) Cells transiently over-expressing Myc-tagged SK2 were treated with the indicated concentrations of (R)-FTY720-OMe ((R)-OMe) or with the vehicle alone (DMSO, 0.1% v/v) for 24 hours. Recombinant SK2 levels were then measured by western blotting analysis using an anti-Myc antibody. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. (b) Western blot showing the effect of proteasomal degradation pathway inhibition on (R)-FTY720-OMe-induced down-regulation of recombinant SK2 expression in LNCaP cells. Cells transiently over-expressing Myc-tagged SK2 were pre-treated with the proteasome inhibitor MG132 (10 μM, 30 minutes) before being treated with (R)-FTY720-OMe (10 μM, 24 hours) or with the vehicle (DMSO, 0.1% v/v). The levels of recombinant SK2 were measured by western blotting analysis using an anti-Myc antibody. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

The effect of (*R*)-FTY720-OMe on proteasome activity was next assessed. Treatment of LNCaP cells with (*R*)-FTY720-OMe did not increase the proteasomal activity against endogenous proteins, as assessed using the Proteasome-Glo™ Cell-Based Assay (Figure 5.7a). SKi, that was previously shown to activate the proteasome (Chapter 3), was used along with (*R*)-FTY720-OMe in this experiment as a positive control, to validate the assay. In agreement with the lack of effect on proteasome activity, cyclin D1 and c-Myc protein levels were not reduced in LNCaP cells treated with (*R*)-FTY720-OMe (Figure 5.7b). In contrast, SKi induced the MG132-sensitive down-regulation of cyclin D1 and c-Myc in these cells (Chapter 3). Thus, these findings indicate that (*R*)-FTY720-OMe does not activate the proteasome in LNCaP cells.

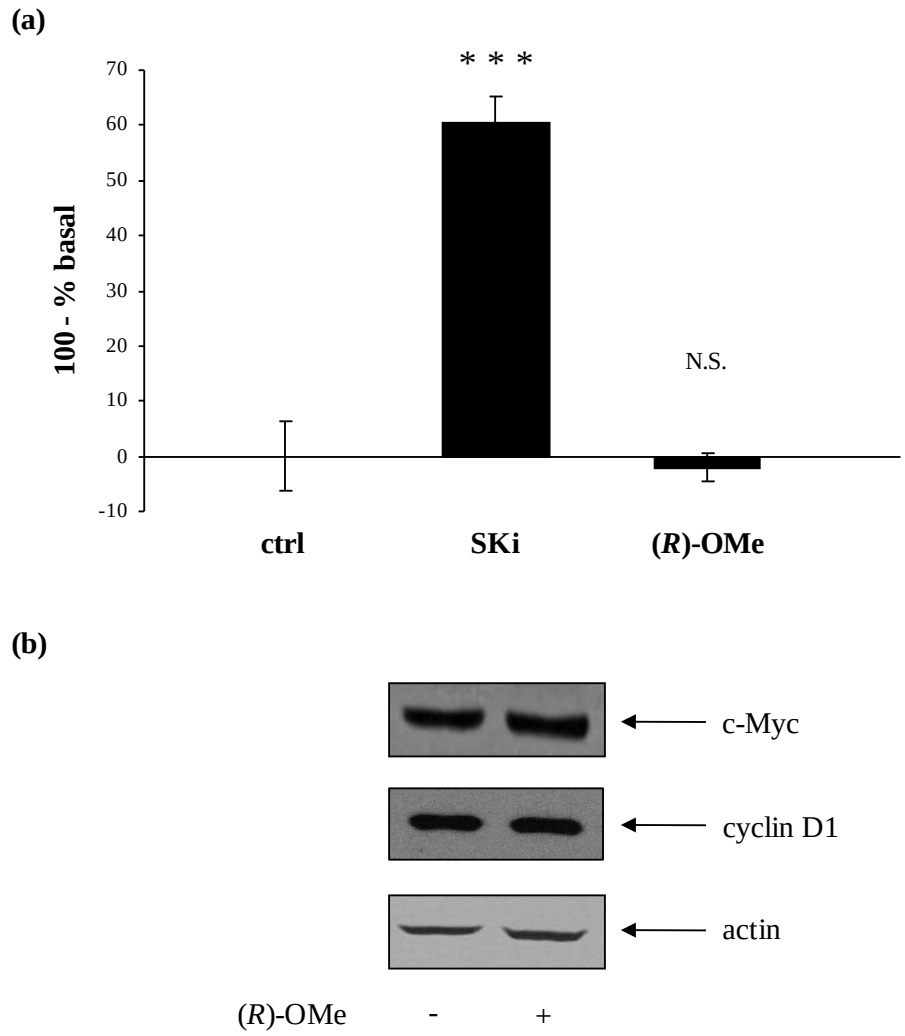



**Figure 5.7** ***Effect of (R)-FTY720-OMe on proteasome activity in LNCaP cells.*** *(a) Proteasomal luminogenic assay showing the effect of (R)-FTY720-OMe ((R)-OMe) (10 μM, 8 hours) on proteasome activity in LNCaP cells. SKi (10 μM, 8 hours) was used as a reference compound to validate the assay. For each treatment, results are expressed as 100-% of proteasomal activity against luminogenic substrate in cells treated with the vehicle alone (ctrl) (DMSO 0.1% v/v) (which corresponds to inhibitor-stimulated activity against endogenous proteins). Data are presented as mean with standard deviation of triplicate assays (n=3). *** denotes a p-value <0.001, while N.S. denotes a p-value >0.05 (not statistically significant) compared with control. (b) Western blots showing the lack of effect of (R)-FTY720-OMe ((R)-OMe) (10 μM, 24 hours) on cyclin D1 and c-Myc expression in LNCaP cells. Blots were stripped and reprobed with anti-actin antibody to ensure comparable protein loading between samples. Results are representative of three separate experiments.*

### 5.2.3 Effect of (*R*)-FTY720-OMe on LNCaP cell survival

Treatment with (*R*)-FTY720-OMe (10 μM) did not promote apoptosis in LNCaP cells, as assessed by the absence of apoptosis-specific 89 kDa cleaved PARP in cells treated with this inhibitor (Figure 5.8). Higher concentrations of (*R*)-FTY720-OMe induced PARP cleavage (Figure 5.8), although this was not associated with a further reduction in SK2 expression (Figure 5.6a), suggesting that apoptosis in response to these concentrations of the inhibitor is an "off-target" effect rather than a consequence of inhibitor-induced removal of SK2.

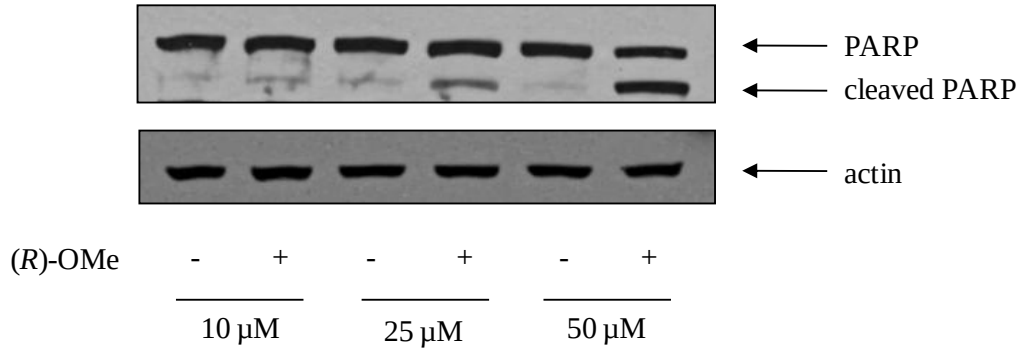



***Figure 5.8 Effect of (R)-FTY720-OMe on LNCaP cell survival.*** *Cells were treated for 24 hours with the indicated concentrations of (R)-FTY720-OMe ((R)-OMe) or with the vehicle alone (DMSO, 0.1% v/v) before measuring cleaved PARP levels by western blotting analysis using an antibody that recognizes both full-length (116 kDa) and cleaved (89 kDa) PARP. Blots were then stripped and reprobed with anti-actin antibody to ensure comparable protein loading. Results are representative of two/three separate experiments.*

## 5.3 Discussion

### 5.3.1 SK inhibitors modulate SK2 expression in LNCaP cells

This study demonstrates that high concentrations of SKi induce a reduction in SK2 expression via a mechanism that does not involve the proteasome. Basal expression of SK2 is likely regulated by the ubiquitin-proteasome pathway, as evidenced by the finding that its levels are increased by treatment of cells with the proteasome inhibitor, MG132. These findings suggest that SKi does not modulate the flux of SK2 through the proteasomal pathway in LNCaP cells, while it increases the proteasome-dependent removal of SK1 in these cells by stimulating proteasomal activity (Chapter 3). This might be accounted for by a different sub-cellular localization of SK1 and SK2, which is consistent with the possibility that SK1 and SK2 might regulate distinct sub-cellular pools of S1P and ceramide, as will be discussed in Chapter 7.

A role for the lysosomal degradation pathway in mediating SKi effect on SK2 expression can also be excluded, based on the finding that the lysosomal cathepsin B specific inhibitor (CA074Me) failed to reverse SKi-induced down-regulation of SK2 in LNCaP cells.
It is not known whether SKi-induced down-regulation of SK2 expression occurs at the transcriptional or post-translational level.

It has been recently shown that SK2 down-regulation occurs in apoptotic cells due to caspase-1-mediated cleavage (Weigert et al., 2010). However, SKi (10 μM) activates apoptosis in LNCaP cells (Chapter 3) while it fails to reduce SK2 expression in these cells, suggesting that SKi does not activate caspase-1 or that SK2 is not a substrate for this enzyme.

Surprisingly, treatment of LNCaP cells with (*S*)-FTY720 vinylphosphonate results in the up-regulation of recombinant SK2 protein levels. This might account for the substantial increase in S1P levels that is observed in LNCaP cells treated with (*S*)-

FTY720 vinylphosphonate, and thus for the apoptotic effect of this inhibitor, as discussed in Chapter 4. Indeed, while low levels of S1P are protective against apoptosis (Cuvillier et al., 1996), very high levels of S1P are pro-apoptotic in prostate cancer cell lines (Mulders et al., 2007).

Further studies are required to establish the mechanism by which (*S*)-FTY720 vinylphosphonate modulates the expression of SK2 and whether this effect is transcriptional or post-translational.

Finally, treatment with the SK2-specific inhibitor (*R*)-FTY720-OMe down-regulates ectopically expressed SK2 in LNCaP cells. A similar effect was reported in HEK 293 cells over-expressing recombinant SK2 (Lim et al., 2011a), indicating that it is not a cell type-specific effect.

The mechanism(s) of SK2 down-regulation in response to (*R*)-FTY720-OMe remains to be elucidated. In particular, it is not known whether this effect is contingent on SK2 activity inhibition and whether (*R*)-FTY720-OMe affects *SK2* gene expression and/or SK2 protein degradation. In this regard, the finding that the proteasome inhibitor MG132 did not reduce the effect of (*R*)-FTY720-OMe on SK2 suggests that (*R*)-FTY720-OMe-induced down-regulation of SK2 does not involve the proteasome. In addition, a role for caspase-1-mediated cleavage can be excluded, since (*R*)-FTY720-OMe does not induce apoptosis in these cells. Alternatively, SK2 might not be a substrate for caspase-1 in these cells.

It is important to appreciate that (*R*)-FTY720-OMe (10 µM) does not down-regulate ectopically expressed SK1 in HEK 293 cells (Lim et al., 2011a). Since the SK1 plasmid used by Lim and colleagues and the SK2 plasmid used for the current study carry the same promoter, this finding provides evidence that (*R*)-FTY720-OMe (10 µM) does not affect CMV promoter activity to reduce the expression of SK2 in LNCaP cells.

(*R*)-FTY720-OMe does not increase the flux of cellular protein through the proteasomal degradation pathway, as demonstrated by the measurement of proteasome activity and by the finding that this inhibitor (in contrast with SKi: Chapter 3) does not induce the proteasomal degradation of cyclin D1 and c-Myc. The

data presented in Chapter 3 support a model in which SKi-induced activation of the proteasome is due to the accumulation of ceramide resulting from acute SK1 activity inhibition. MS analysis of sphingolipid levels showed that chronic treatment of LNCaP cells with (*R*)-FTY720-OMe does not result in increased ceramide levels (see below). Thus, it is likely that acute inhibition of SK2 activity by (*R*)-FTY720-OMe might also fail to elevate intracellular ceramide mass in these cells. This would provide an explanation as to why this inhibitor does not activate the proteasome in LNCaP cells. Whether the lack of increased ceramide formation is due to an off-target effect of (*R*)-FTY720-OMe (i.e. inhibition of ceramide synthase) remains to be determined.

### 5.3.2 Effect of (*R*)-FTY720-OMe on sphingolipid levels in LNCaP cells

To assess the effect of SK2 ablation on sphingolipid metabolism in LNCaP cells, we measured the sphingolipid levels in cells treated with (*R*)-FTY720-OMe by LC-MS analysis (in collaboration with Dr E. Berdyshev, University of Illinois at Chicago) (unpublished data; Supplementary data, Figure S8). Treatment with (*R*)-FTY720-OMe (10 μM, 24 hours), under conditions that remove ectopically expressed SK2, had no significant effect on ceramide levels, while it increased sphingosine levels and decreased S1P levels. These data suggest that there is not an active flux from ceramide to sphingosine in the ceramide-sphingosine-S1P pathway regulated by SK2. This can be accounted for by a model in which SK2-derived S1P is not accessible to S1P lyase, and sphingosine and S1P are rapidly recycled by the combined action of SK2 and S1P phosphatase. This model is supported by a study from Spiegel and colleagues demonstrating that SK2 acts in concert with S1P phosphatase in a sphingosine-S1P recycling pathway in HEK 293 cells (Maceyka et al., 2005). In these cells, sphingosine formed from S1P dephosphorylation is then converted back to ceramide by the action of ceramide synthase (Maceyka et al., 2005). In contrast, the lack of effect of (*R*)-FTY720-OMe on ceramide levels in LNCaP cells suggests that the back-conversion of sphingosine to ceramide is not operative in the ceramide-sphingosine-S1P network regulated by SK2 in these cells.

An alternative possibility to explain the lack of ceramide accumulation in LNCaP cells treated with (*R*)-FTY720-OMe is that this compound might prevent the back-conversion of sphingosine to ceramide by inhibiting ceramide synthase. Indeed, FTY720 which is structurally similar to (*R*)-FTY720-OMe inhibits ceramide synthase activity *in vitro* and *in vivo* (Berdyshev et al., 2009; Lahiri et al., 2009). (*R*)-FTY720-OMe might therefore be expected to reduce dihydroceramide and ceramide levels due to inhibition of the *de novo* pathway. However, this was not the case.

The analysis of sphingolipid levels in LNCaP cells where SK2 expression has been selectively knocked-down by siRNA treatment would assist in defining how SK2 modulates sphingolipid metabolism in these cells.

In HEK 293 cells, the SK2-mediated generation of ceramide is partly responsible for the pro-apoptotic function of SK2 in these cells (Maceyka et al., 2005). Thus, over-expression of SK2 results in ceramide accumulation and activation of apoptosis (Maceyka et al., 2005). In contrast, in the absence of the back-conversion of sphingosine to ceramide, SK2 might function to promote S1P formation and therefore a pro-growth signal. In this regard, it has been shown that the SK2 inhibitor, ABC294640 exerts an anti-proliferative effect in DU145 prostate cancer cells (French et al., 2010), suggesting that SK2 might promote prostate cancer cell proliferation. Future experiments will be aimed at characterising the function of SK2 in regulating LNCaP cell proliferation.

### 5.3.3 SK2 regulation of LNCaP cell survival

There are currently no published reports regarding the role of SK2 in regulating prostate cancer cell survival. The data presented in this Chapter demonstrate that (*R*)-FTY720-OMe fails to promote apoptosis at a concentration which reduces SK2 expression in LNCaP cells. This finding indicates that in contrast with SK1 (Chapter 3; Chapter 4), SK2 is not involved in controlling the apoptotic status of LNCaP cells. To confirm this, it will be necessary to examine whether siRNA-mediated ablation of SK2 activates the onset of apoptosis in LNCaP cells.

The failure of (*R*)-FTY720-OMe to induce apoptosis in LNCaP cells is consistent with the finding that treatment with this inhibitor does not increase ceramide levels in these cells. Indeed, apoptosis in LNCaP and LNCaP-AI cells in response to SKi or (*S*)-FTY720-vinylphosphonate, respectively, is associated with the accumulation of ceramide (Chapter 3; Chapter 4). These data are also in agreement with the finding that SK1 knock-down sensitises prostate cancer cells to γ-irradiation by elevating the intracellular levels of ceramide (Nava et al., 2000b; Pchejetski et al., 2010), while SK2 knock-down fails to do so (Pchejetski et al., 2010).

Although SK2 does not appear to regulate the apoptotic status of LNCaP cells, it cannot be excluded that SK2 might control LNCaP cell survival. Indeed, the data presented in the current study suggest that SK2 might inhibit autophagy in LNCaP cells, as discussed in Chapter 3. Evidence exists that autophagy might represent a pro-survival pathway in tumour cells, including prostate cancer cells (Lavieu et al., 2006; Li et al., 2008). Thus, by inhibiting autophagy, SK2 might reduce LNCaP cell viability. Additionally, it has been shown that the activation of autophagy maintains the survival of LNCaP cells subjected to hormone ablation (Li et al., 2008; Chhipa et al., 2011), thereby favouring the transition of these cells to androgen independence. Therefore, SK2 might hinder androgen escape by preventing the activation of this pro-survival pathway. Evaluating these hypotheses requires further investigation.

Taken together, these findings support a model in which SK1 and SK2 regulate distinct functional pools of sphingolipids in prostate cancer cells, as will be further discussed in Chapter 7.

## 5.4 Summary

This study shows that SK2 expression is modulated by SK inhibitors in LNCaP cells. Further studies are required to elucidate the mechanisms involved.

The selective elimination of SK2 from LNCaP cells has distinct effects on sphingolipid levels and cell survival compared with SK1, providing evidence that the two enzymes might regulate different pools of sphingolipids in these cells. In particular, the data presented in this Chapter provide evidence that SK2 is not involved in regulating proteasome activity and the apoptotic status of LNCaP cells. This is associated with the failure to increase intracellular ceramide levels, when SK2 is eliminated from these cells in response to (*R*)-FTY720-OMe.

The functional significance of the modulation of SK2 expression by SK1 inhibitors in prostate cancer cells (i.e. whether this is favourable to the anticancer activity of SK1 inhibitors) remains to be determined.

# CHAPTER 6:

# CHARACTERISATION OF SK REGULATION OF ANDROGEN RECEPTOR EXPRESSION IN PROSTATE CANCER CELL MODELS

# 6. CHARACTERISATION OF SK REGULATION OF ANDROGEN RECEPTOR EXPRESSION IN PROSTATE CANCER CELL MODELS

## 6.1 Introduction

As discussed in Chapter 1, a functional androgen receptor (AR) is required for prostate tumorigenesis and aberrant AR signalling is a critical factor in the resistance to antiandrogen therapy and in the progression of prostate cancer to a hormone refractory state (Buchanan et al., 2001; Chen et al., 2004; Dehm and Tindall, 2006). Thus, abrogating AR signalling represents a promising therapeutic strategy in the management of this disease.

Interestingly, SK1 is linked with AR signalling pathway in prostate cancer cells. Indeed, dihydrotestosterone induces a rapid and transient stimulation of SK1 activity in LNCaP cells in an AR-dependent manner (Dayon et al., 2009). This effect is mediated by PI3K/Akt and is required for androgen-stimulated proliferation of hormone-sensitive prostate cancer cells (Dayon et al., 2009). Significantly, the anti-proliferative effect which results from androgen deprivation in *in vitro* and *in vivo* models of hormone-sensitive prostate cancer is reversed by SK1 over-expression, confirming a role for SK1 in sustaining prostate cancer cell growth.

SK1 is also involved in the response of androgen-dependent prostate cancer cells to hormone deprivation. Interestingly, androgen withdrawal exerts a dual effect on SK1 in these cells (Dayon et al., 2009). Thus, short-term androgen depletion induces SK1 activity inhibition whereas long-term removal of androgen results in a sustained up-regulation in SK1 expression and activity, which is associated with the progression of prostate cancer cells to androgen independence (Dayon et al., 2009). Significantly, inhibiting SK1 prevents the transition of prostate cancer cells to a hormone refractory state during chronic androgen depletion (Dayon et al., 2009), providing evidence of an instrumental role for SK1 in this process.

In contrast with SK1, an interaction between SK2 and AR signalling in prostate cancer cells has not been reported in the literature.

Having established that SK inhibitors induce a chemical knock-down of SK1/2 (Chapter 3; Chapter 4; Chapter 5), these agents were employed to investigate the effects of SK inhibition/down-regulation on AR expression in LNCaP and LNCaP-AI cells.

## 6.2 Results

### 6.2.1 Analysis of AR expression in LNCaP and LNCaP-AI cells

The expression of AR protein was examined in LNCaP and LNCaP-AI cells by western blotting analysis, which confirmed that both cell lines express AR (Figure 6.1a). AR protein levels in LNCaP-AI cells were higher than those in the parental cell line (25±17% increase *vs* LNCaP cells, p value <0.001), indicating that the transition of LNCaP cells to androgen independence is associated with a modulation of AR expression.

In addition to full-length AR, which has a molecular mass of 110 kDa, the anti-AR antibody that was used for western blotting analysis detected an additional immunoreactive band, with an apparent molecular mass of ~100 kDa (Figure 6.1a). This band might correspond to a truncated isoform of AR, although its identity needs to be confirmed using an AR specific siRNA. SK inhibitors had a similar effect on the expression levels of full-length AR and of this putative AR truncated variant (see below), and the AR annotation is therefore used herein to indicate both.

### 6.2.2 Effect of SKi on AR expression in LNCaP and LNCaP-AI cells

The treatment of LNCaP and LNCaP-AI cells with SKi induced a substantial reduction in AR protein levels (Figure 6.1a; supplementary data, Figures S9, S10), an effect that was detectable within 8 hours of treatment with the inhibitor (Figure 6.5a; supplementary data, Figure S9).

The change in AR protein expression in LNCaP cells treated with SKi was associated with a significant reduction in AR mRNA levels (70±5% decrease *vs* control, p value <0.05), as assessed by quantitative Real Time-PCR (Figure 6.1b), indicating that SKi affects *AR* gene expression. However, the effect of SKi on AR protein expression persisted in LNCaP cells pre-treated with the protein synthesis inhibitor cycloheximide (Figure 6.2; supplementary data, Figure S9).

The effect of siRNA-mediated SK1 silencing on AR expression was also assessed. As shown in Figure 6.3, a 72 hours treatment with SK1 siRNA, which knocks-down SK1a in LNCaP and LNCaP-AI cells without however reducing SK1b expression (Chapter 3), had no significant effect on AR protein levels in the two cell lines (SK1 siRNA *vs* scrambled siRNA, p value >0.05 in both cell lines).

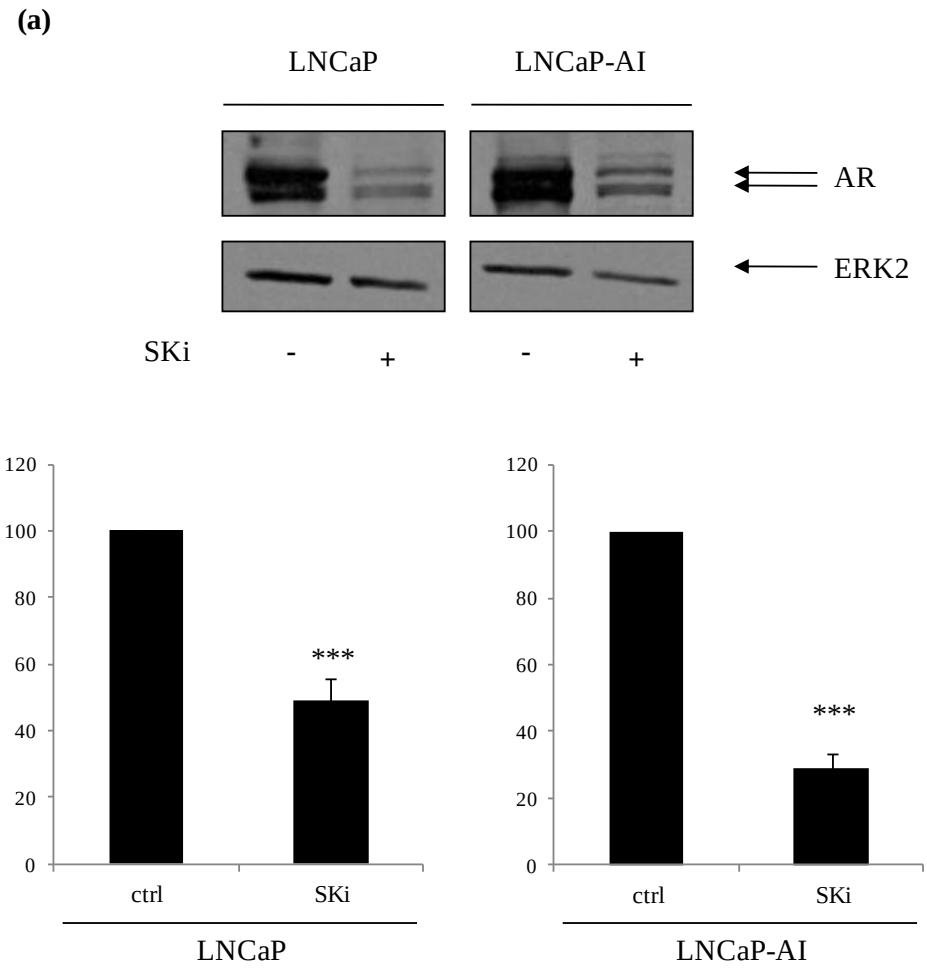



**Figure 6.1** ***Effect of SKi on AR expression in LNCaP and LNCaP-AI cells.*** *(a) Western blotting analysis of AR protein levels in LNCaP and LNCaP-AI cells treated for 48 hours with SKi (10 μM). Blots were then stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of at least three separate experiments. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). *** denotes a p value <0.001. (b) Quantitative Real Time-PCR analysis of AR mRNA levels in LNCaP cells treated with SKi (10 μM, 24 hours) or with the vehicle (DMSO 0.1% v/v, ctrl). GAPDH was used as a reference gene. Data are expressed as relative AR mRNA expression, normalized to GAPDH mRNA levels. Bars represent mean of triplicate values ± S.E. and are expressed as % of control (vehicle-treated cells). * denotes a p-value <0.05 compared with control (vehicle-treated cells).*

**(b)**

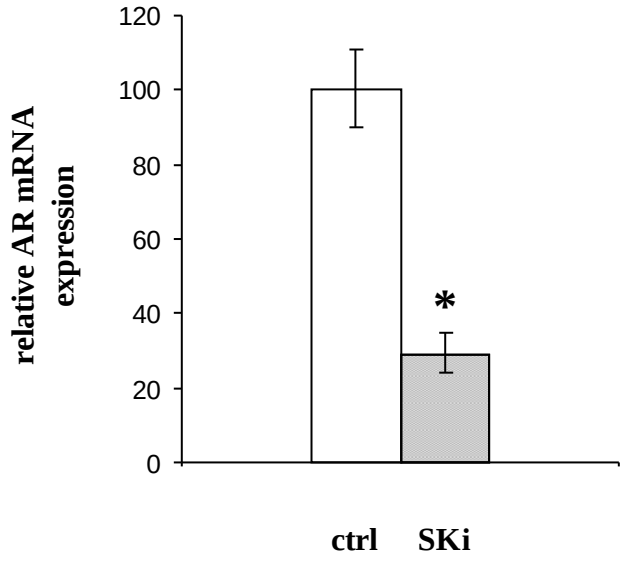



**Figure 6.1** ***Effect of SKi on AR expression in LNCaP and LNCaP-AI cells (continued).***

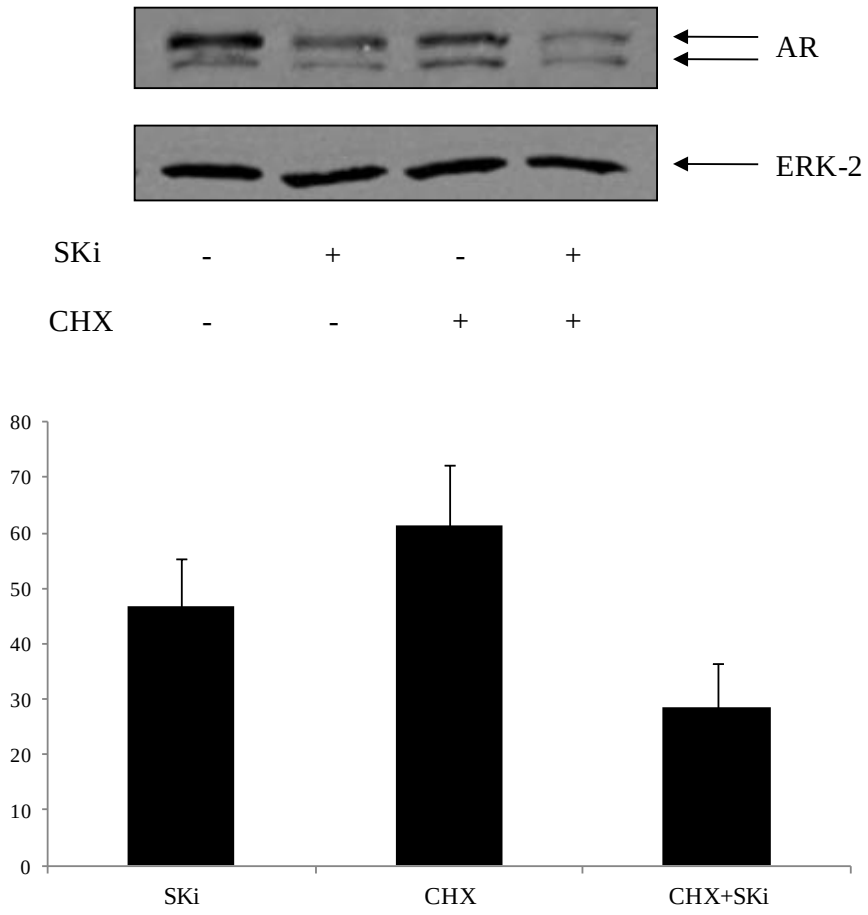



**Figure 6.2** ***Effect of protein synthesis inhibition on SKi-induced down-regulation of AR in LNCaP cells.*** *Cells were incubated for 30 minutes with the protein synthesis inhibitor cycloheximide (CHX) (5 µg/ml, 30 minutes pre-treatment) before being treated with SKi (10 µM, 24 hours). AR protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001; CHX vs control, <0.01; CHX+SKi vs SKi, N.S. N.S. denotes not statistically significant (p value >0.05).*

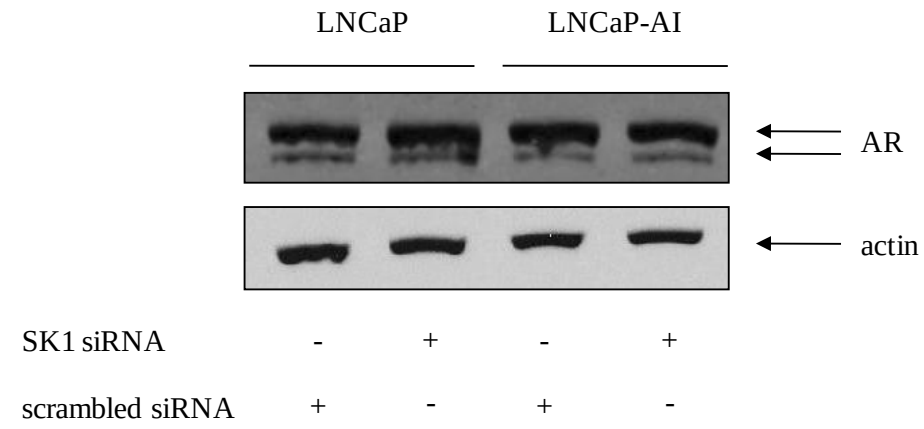



**Figure 6.3** ***Effect of transfection with siRNA oligonucleotides targeting SK1 on AR expression in LNCaP and LNCaP-AI cells.*** *Western blotting analysis of AR levels in LNCaP and LNCaP-AI cells transfected with siRNA oligonucleotides targeting SK1 (200 nM) or with scrambled siRNA oligonucleotides (200 nM) for 72 hours. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Samples used for western blotting analysis are the same as for Figure 3.25b.*

Having established that SKi reduces AR expression in prostate cancer cells, the study focused on characterising the mechanism involved.

Pharmacological inhibition of the executioner caspase-3 and -7 using Ac-DEVD-CHO did not block AR down-regulation in response to SKi (Figure 6.4a), nor did the caspase-2 inhibitor ICH-1 (Figure 6.4b).

Since SKi promotes the degradation of SK1 and other proteins through the proteasomal pathway in LNCaP and LNCaP-AI cells (Chapter 3), the involvement of this proteolytic route in SKi-induced down-regulation of AR was investigated, using the proteasomal inhibitor MG132.
In line with previous reports (Lin et al., 2002; Yang et al., 2008), MG132 itself induced a reduction in AR protein levels in LNCaP cells treated for 48 hours with this compound (not shown). A short time-point (8 hours) was therefore chosen for these experiments, where AR expression was still down-regulated by SKi, without however being affected by MG132 alone. MG132 did not inhibit the effect of SKi on AR protein levels in LNCaP cells while it reversed SKi-induced down-regulation of c-Myc under the same conditions (Figure 6.5a; supplementary data, Figure S9), indicating that MG132 was effectively inhibiting the proteasome in these experiments.

Pre-treatment with the cathepsin B inhibitor CA074Me also failed to reverse SKi-induced down-regulation of AR in LNCaP cells (Figure 6.5b), thereby excluding a role for the lysosomal degradation pathway in mediating AR proteolysis.

Since AR contains a calpain cleavage site and is a substrate for calpain-catalysed proteolysis (Pelley et al., 2006; Libertini et al., 2007), a role for this protease in mediating the degradation of AR in response to SKi was tested. As shown in Figure 6.5c, pre-treatment of LNCaP cells with the calpain 1/2 inhibitor PD150606 did not reverse SKi effect on AR expression.

AR has also been reported to be a substrate for serine protease-catalysed cleavage (de Boer et al., 1987). However, the serine protease inhibitor phenylmethylsulfonyl fluoride did not block AR down-regulation in SKi-treated LNCaP cells (Figure 6.5d).

A recent study has shown that the activation of ERK-1/2 leads to AR mRNA and protein down-regulation in LNCaP cells (Hong et al., 2011), demonstrating a role for this pathway in modulating AR expression in prostate cancer cells. Since SKi induces ERK-1/2 activation in LNCaP cells (Chapter 3), a role for this signalling cascade in mediating AR down-regulation was tested. For this purpose, LNCaP cells were pre-treated with the MEK inhibitor PD98059, which completely abrogates SKi-induced ERK-1/2 phosphorylation in these cells (Chapter 3). However, the effect of SKi on AR protein expression in LNCaP cells persisted in the presence of PD98059 (Figure 6.6). This is consistent with the finding that SKi reduces AR levels in LNCaP-AI cells, where ERK-1/2 is not activated in response to the treatment with this inhibitor (Chapter 3).

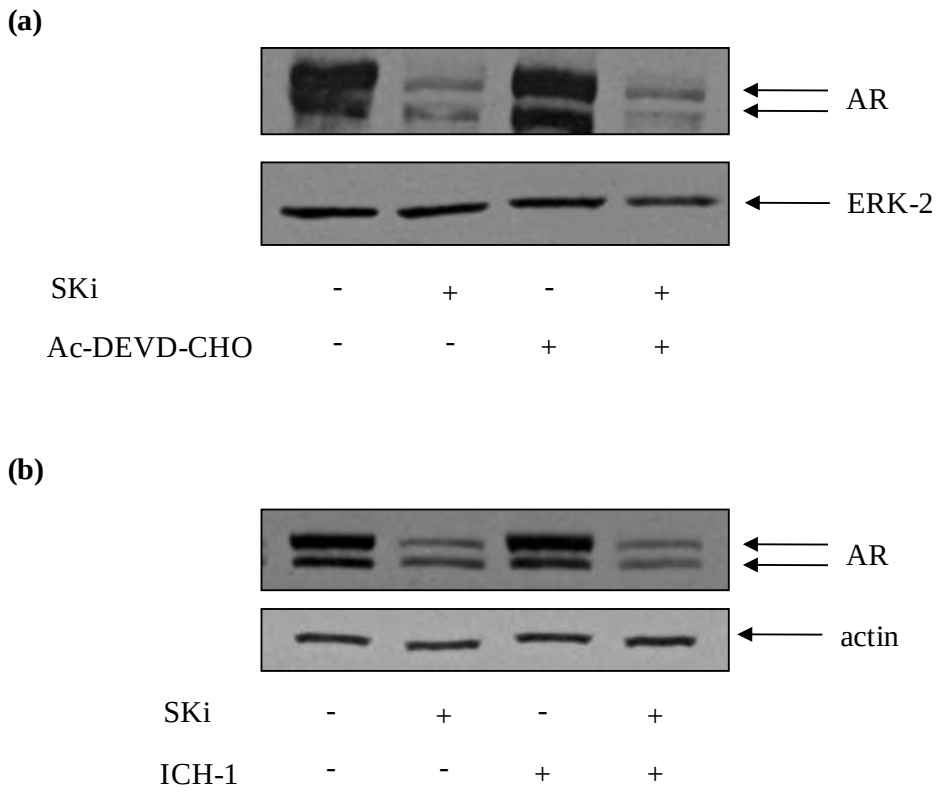



**Figure 6.4** ***Effect of caspase inhibitors on SKi-induced down-regulation of AR in LNCaP cells.*** *Western blotting analysis of AR expression in lysates from (a) cells incubated for 30 minutes with the caspase-3/7 inhibitor Ac-DEVD-CHO (100 μM) before being treated for 48 hours with SKi 10 μM; (b) cells incubated with the caspase-2 inhibitor ICH-1 (40 μM, 1 hour pre-treatment) before being treated with SKi (10 μM, 24 hours). Blots were then stripped and re-probed with anti-ERK-2 or anti-actin antibodies to ensure comparable protein loading. Results are representative of three separate experiments.*

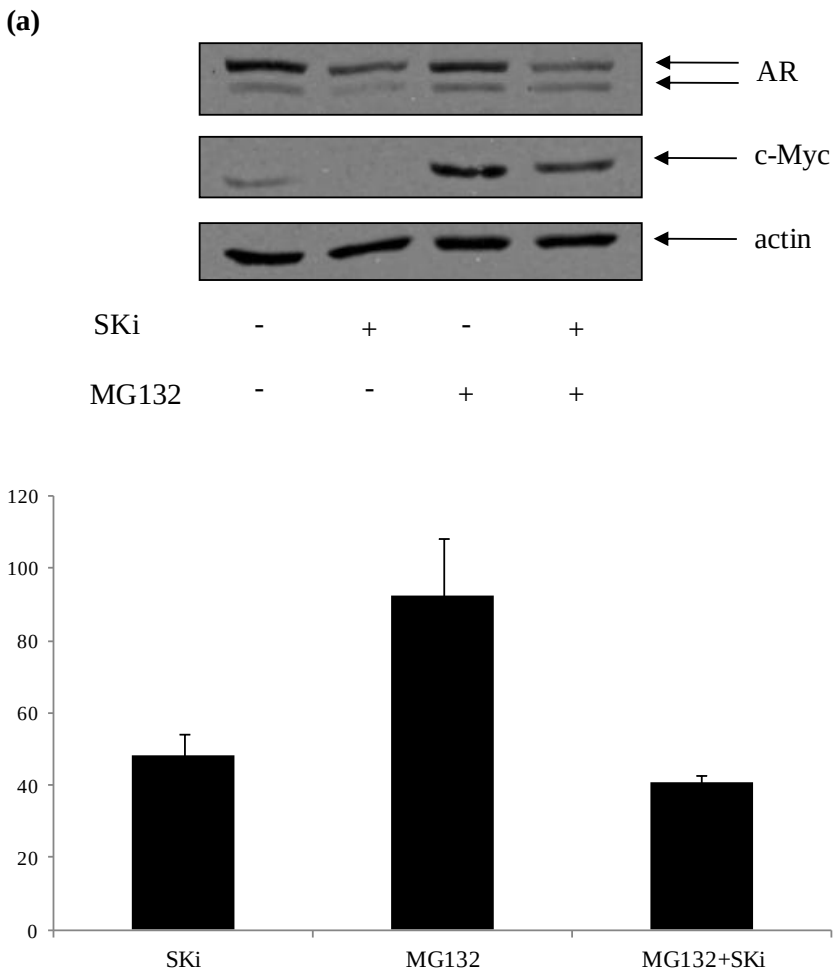



**Figure 6.5** ***Effect of the inhibition of various proteolytic pathways on SKi-induced downregulation of AR expression in LNCaP cells.*** *Western blotting analysis of AR expression in lysates from: (a) cells incubated with the proteasomal inhibitor MG132 (10 μM, 30 minutes pre-treatment) before being treated with SKi (10 μM, 8 hours). Also shown is a western blot showing c-Myc expression under these conditions. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001; MG132 vs control, N.S.; MG132+SKi vs SKi, N.S. N.S. denotes not statistically significant (p value >0.05). (b) cells incubated with the cathepsin B inhibitor CA074Me (10 μM, 30 minutes pre-treatment) before being treated with SKi (10 μM, 48 hours); (c) cells incubated with the calpain inhibitor PD150606 (250 μM, 4 hours pre-treatment) before being treated with SKi (10 μM, 20 hours). (d) cells incubated with the serine proteases inhibitor phenylmethylsulphonyl fluoride (PMSF) (1 mM, 4 hours pre-treatment) before being treated with SKi (10 μM, 20 hours). Blots were then stripped and re-probed with anti-ERK-2 or anti-actin antibodies to ensure comparable loading between samples. Results are representative of three separate experiments.*

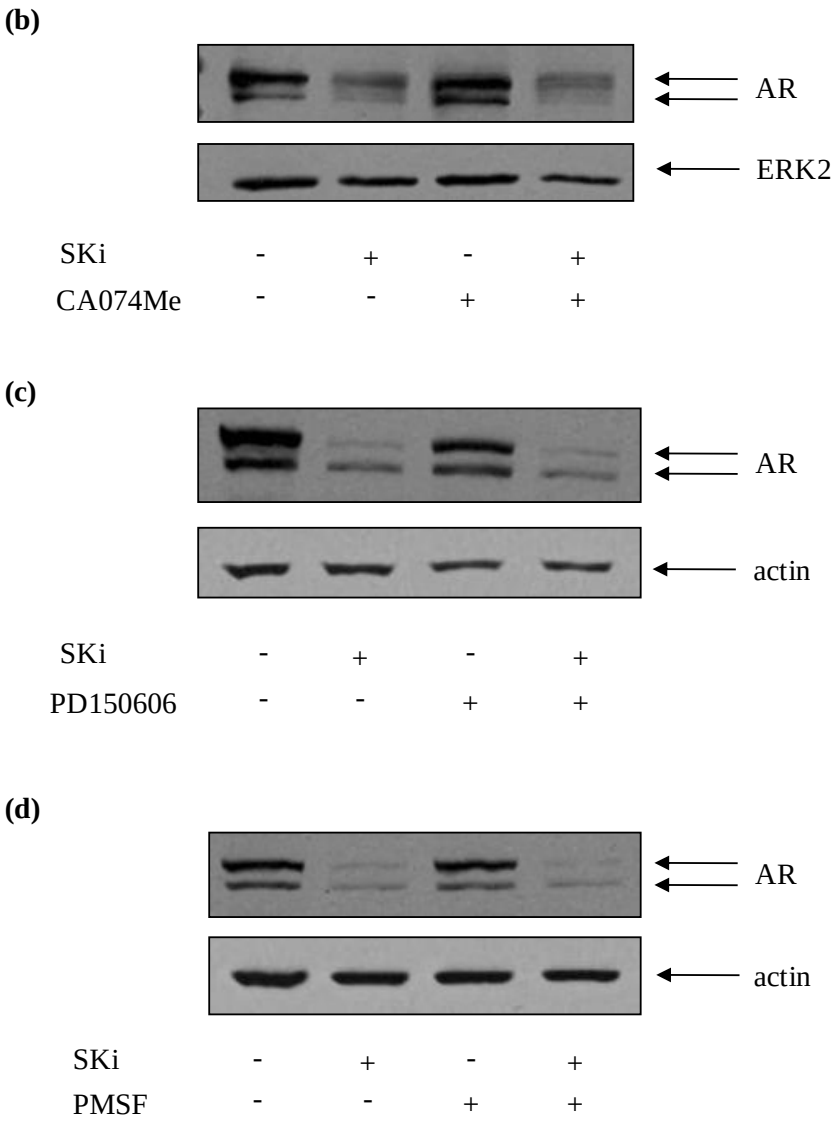



**Figure 6.5** ***Effect of the inhibition of various proteolytic pathways on SKi-induced downregulation of AR expression in LNCaP cells (continued).***

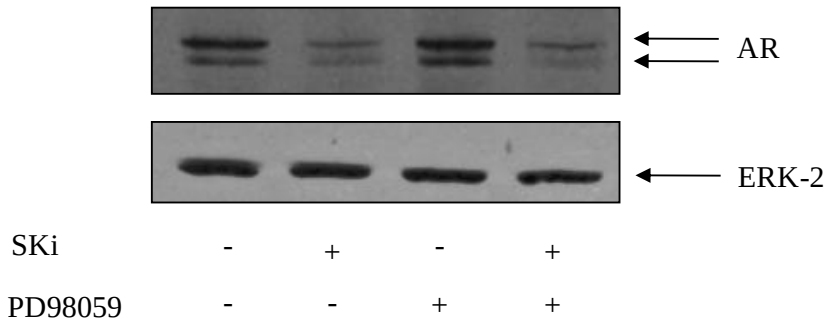



**Figure 6.6** ***Effect of MEK inhibition on SKi-induced down-regulation of AR in LNCaP cells.*** *Cells were incubated for 30 minutes with PD98059 (10 μM), before being treated with SKi (10 μM, 48 hours). AR protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-ERK-2 antibody to ensure comparable protein loading. Results are representative of three separate experiments.*

The effect of SKi on AR expression in LNCaP cells might be due to the inhibition/down-regulation of SK1, which results in an increase in ceramide and a decrease in S1P intracellular levels (Chapter 3; Loveridge et al., 2010).

In order to test a role for ceramide in mediating SKi-induced down-regulation of AR expression, LNCaP cells were treated with a cell permeable analogue of ceramide, C2-ceramide. C2-ceramide reduced AR protein levels, an effect that was detectable within 8 hours of treatment (Figure 6.7; supplementary data, Figure S9). The down-regulation of AR expression induced by C2-ceramide was reversed by pre-treating cells with the proteasome inhibitor MG132 (Figure 6.7b; supplementary data, Figure S9), indicating that C2-ceramide promotes AR degradation via the proteasomal pathway.

It was then assessed whether inhibiting the SKi-induced increase in intracellular ceramide levels could prevent the down-regulation of AR expression.
Pre-treatment of LNCaP cells with fumonisin B1, which inhibits ceramide synthase and therefore the back-conversion of sphingosine to ceramide, did not reverse the reduction in AR protein levels promoted by SKi (Figure 6.8b). Additionally, inhibition of serine palmitoyltransferase with myriocin failed to reverse the SKi-induced down-regulation of AR in LNCaP cells (Figure 6.8a), excluding a role for *de novo*-synthesized ceramide in mediating SKi effect on AR expression.

To test the possibility that AR down-regulation is due to a reduction in S1P, LNCaP cells were co-treated with S1P and SKi. Figure 6.8c shows that the addition of exogenous S1P to the cells did not rescue the SKi-induced down-regulation of AR.

Since the treatment of LNCaP cells with SKi also results in increased intracellular levels of dihydrosphingosine and of various dihydroceramide species (Loveridge et al., 2010), the effect of these sphingolipids on AR expression was tested. However, neither C2-dihydroceramide, a cell permeable analogue of dihydroceramide, nor dihydrosphingosine significantly reduced AR expression in LNCaP cells (control *vs* treated cells, p value >0.05 for both treatments) (Figure 6.7a).

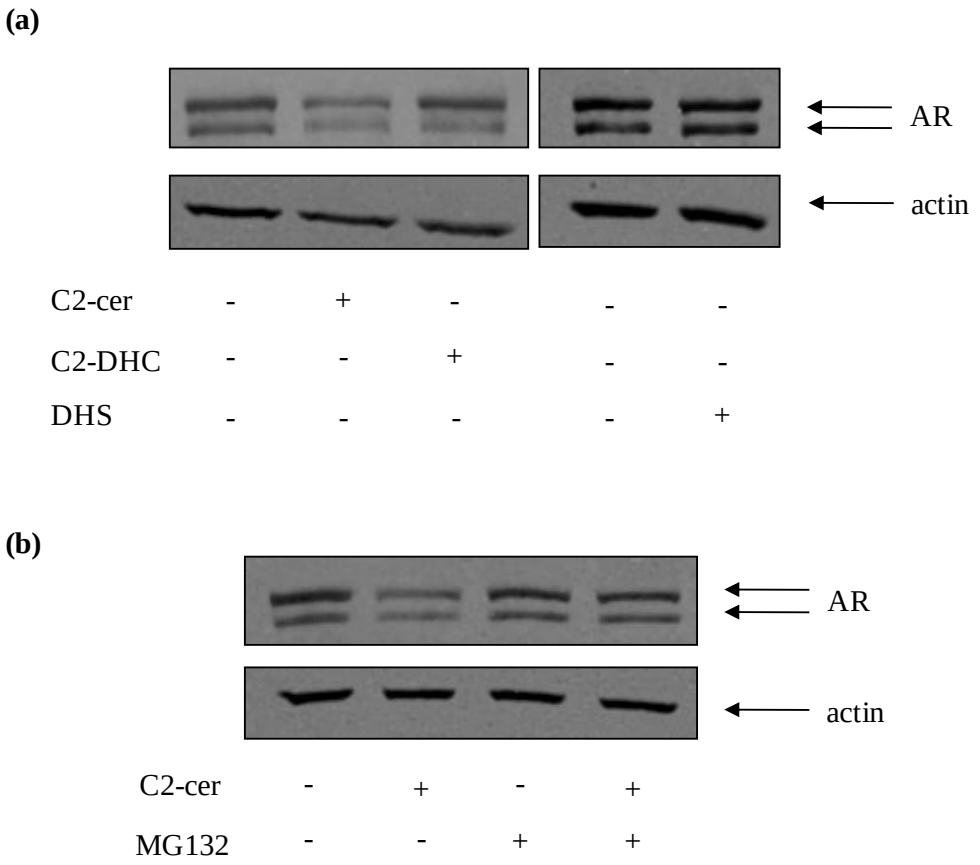



**Figure 6.7** ***Effect of C2-ceramide, C2-dihydroceramide and dihydrosphingosine on AR expression in LNCaP cells.*** *(a) Cells were treated for 24 hours with C2-ceramide (C2-cer, 50 µM), C2-dihydroceramide (C2-DHC, 50 µM), or dihydrosphingosine (DHS, 25 µM) and AR protein levels were measured by western blotting analysis. (b) Western blot showing the effect of the proteasome inhibitor MG132 (10 µM, 30 minutes pre-treatment) on C2-ceramide (C2-cer) (50 µM, 8 hours)-induced down-regulation of AR in LNCaP cells. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: C2-ceramide (24h) vs control, <0.001; C2-ceramide (8h) vs control, <0.001; MG132 (8h) vs control, N.S.; MG132+C2-ceramide vs C2-ceramide, <0.01. N.S. denotes not statistically significant (p value >0.05).*

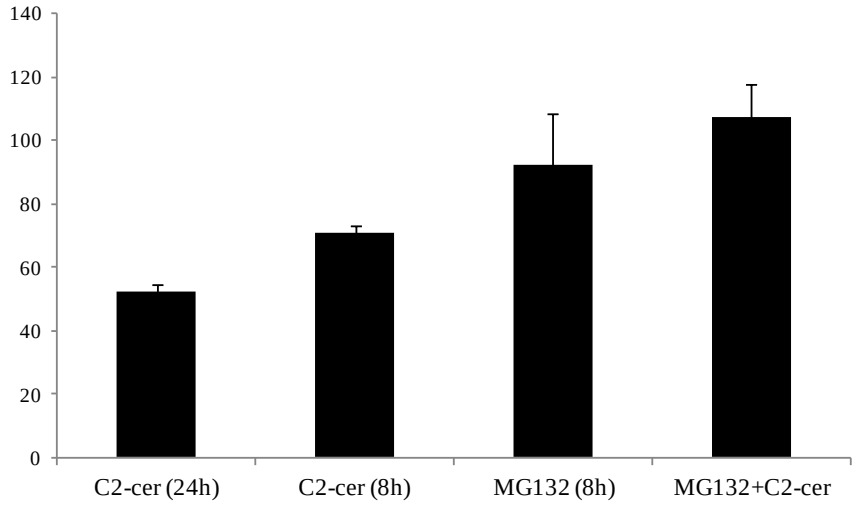



**Figure 6.7** ***Effect of C2-ceramide, C2-dihydroceramide and dihydrosphingosine on AR expression in LNCaP cells (continued).***

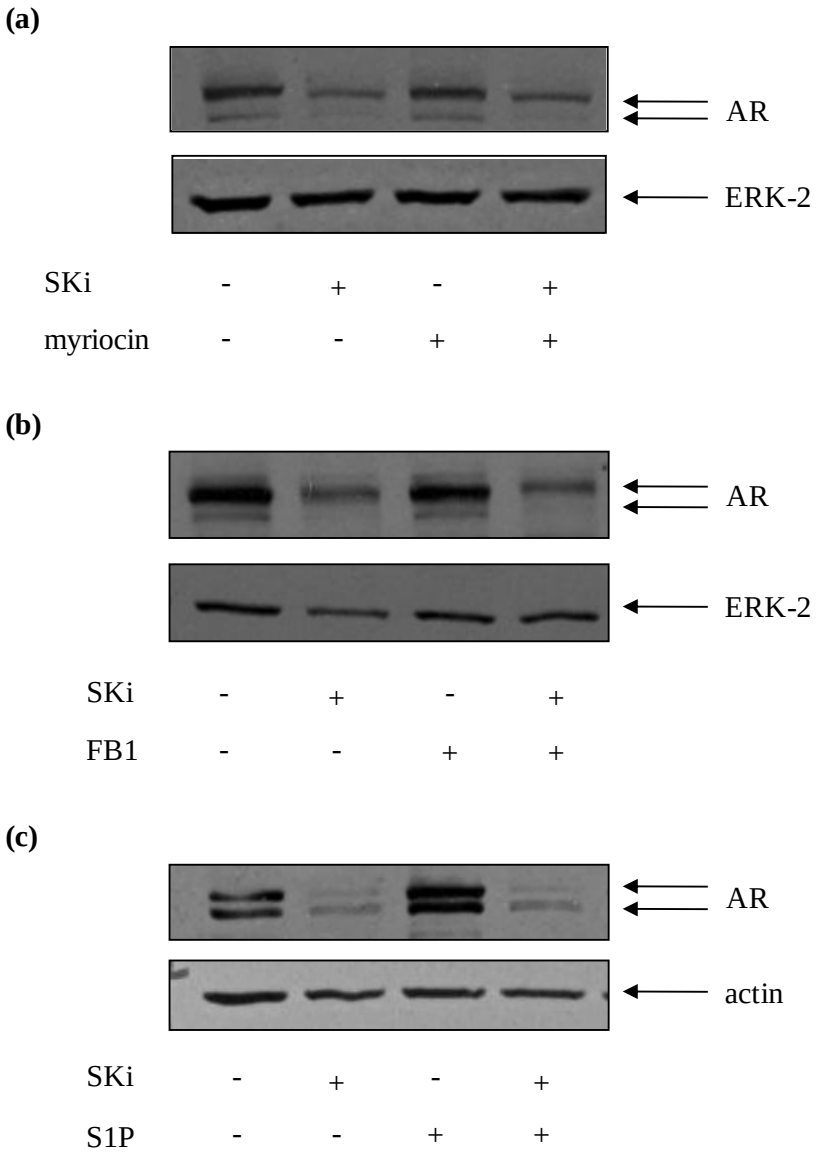



**Figure 6.8** ***Effect of ceramide synthesis inhibition or exogenous S1P on SKi-induced down-regulation of AR in LNCaP cells.*** *Western blots showing the effect of SKi (10 μM, 24 hours) on AR protein levels in LNCaP cells pre-treated with (a) myriocin (100 nM, 2 hours pre-treatment) or (b) fumonisin B1 (FB1) (100 μM, 1 hour pre-treatment), to inhibit the de novo ceramide synthesis or the sphingosine recycling pathway, respectively. (c) Western blot showing the effect of exogenously added S1P (10 μM, 48 hours) on AR expression in cells treated with SKi (10 μM, 48 hours). Blots were then stripped and re-probed with anti-ERK-2 or anti-actin antibodies to ensure comparable loading. Results are representative of two/three separate experiments.*

### 6.2.3 Role of oxidative stress response in SKi-induced AR down-regulation

Fajardo and co-workers demonstrated the oxidative stress-induced down-regulation of AR expression in several prostate cancer cell lines, including LNCaP cells (Fajardo et al., 2011). A mass spectrometry-based metabolome analysis revealed that chronic treatment of LNCaP and LNCaP-AI cells with SKi leads to a pronounced oxidative stress response (see below). Therefore, it was of interest to determine whether the down-regulation of AR expression is linked to the activation of oxidative stress by SKi. For this purpose, LNCaP cells were pre-treated with the reactive oxygen species (ROS) scavenger, N-acetyl L-cysteine (NAC), before being challenged with SKi. SKi-induced down-regulation of AR protein levels was partially but significantly reversed by NAC (Figure 6.9; supplementary data, Figure S9), indicating that the effect of SKi on AR expression is mediated by a ROS-dependent pathway. Higher concentrations of NAC were tested, but could not be used because they resulted in cytotoxicity (data not shown).

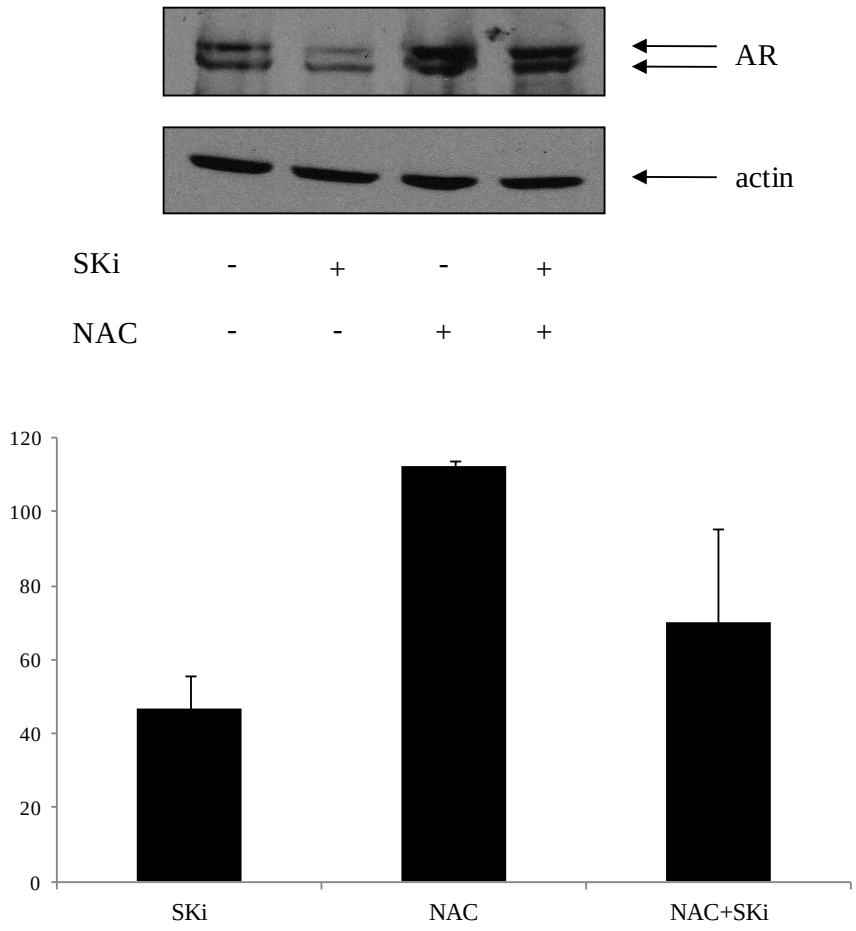



**Figure 6.9** ***Effect of ROS scavenging on SKi-induced down-regulation of AR expression in LNCaP cells.*** *Cells were treated with SKi (10 µM, 24 hours) in the absence or presence of the ROS scavenger N-acetyl L-cysteine (NAC) (10 mM, 1 hour pre-treatment). AR expression was measured by western blotting analysis. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: SKi vs control, <0.001; NAC vs control, N.S.; NAC+SKi vs SKi, <0.05. N.S. denotes not statistically significant (p value >0.05).*

Alimirah and colleagues demonstrated that p53 negatively regulates AR expression in LNCaP cells (Alimirah et al., 2007). Additionally, p53 modulates ROS generation in mammalian cells (Liu et al., 2008). In particular, the accumulation of p53 leads to the activation of oxidative stress (Liu et al., 2008). Therefore, in an attempt to interrogate a role for p53 in the ROS-dependent AR down-regulation induced by SKi, the effect of SKi treatment on p53 expression in LNCaP and LNCaP-AI cells was assessed.

Both LNCaP and LNCaP-AI cells express p53 (Figure 6.10). As expected for a protein which is rapidly turned-over through the ubiquitin-proteasomal pathway (Maki et al., 1996), treatment with MG132 led to a marked increase in p53 levels in both LNCaP and LNCaP-AI cells (Figure 6.10), in line with previous studies showing that p53 accumulates in cells treated with proteasome inhibitors (Maki et al., 1996). Therefore, lysates from LNCaP or LNCaP-AI cells treated with MG132 were used as positive controls when immunoblotting for p53.

Treatment of LNCaP-AI cells with SKi induced a substantial increase in p53 expression (Figure 6.10). In contrast, SKi failed to increase p53 levels in LNCaP cells (Figure 6.10), while it reduced AR expression under the same conditions (Figure 6.1a; Figure 6.2). These findings indicate that p53 might not be involved in the regulation of AR expression by SKi.

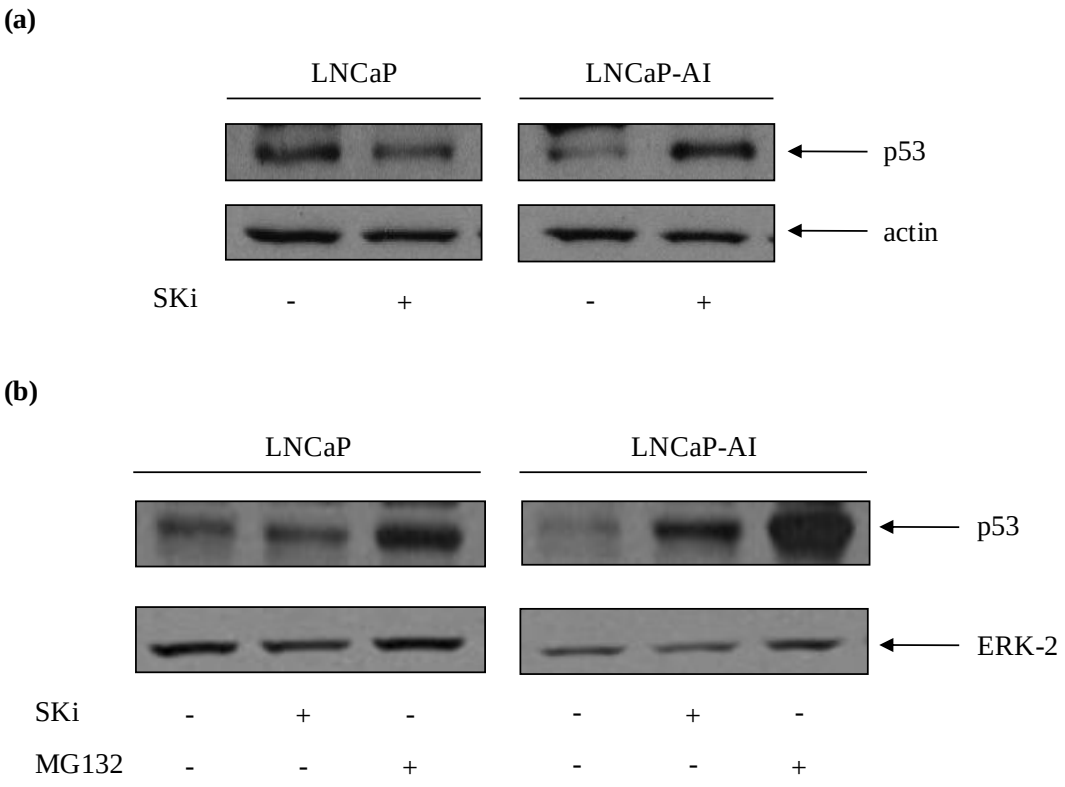



**Figure 6.10** ***Effect of SKi on p53 protein levels in LNCaP and LNCaP-AI cells.*** *Western blotting analysis of p53 expression in LNCaP and LNCaP-AI cells treated with SKi (10 μM) for 24 (a) or 48 (b) hours. For each cell line, lysates from cells treated with MG132 (10 μM, 48 hours) were used as a positive control. Blots were stripped and re-probed with anti-ERK-2 or anti-actin antibodies to ensure comparable protein loading. Results are representative of two/three separate experiments.*

### 6.2.4 Effect of FTY720 and (*S*)-FTY720 vinylphosphonate on AR expression in LNCaP-AI cells

Having established that the chronic treatment with SKi reduces AR protein levels in LNCaP and LNCaP-AI cells, it was assessed whether different SK1 inhibitors, structurally unrelated to SKi, have the potential to modulate AR expression in prostate cancer cells. In particular, we tested the effect of FTY720 and (*S*)-FTY720 vinylphosphonate on AR expression in LNCaP-AI cells, since these inhibitors down-regulate SK1 in these cells (Chapter 4).

Both FTY720 and (*S*)-FTY720 vinylphosphonate reduced AR protein levels in LNCaP-AI cells (Figure 6.11; supplementary data, Figure S10).

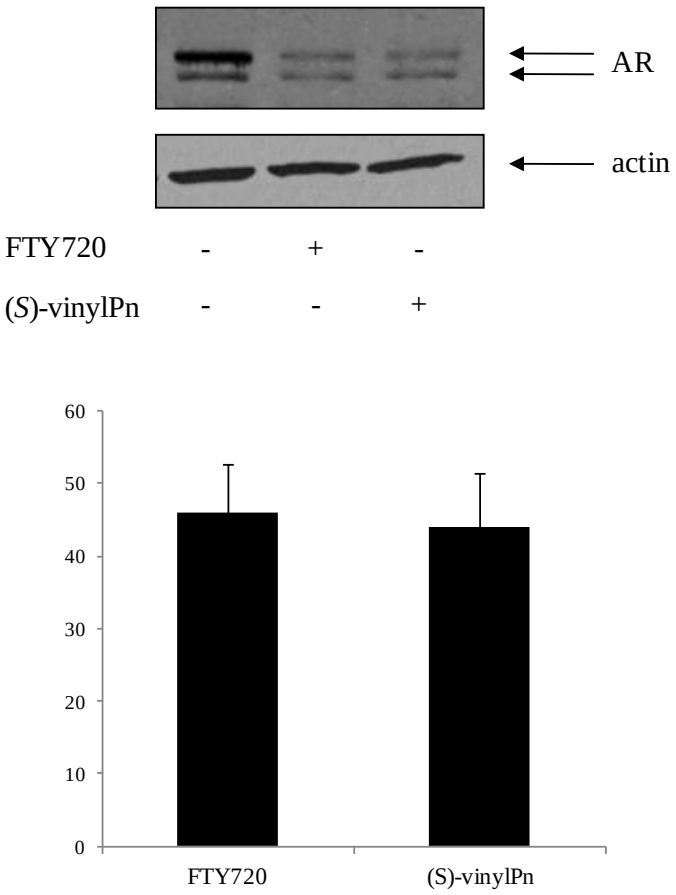



**Figure 6.11** ***Effect of FTY720 and (S)-FTY720 vinylphosphonate on AR expression in LNCaP-AI cells.*** *Western blot showing the effect of FTY720 or (S)-FTY720 vinylphosphonate ((S)-vinylPn) (both 10 μM, 48 hours) on AR protein levels in LNCaP-AI cells. Blots were then stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of three separate experiments. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). p values were as follows: FTY720 vs control, <0.001; (S)-FTY720-vinylphosphonate, <0.001.*

### 6.2.5 Effect of a SK2-specific inhibitor on AR expression in LNCaP cells

To investigate whether SK2 has a role in regulating AR expression in prostate cancer cells, an SK2-selective inhibitor, (*R*)-FTY720-OMe, was used.

(*R*)-FTY720-OMe did not significantly reduce AR protein levels in LNCaP cells (Figure 6.12; supplementary data, Figure S9), while it completely down-regulated ectopically expressed SK2 in these cells under the same conditions (Chapter 5). Higher concentrations of (*R*)-FTY720-OMe reduced AR protein levels (Figure 6.12) without however further down-regulating SK2 expression (Chapter 5), suggesting that the effect of this inhibitor on AR expression is independent of SK2.

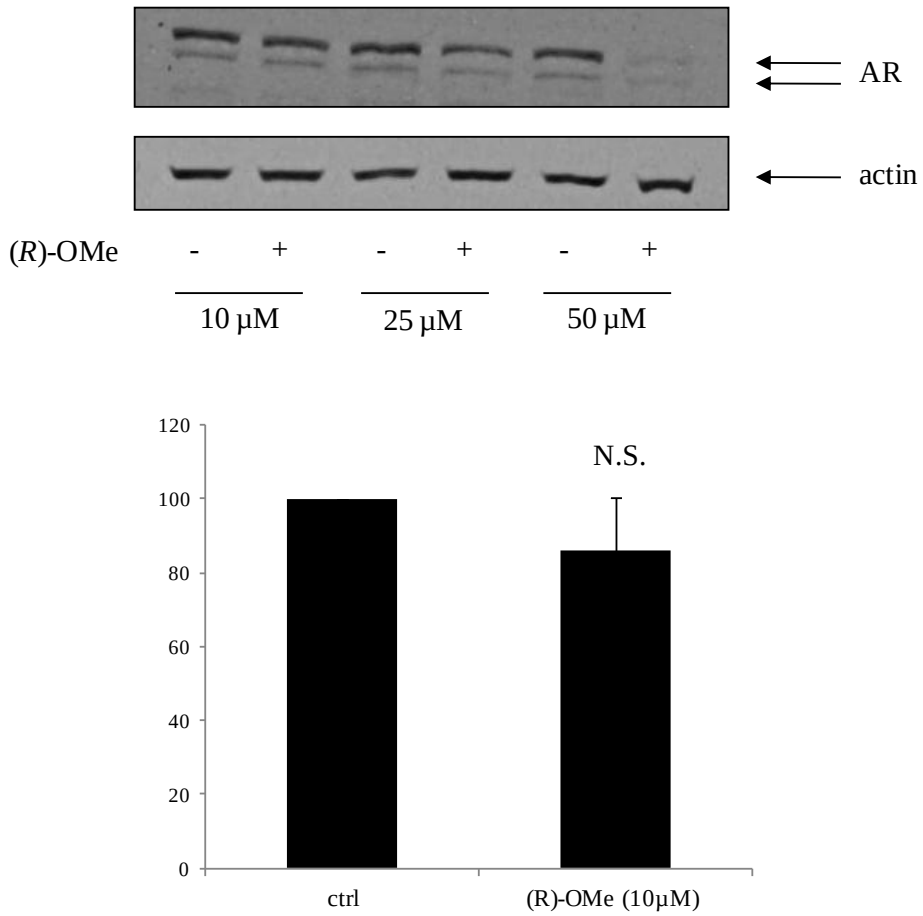



**Figure 6.12** ***Effect of (R)-FTY720-OMe on AR expression in LNCaP cells.*** *Cells were treated for 24 hours with the indicated concentrations of (R)-FTY720-OMe ((R)-OMe). AR protein levels were then measured by western blotting analysis. Blots were stripped and re-probed with anti-actin antibody to ensure comparable protein loading. Results are representative of two/three separate experiments. Also shown is the densitometric quantification of AR immunoreactivity, expressed as a percentage of the control (DMSO) (ctrl=100%). N.S. denotes not statistically significant (p value >0.05).*

## 6.3 Discussion

### 6.3.1 AR protein expression is up-regulated in LNCaP-AI cells compared with LNCaP cells

This study confirms that LNCaP and LNCaP-AI cells express AR and shows that AR protein levels are up-regulated in LNCaP-AI cells compared with LNCaP cells, as assessed by western blotting analysis. Similarly, other studies reported an up-regulation of AR expression in LNCaP cells that have progressed to androgen-independence following long-term culture in androgen deprivation conditions (Kokontis et al., 1994; Halkidou et al., 2003), which is accompanied by an increase in AR transcriptional activity upon androgen stimulation (Kokontis et al., 1994). Thus, AR up-regulation might represent a compensatory mechanism that allows LNCaP cells to adapt to a hormone-deprived environment by increasing their sensitivity to androgens (Linja et al., 2001). Additionally, it has been shown that the up-regulation of AR decreases the specificity of this receptor for its ligand, allowing its activation by other steroids (Chen et al., 2004). Furthermore, it has been reported that high expression of AR results in a ligand-independent activation of this receptor (Huang et al., 2002).

The molecular mechanism underlying the up-regulation of AR protein expression in LNCaP-AI cells compared with LNCaP cells remains to be investigated. In this regard, Kokontis and colleagues demonstrated an increase in AR mRNA levels in LNCaP cells subjected to androgen withdrawal (Kokontis et al., 1994). It is therefore likely that the up-regulation of AR protein observed in LNCaP-AI cells used in the current study is also the result of an increase in the levels of the corresponding mRNA.

Among the molecules that positively regulate *AR* gene expression, cyclic adenosine monophosphate (cAMP) (acting via the transcription factor cAMP response element-binding protein) and Myc have been shown to be up-regulated in LNCaP cells that have progressed to androgen independence (Mizokami et al., 1994; Dayon et al.,

2009; Lee et al., 2009; Kokontis et al., 1994; Karan et al., 2002). It would be interesting to test whether these molecules mediate the increase in AR expression in LNCaP-AI cells.

Additionally, the data presented in this study show that the up-regulation of AR in LNCaP-AI cells compared with LNCaP cells correlates with increased expression of SK1. The finding the SK1 inhibitors down-regulate AR in LNCaP and LNCaP-AI cells is consistent with the hypothesis that SK1 modulates AR expression in these cells. In order to test a role for SK1 in driving AR up-regulation, it will be necessary to assess whether SK1 over-expression in LNCaP cells results in an increase in AR protein levels.

Numerous studies have shown that AR is up-regulated in most hormone-refractory prostate cancers compared with hormone-dependent cancers (Van der Kwast et al., 1991; Ruizeveld de Winter et al., 1994; Latil et al., 2001; Linja et al., 2001; Edwards et al., 2003). Thus, in addition to their ability to grow in the absence of androgens and their responsiveness to hormone stimulation, LNCaP-AI cells share another common feature with hormone-refractory tumour cells that originate in patients following androgen ablation therapy, i.e. the over-expression of AR. These characteristics support the relevance of LNCaP-AI cells as an *in vitro* model for androgen-independent prostate cancer. In contrast, PC-3 and DU145 cells do not express AR (Tilley et al., 1990; Trapman et al., 1990) and therefore their behaviour might not resemble the human tumours.

### 6.3.2 SKi activates oxidative stress in LNCaP and LNCaP-AI cells

Treatment of LNCaP and LNCaP-AI cells with SKi modulates the metabolome, as assessed by the detection of changes in the intracellular levels of various metabolites measured using liquid chromatography-mass spectrometry (LC-MS) (in collaboration with Dr D. Watson, University of Strathclyde; unpublished data) (Supplementary data, Table 1). In particular, SKi induces a pronounced oxidative stress response in these cells, as indicated by marked changes in the levels of

glutathione, NADPH, and various intermediates of the pentose phosphate and the glycolytic pathways.

LNCaP and LNCaP-AI cells treated with SKi show an increased ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH), which is indicative of oxidative stress. In fact, GSH functions as an antioxidant to remove hydrogen peroxide, thereby preventing the formation of free radicals and their potential damaging effects. Thus, GSSG is formed from the reaction between GSH and hydrogen peroxide, which is catalysed by glutathione peroxidase.

Treatment of LNCaP and LNCaP-AI cells with SKi results in a substantial decrease in NADPH levels, which is consistent with the activation of an oxidative stress response by SKi and which might occur via different mechanisms. First, NADPH functions as a reducing agent to remove hydrogen peroxide, in a reaction catalysed by catalase. Second, NADPH is needed to convert GSSG back into GSH by the action of glutathione reductase. Third, SKi treatment might activate NADPH oxidase, which utilizes NADPH to produce superoxide. This hypothesis is in line with a recent study reporting that genetic knock-down of SK1 promotes ROS generation in human carcinoma cell lines, an effect which is partially reversed by an NADPH oxidase inhibitor (Huwiler et al., 2011).

Treatment of LNCaP and LNCaP-AI cells with SKi leads to an increased flux through the pentose phosphate pathway, as indicated by the accumulation of phosphogluconate and ribulose 5-phosphate, an intermediate and an end-product, respectively, of this metabolic cascade. This is suggestive of an attempt by the cells to maintain NADPH levels using the pentose phosphate shunt. Indeed, the pentose phosphate pathway is a major source of cellular NADPH. The activation of the pentose phosphate shunt might be accomplished by enhanced glycolysis. Indeed, the levels of various intermediates of the glycolytic pathway (fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate), as well as the levels of S-lactoylglutathione, a derivative of methylglyoxal (a side product of this pathway), are elevated in cells treated with SKi. In this regard, it has been previously proposed that an increased

flux through the glycolytic pathway might protect cells against oxidative stress via the activation of the pentose phosphate pathway (Kondoh, 2008). Additionally, it has been hypothesized that enhanced glycolysis might be accompanied by a reduction in mitochondrial respiration, which would result in a decreased generation of ROS (Kondoh, 2008).
Interestingly, it has been shown that ROS up-regulate glycolytic activity in hepatoma cells, an affect that might be mediated by HIF1-α (Shi et al., 2009), providing a link between oxidative stress and glycolysis.

Similarly to LNCaP and LNCaP-AI cells, SKi modulates the intracellular levels of various metabolites and activates oxidative stress in MCF-7 breast cancer cells (unpublished data), providing evidence that SKi regulates the metabolome of cancer cells.

The activation of an oxidative stress response that we observed in SKi-treated LNCaP, LNCaP-AI and MCF-7 cells is recapitulated by SK1 genetic knock-down in various carcinoma cells (Huwiler et al., 2011), providing evidence that this does not represent an off-target effect of SKi. To further prove this, ongoing experiments are aimed at assessing whether forced over-expression of SK1 by stable transfection of LNCaP cells is protective against SKi-induced oxidative stress. Additionally, the effect of an SK2-specific inhibitor, (*R*)-FTY720-OMe, will be tested, to establish whether SKi modulates the metabolome by inhibiting/down-regulating SK2.

The mechanism by which SK1 knock-down leads to increased ROS formation is currently unknown. As mentioned above, the study by Huwiler et al. suggests that the activation of NADPH oxidase in cells were SK1 is down-regulated might be responsible for the onset of an oxidative stress response (Huwiler et al., 2011). Indeed, NADPH oxidase plays a major role in generating ROS.
The activation of oxidative stress in cells in which SK1 function is compromised, either by genetic knock-down or by pharmacological inhibition, might result from the disruption of the sphingolipid rheostat. In this regard, ceramide has a well established role in promoting ROS formation, either by activating ROS-generating

enzymes or by interacting with the mitochondrial electron transport chain (Li et al., 2010).

Importantly, the activation of oxidative stress in LNCaP and LNCaP-AI cells is linked with the effect of SKi on AR expression (see below).

### 6.3.3 SKi modulates AR expression in LNCaP cells via an oxidative stress-dependent mechanism

The data presented provide evidence that SKi reduces AR expression in LNCaP cells by activating an oxidative stress response, as proven by the demonstration that AR down-regulation in response to SKi is attenuated by the ROS scavenger N-acetylcysteine. The oxidative stress-mediated down-regulation of AR expression in prostate cancer cells has been recently reported by others, although the proteolytic pathway involved has not been defined (Fajardo et al., 2011).
It is important to appreciate that N-acetylcysteine might not be able to completely abrogate SKi-induced increase in ROS levels in LNCaP cells. Indeed, the metabolome analysis revealed that SKi treatment causes high levels of oxidative stress in LNCaP and LNCaP-AI cells (section 6.3.2). Thus, ROS levels in cells co-treated with SKi and N-acetylcysteine might be still sufficient to promote AR down-regulation to some extent and this might explain the partial recovery of AR expression in the presence of the ROS scavenger. To address this issue, it will be useful to assess whether pre-treatment with N-acetylcysteine can completely block ROS production in SKi-treated LNCaP cells, as well as to test whether the SKi effect on AR expression can be fully recovered by a different, more potent antioxidant.
Alternatively, the partial effect of N-acetylcysteine might suggest the involvement of additional mechanism(s), oxidative stress-independent, in mediating SKi-induced down-regulation of AR.

The oxidative stress-dependent pathway that mediates AR down-regulation in SKi-treated prostate cancer cells is currently unknown, although the data presented here exclude a role for lysosomal cathepsin B, calpain and serine proteases.

It has been reported that various apoptotic stimuli decrease AR protein levels in LNCaP cells (Yang et al., 2008). However, the finding that SKi reduces AR expression in LNCaP-AI cells, which do not undergo apoptosis upon treatment with this inhibitor (Chapter 3), suggests that AR down-regulation does not occur downstream of the activation of apoptotic cell death. Indeed, the pharmacological inhibition of executioner caspase-3 and -7 or caspase-2, which has been recently shown to have an important function in apoptosis (Bouchier-Hayes, 2010), failed to reverse the effect of SKi on AR expression. These results are consistent with the fact that AR does not contain consensus sequences for caspase cleavage, as assessed using the PeptideCutter on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

The proteasome inhibitor MG132 failed to reverse SKi-induced decrease in AR expression in LNCaP cells. It is important to appreciate that this finding does not conclusively exclude a role for the proteasomal pathway in mediating AR down-regulation. Indeed, the data presented in Chapter 3 provide evidence that SKi activates the proteasome, thereby partially countering the block of the proteasome by MG132. This results in an incomplete inhibition of this degradation pathway. Thus, the residual proteasome activity in LNCaP cells co-treated with SKi and MG132 might be sufficient to induce AR degradation. This possibility is further supported by the finding that p53 (which is processed through the ubiquitin-proteasome pathway: Maki et al., 1996) accumulates in LNCaP cells treated with MG132, while p53 levels in LNCaP cells co-treated with SKi and MG132 are not different than those in untreated cells (data not shown), indicating that the proteasome activity in these cells is (at least to some degree) restored.
Since ubiquitination represents the first step in protein processing via the proteasome system, investigating whether AR is ubiquitinated in response to SKi treatment may assist in understanding whether SKi promotes the proteasomal degradation of AR.

The change in AR protein expression upon treatment with SKi was associated with a reduction in AR mRNA as assessed by quantitative PCR. However, SKi appears to modulate AR protein expression principally via a post-translational mechanism as

the response persisted in cells treated with cycloheximide, which inhibits *de novo* protein synthesis. It is, therefore, possible that the down-regulation of AR protein expression in response to SKi reduces AR-dependent transcriptional regulation of the *AR* gene. This possibility is supported by studies showing that AR functions as a transcription factor to modulate the expression of *AR* gene, in an auto-regulatory loop. In particular, androgen treatment up-regulates AR mRNA and protein levels in PC-3 cells ectopically expressing AR (Dai et al., 1996).

A hypothesis that is being currently tested is that the accumulation of methylglyoxal in SKi-treated LNCaP and LNCaP-AI cells might be responsible for AR down-regulation. Indeed, methylglyoxal has been shown to reduce HIF-1α expression by promoting its polyubiquitination and proteasomal degradation (Bento et al., 2010), and it is therefore possible that other cellular proteins are similarly affected in response to methylglyoxal.

Interestingly, N-acetylcysteine forms a mercaptal with methylglyoxal that can be detected by LC-MS (Dr David Watson, personal communication). Thus, N-acetylcysteine might attenuate the effect of SKi on AR expression by "inactivating" methylglyoxal. In order to test this hypothesis, LC-MS analysis will be used to assess whether a mercaptal is formed in LNCaP cells co-treated with N-acetylcysteine and SKi. Additionally, it will be necessary to test whether methylglyoxal treatment down-regulates AR in LNCaP cells and whether the over-expression of glyoxilase I, to accelerate methylglyoxal elimination, inhibits SKi effect on AR expression.

### 6.3.4 Role of the sphingolipid rheostat in mediating SKi-induced AR down-regulation

As discussed in Chapter 3, treatment of LNCaP cells with SKi has a profound effect on sphingolipid levels, resulting in ceramide accumulation and S1P reduction as a consequence of the acute inhibition of SK1 activity and the subsequent proteasomal degradation of SK1. The SKi-induced down-regulation of AR in LNCaP cells, however, is not blocked by the ceramide synthase inhibitor fumonisin B1 nor by myriocin, which inhibits ceramide *de novo* synthesis, suggesting that ceramide that

accumulates as a consequence of SK1 inhibition/down-regulation is not involved in regulating AR expression. It is important to note, however, that the data presented in Chapter 3 provide evidence that fumonisin B1 does not completely block the back-conversion of sphingosine to ceramide in SKi-treated LNCaP cells. Thus, the intracellular levels of ceramide in cells co-treated with SKi and fumonisin B1 might be sufficient to promote AR down-regulation.

Although ceramide might not mediate SKi-induced down-regulation of AR, C2-ceramide induces an MG132-sensitive degradation of AR in LNCaP cells. This finding is consistent with the demonstration that C2-ceramide stimulates proteasome activity in these cells (Chapter 3). Indeed, AR is processed by the ubiquitin-proteasomal pathway under basal conditions (Sheflin et al., 2000; Lin et al., 2002) and C2-ceramide, by activating the proteasome, is likely to accelerate the rate at which AR is removed from cells.

It is also important to appreciate that the lack of effect of exogenously added S1P in restoring AR expression in SKi-treated LNCaP cells does not necessarily exclude the possibility that AR down-regulation is linked to the reduction in S1P induced by SKi. In fact, S1P might modulate AR expression by acting on intracellular targets and exogenous S1P, which enters cells only poorly, might not be able to reach them.

An alternative possibility is that AR expression is regulated by non-catalytic activities of SK1. Indeed, by acutely inhibiting SK1 activity and consequently down-regulating SK1 expression, SKi impairs both catalytic and non-catalytic functions of SK1. To address this issue, it would be useful to assess whether SK1 expression is reduced by an 8 hours treatment with SKi, which was found to down-regulate AR.
In this case, the lack of effect of fumonisin B1 in reversing SKi-induced down-regulation of AR might be explained by the finding that fumonisin B1 only partially restores the expression of SK1 in SKi-treated cells (Chapter 3). Thus, SK1 levels in LNCaP cells co-treated with fumonisin B1 and SKi might fall below a threshold required to prevent AR down-regulation.

Since SKi down-regulates AR in both LNCaP and LNCaP-AI cells, while SK1b is retained in LNCaP-AI cells under the same conditions (Chapter 3), it might be argued that AR expression is regulated by SK1a only. However, the finding that treatment with SK1 siRNA, which removes SK1a but not SK1b from LNCaP and LNCaP-AI cells (Chapter 3), does not result in AR down-regulation suggests that AR expression is likely to be modulated by both SK1a and SK1b activity/ expression levels.

SKi inhibits the activity and down-regulates the expression of both SK1 and SK2 (Chapter 3; Chapter 5). However, the finding that the SK2-specific inhibitor (*R*)-FTY720-OMe did not down-regulate AR in LNCaP cells when used at a concentration that eliminates SK2 from these cells indicates that this enzyme is not involved in the regulation of AR expression in prostate cancer cells and that the major effect of SKi on AR is via inhibition/down-regulation of SK1.

To definitively prove that SKi down-regulates AR by reducing SK1 activity/expression it will be necessary to test whether the effect of SKi on AR is reversed by enforced expression of SK1.

Antoon and colleagues have recently shown that SKi binds the estrogen receptor (ER), thereby inhibiting ER signalling in human breast cancer cells and consequently reducing cell viability, survival and proliferation (Antoon et al., 2011). In contrast with this "off-target" effect of SKi, however, the data presented in the current study provide evidence that SKi reduces AR expression through a different mechanism, i.e. by inducing ROS generation. Additionally, two other SK1 inhibitors, FTY720 and (*S*)-FTY720 vinylphosphonate, which are structurally dissimilar to SKi, reduce AR protein levels in LNCaP-AI cells, suggesting that the ability to modulate AR expression is a common feature of SK1 inhibitors. Significantly, (*S*)-FTY720 vinylphosphonate fails to down-regulate AR in LNCaP cells, and this is correlated with the lack of effect of this inhibitor on SK1 expression in these cells (data not shown).

It remains to be established whether FTY720 and (*S*)-FTY720 vinylphosphonate reduce AR expression via the same mechanism as SKi, i.e. by activating an oxidative

stress response. In this regard, it is interesting to note that FTY720 has been reported to induce oxidative stress in hepatocellular carcinoma cells (Hung et al., 2008) and acute lymphoblastic leukemia cells (Wallington-Beddoe et al., 2011).

In addition to the inhibitors tested in the present study, resveratrol, which has been recently shown to inhibit SK1 *in vitro* (Lim et al., 2012a) and to reduce SK1 activity and expression in PC-3 cells (Brizuela et al., 2010), down-regulates AR mRNA and protein levels in LNCaP cells, thereby impairing AR transcriptional activity (Mitchell et al., 1999; Kai and Levenson, 2011).

### 6.3.5 Significance of AR down-regulation by SK1 inhibitors

It is well established that both androgen-dependent and androgen-independent prostate cancers rely on a functional AR pathway for their growth and survival (Zegarra-Moro et al., 2002; Chen et al., 2008) and that genetic knock-down of AR results in growth inhibition and tumour regression in *in vitro* and *in vivo* models of prostate cancer (Eder et al., 2000; Haag et al., 2005; Snoek et al., 2009). The requirement of prostate cancer cells for AR is exploited in the clinic. Indeed, the standard therapeutic strategies for the treatment of advanced prostate cancer aim at antagonizing AR action via various approaches, as discussed in Chapter 1. Additionally, AR remains an important therapeutic target in androgen-independent prostate cancer.

Remarkably, SK1 inhibitors, by removing AR from prostate cancer cells, have the potential to completely abrogate AR-mediated signalling. This suggests that the therapeutic efficacy of SK1 inhibitors might be superior to that of the currently available AR-targeted clinical options. In this context, the ability of SK1 inhibitors to remove AR from androgen-independent prostate cancer cells is particularly important, given the lack of curative therapies for hormone-refractory prostate cancer.

## 6.4 Summary

The present study demonstrates that SK1 inhibitors, namely SKi, FTY720 and (S)-FTY720 vinylphosphonate, down-regulate AR in androgen-dependent and androgen-independent prostate cancer cells, providing evidence for a role for SK1 in modulating AR expression. In contrast, eliminating SK2 from LNCaP cells did not affect AR protein levels. In particular, the data presented in this Chapter demonstrate that SKi reduces AR expression via an oxidative stress-induced mechanism in LNCaP cells. Current studies are aimed at establishing whether the effect of SKi on the prostate cancer cell metabolome and AR is contingent on the inhibition of SK1 activity/expression.

This study can be progressed by elucidating the exact mechanism(s) by which SK1 inhibitors reduce AR protein levels and integrating the post-translational down-regulation of AR with the effect on *AR* gene transcription. Also, it will be important to test additional prostate cancer cell lines in order to assess the generality of the effect of SK1 inhibitors on AR expression.

Removal of AR from prostate cancer cells provides a means for inhibiting prostate cancer cell growth and could represent an additional therapeutic value of SK1 inhibitors along with their ability to affect the expression of SK1 thereby inducing apoptosis (Chapter 3; Chapter 4). Further work is required to prove the functional significance of AR down-regulation by SK1 inhibitors, i.e. whether this does actually result in an effective block of AR transcriptional activity and in prostate cancer cell growth inhibition.

# CHAPTER 7:

# GENERAL DISCUSSION

# 7. GENERAL DISCUSSION

There is considerable evidence implicating the SK1/S1P signalling pathway in human cancer (discussed in Chapter 1 and reviewed in Shida et al., 2008 and Pyne and Pyne, 2010). Clinical studies have shown that SK1 expression is elevated in various types of cancer and correlates with adverse clinical features such as reduced cancer patient survival, acquisition of resistance to therapeutic agents and increased disease recurrence (French et al., 2003; Johnson et al., 2005; Van Brocklyn et al., 2005; Kawamori et al., 2006; Ruckäberle et al., 2008; Li et al., 2009; Long et al., 2010a; Watson et al., 2010; Ohotski et al., 2012). This is consistent with a plethora of *in vitro* and *in vivo* studies showing that the SK1/S1P axis regulates many processes that are fundamental for cancer development and progression. In particular, aberrations in this pathway promote enhanced proliferation, reduced apoptosis and increased invasiveness of tumour cells (Nava et al., 2002; Bektas et al., 2005; Sarkar et al., 2005; Van Brocklyn et al., 2005; Bonhoure et al., 2008; Sukocheva et al., 2009; Pchejetski et al., 2008; Brizuela et al., 2010; Van Brocklyn et al., 2003; Park et al., 2007; Kim et al., 2011). In addition, acting on endothelial cells S1P potently stimulates angiogenesis and therefore tumour vascularisation (Nava et al., 2002; Anelli et al., 2010).

These observations provide compelling evidence that targeting SK1 might represent an effective therapeutic strategy to treat cancer, by affecting multiple hallmarks of cancer. Significantly, *in vivo* studies provided validation of SK1 as a therapeutic target, demonstrating that SK1 inhibition exhibits potent anti-tumour activity (reducing tumour growth and metastasis) in animal models of cancer (French et al., 2006; Brizuela et al., 2010; Pchejetski et al., 2010). In addition, inhibition of SK1 sensitizes tumour cells to apoptosis induced by anti-cancer therapeutic agents (Baran et al., 2007; Pchejetski et al., 2008; Guillermet-Guibert et al., 2009; Pchejetski et al., 2010). The results of a Phase I clinical trial of the SK inhibitor Safingol (L-*threo*-dihydrosphingosine) in combination with cisplatin for the treatment of advanced

tumours have been recently published and show that the drug combination is effective in inducing some degree of tumour regression (Dickson et al., 2011).

Notably, targeting SK1 offers two advantages: 1) limitation of the availability of S1P at its membrane receptors and intracellular targets, and 2) elevation of ceramide and sphingosine levels to promote a pro-apoptotic signal (Ogretmen and Hannun, 2004). Given the potential of SK1 as a therapeutic target, the design of effective SK1 inhibitors for anti-cancer clinical use is a primary goal of drug discovery in this research area.

## 7.1 SK1 inhibitors induce the proteasomal degradation of SK1: potential for translation into the clinic

A major finding of the current study is the identification of an entirely new mechanism of action of SK1 inhibitors. In particular, we demonstrated that various SK1 inhibitors (SKi, FTY720, (*S*)-FTY720 vinylphosphonate) have the ability to remove SK1 from prostate cancer cells by inducing its proteasomal degradation. Similarly, these inhibitors promote SK1 proteasomal degradation in other cell types (Tonelli et al., 2010; Loveridge et al., 2010; Lim et al., 2011b).
Interestingly, resveratrol, which is structurally similar to SKi and inhibits purified SK1 activity *in vitro* (Lim et al., 2012a), reduces SK1 protein levels in PC-3 cells (Brizuela et al., 2010). This effect is temporally preceded by SK1 activity inhibition and ceramide accumulation and is reversed by enforced over-expression of SK1 (Brizuela et al., 2010). Resveratrol-induced down-regulation of SK1 expression was also observed in MCF-7 breast cancer cells (Lim et al., 2012a). Thus, the down-regulation of SK1 expression might be a common feature of SK1 inhibitors.

The finding that SK1 inhibitors have the potential to create chemical knock-down of SK1 is particularly significant in that it might help to overcome two major obstacles that have thus far hampered the translation of SK1 inhibitors into the clinic: poor inhibitor potency and limited duration of sphingolipid levels modulation. In fact, it is

anticipated that SK1 inhibitors that induce the removal of SK1 from cancer cells will exhibit significantly improved efficacy and duration of action compared with those that simply reversibly inhibit SK1 activity. Indeed, removal of SK1 in response to SK1 inhibitors results in a sustained perturbation of sphingolipid metabolism and subsequent activation of apoptosis in prostate cancer cells.
Thus, these findings provide impetus for the development of SK1 inhibitors that exploit this mechanism of action (i.e. activation of SK1 proteasomal degradation) to be used as anti-cancer therapeutics.

SK1 inhibitors that promote the proteasomal degradation of SK1 and consequently a durable accumulation of ceramide could also prove useful to overcome the resistance of cancer cells to therapy. Indeed, a number of studies provide evidence that SK1 protects cancer cells from apoptosis induced by therapeutic agents by countering the elevation of ceramide induced by these treatments. Thus, SK1 inhibition sensitizes cancer cells to therapeutic treatments (chemotherapy and radiation therapy) by elevating the cellular content of ceramide (Nava et al., 2000b; Baran et al., 2007; Scarlatti et al., 2007; Pchejetski et al., 2008; Guillermet-Guibert et al., 2009, Pchejetski et al., 2010). These findings strongly support the rationale for the employment of SK1 inhibitors that remove SK1 from cancer cells in combination with conventional therapeutic regimens for the treatment of chemo- and radio-therapy resistant cancers.

Significantly, this study demonstrates that the SK1 isoforms, SK1a and SK1b exhibit different regulatory properties (that modulate their sensitivity to proteasomal degradation) and uncovered the importance of SK1b in maintaining prostate cancer cell survival. In particular, the current study demonstrates that removing SK1b from these cells is necessary to disrupt the ceramide/sphingosine/S1P rheostat and to therefore induce ceramide-dependent apoptosis. Thus, this study provides the first evidence that SK1b might control chemotherapeutic resistance of prostate cancer cells.
These findings highlight the necessity to effectively target SK1b to kill cancer cells. In this regard, understanding the mechanisms that regulate the sensitivity of SK1 to

SK1 inhibitors-induced degradation would definitely help in the design of more effective SK1 inhibitors for therapeutic intervention.

## 7.2 SK1 inhibitors affect multiple targets: potential for a multi-pronged attack on prostate cancer

The current study, in line with previous reports (Pchejetski et al., 2005; Akao et al., 2006; Brizuela et al., 2010; Pchejetski et al., 2010), demonstrates that SK1 ablation is associated with the induction of apoptosis in prostate cancer cells.
In addition, others have shown that knock-down of SK1 induces growth inhibition in prostate cancer cells, providing evidence that SK1 has a key role in prostate cancer cell proliferation (Akao et al., 2006; Dayon et al., 2009). Furthermore, S1P promotes prostate cancer cell migration and invasion (Sekine et al., 2011). These findings suggest that SK1 inhibitors might also suppress prostate cancer cell growth and metastatic potential, in addition to their pro-apoptotic effect. Thus, acute inhibition of SK1 activity by SK1 inhibitors might reduce S1P levels to suppress cell proliferation and migration, whereas the sustained dysregulation of the sphingolipid rheostat resulting from SK1 removal might result in the induction of apoptosis.

Moreover, evidence exists that SK1 might be involved in the acquisition of androgen independence. Indeed, androgen-dependent prostate cancer cells progress to an androgen-independent state when subjected to chronic androgen deprivation, which mimics the development of a castration-resistant prostate cancer in patients treated with androgen ablation therapy (Halkidou et al., 2003; Shi et al., 2004; Dayon et al., 2009). Significantly, inhibition of SK1 prevents the development of androgen independence *in vitro* (Dayon et al., 2009). Hence, SK1 inhibitors might be a valuable therapeutic option for preventing androgen escape in human prostate cancer, a major issue in the clinical management of this pathology.

Interestingly, the current study identified novel effects of SK1 inhibitors that might offer additional therapeutic advantages. In particular, SK1 inhibitors substantially

reduce the expression of androgen receptor (AR) in prostate cancer cells. AR is essential not only in androgen-dependent prostate cancer cells but also in hormone-refractory cells, where aberrant activation of AR mediates a pro-growth signal in the absence of androgen stimulation (Buchanan et al., 2001; Chen et al., 2008). Significantly, *in vivo* studies demonstrated that genetic knock-down of AR results in growth inhibition and regression of both androgen-dependent and androgen-independent prostate (Eder et al., 2002; Snoek et al., 2009). Thus, the removal of AR by SK1 inhibitors would deprive prostate cancer cells of an essential signalling pathway and might therefore represent an additional means for suppressing prostate cancer growth. This could represent a further facet for enhancing the efficacy of SK1 inhibitors as anti-cancer agents.

In addition, the SK1 inhibitor SKi reduces autophagy in prostate cancer cells. Evidence exists that the activation of autophagy might represent a pro-survival mechanism in prostate cancer cells that might favour the transition to androgen independence (Li et al., 2008a; Chhipa et al., 2011). Thus, the ability of SK1 inhibitors to block autophagy to remove this adaptive survival response of cancer cells might represent an added therapeutic value of these compounds.

Among the SK1 inhibitors used in the current study, (*S*)-FTY720 vinylphosphonate has properties that might be particularly advantageous for its employment as an anti-cancer agent. In fact, besides acting on SK1 (to induce its proteasomal degradation) and down-regulating AR, this compound is also a pan S1P receptor antagonist (Valentine et al., 2010). Thus, (*S*)-FTY720 vinylphosphonate has the potential to interrupt S1P inside-out signalling in tumour cells to inhibit their proliferation, survival and migration. Significantly, clinical studies demonstrated that high expression of $S1P_1$, $S1P_3$ and $S1P_4$ in breast cancer tumours correlates with poor prognosis (Watson et al., 2010; Ohotski et al., 2012), highlighting the functional relevance of S1P receptors in human cancer.
Importantly, S1P released by cancer cells can also act on endothelial cells via a paracrine mechanism to promote angiogenesis (Anelli et al., 2010). Thus, interfering with S1P inside-out signalling, either via genetic knock-down of $S1P_1$ or using an

anti-S1P antibody, strongly inhibits angiogenesis and therefore tumour growth *in vivo* (Chae et al., 2004; Visentin et al., 2006). Therefore, (*S*)-FTY720 vinylphosphonate might also be effective in blocking tumour neovascularisation by virtue of antagonism of S1P receptors.

Significantly, (*S*)-FTY720 vinylphosphonate can overcome the resistance of SK1b to proteasomal degradation that is observed with SKi in LNCaP-AI cells to induce apoptosis. In this regard, (*S*)-FTY720 vinylphosphonate does not appear to induce activation of the proteasome to accelerate degradation of SK1b. Thus, this inhibitor might promote SK1b proteasomal degradation 1) by inducing conformational changes in this protein that increase its susceptibility to be removed by the proteasome, and/or 2) by activating the E3 ubiquitin ligase specific for SK1b. Indeed, (*S*)-FTY720 vinylphosphonate is structurally similar to S1P, which binds to the RING domain of TRAF2 to stimulate its E3 ubiquitin ligase activity (Alvarez et al., 2010).

This finding is remarkable as (*S*)-FTY720 vinylphosphonate might represent the prototype for the development of chemotherapeutic drugs effective in the treatment of androgen-independent prostate cancer, which at present are still lacking.

The fact that SK1 inhibitors have the potential to affect multiple pathways is particularly significant from the standpoint of translation to the clinic, as it is anticipated that the combined inhibition of multiple targets in cancer cells would offer increased therapeutic efficacy.

## 7.3 SK1 and SK2 have distinct functions in prostate cancer cells

SK1 and SK2 appear to have redundant functions in normal, non-cancerous cells, i.e. they may compensate for a deficiency in each other function (section 7.4). The redundancy for SK1 seems to be lost in some cancer cells (section 7.4). However,

SK1 and SK2 might have distinct functions in other cancer cell types, as discussed below for prostate cancer.

The data presented in the current study demonstrate that SK1 and SK2 have distinct and non-redundant functions in prostate cancer cells. This conclusion is based on the finding that inhibition of the two enzyme isoforms has distinct effects on intracellular sphingolipid levels in LNCaP cells, which is translated into different responses in these cells. In particular, treatment of LNCaP cells with SKi induces ceramide-dependent apoptosis and inhibits autophagy, whereas a specific inhibitor of SK2, (*R*)-FTY720-OMe fails to promote apoptosis while stimulating autophagy in these cells. In addition, SKi induces the ceramide-mediated activation of the proteasome while (*R*)-FTY720-OMe fails to do so.

Taken together, these findings support a model in which SK1 and SK2 regulate distinct functional pools of sphingolipids in LNCaP cells. This might be accomplished by differential employment of S1P metabolizing enzymes by SK1 and SK2 (S1P lyase *versus* S1P phosphatase, respectively). A major consequence is that SK1 functions to remove ceramide from prostate cancer cells, while SK2 appears to have a minor role in this regard. It remains to be defined whether SK2 controls a pool of S1P that has a more proactive role in signalling.

The functional coupling of SK1 and SK2 with different S1P-metabolizing enzymes might be dependent on their localization to distinct intracellular compartments.

Additionally, the functional differences observed for SK1 and SK2 might be due to a distinct subcellular localization of the two enzymes in that the site where S1P is generated might dictate its interaction with distinct downstream effectors to modulate different signalling pathways.

The significance of SK1 and SK2 subcellular localization in regulating the functions of these enzymes in prostate cancer cells requires formal investigation.

These findings are entirely consistent with previous studies showing that SK1 and SK2 are functionally different with respect to the regulation of sphingolipid metabolism and their knock-down is associated with distinct effects on cells, and with the notion that the functional differences between SK1 and SK2 might reflect a

different subcellular localization of the two enzymes (Maceyka et al., 2005; Wattenberg et al., 2006; Le Stunff et al., 2007; Gao and Smith, 2011; Pitson, 2011).

In this regard, Spiegel and colleagues have shown that SK2, but not SK1, is involved in the recycling of sphingosine into ceramide, and this is accomplished by the concerted action with S1P phosphatase (Maceyka et al., 2005; Le Stunff et al., 2007). This function of SK2 is dependent on its localization to the endoplasmic reticulum and underlies the ability of SK2 to induce apoptosis (Maceyka et al., 2005). Indeed, targeting SK1 to the endoplasmic reticulum converts this enzyme from a pro-survival to a pro-apoptotic molecule (Maceyka et al., 2005).

Interestingly, Gao and Smith (2011) demonstrated that SK2 knock-down results in SK1 up-regulation and subsequent increase in intracellular S1P in various cancer cell lines. However, SK1-derived S1P was not able to compensate the anti-proliferative and anti-migratory effects due to SK2 knock-down in these cells (Gao and Smith, 2011). These observations provide strong evidence that SK1 and SK2 regulate functionally distinct pools of S1P in the cancer cell types studied by Gao and Smith, hence the lack of redundancy.

In addition to the regulation of distinct pools of sphingolipids, another possible explanation for the lack of redundancy is that some of the isoform-specific effects of SK1/SK2 in cancer cells might be due to yet unidentified non-catalytic activities of these enzymes, where the functionality of these enzymes is not related to their kinase activity. For instance, SK1 appears to regulate EGF signalling independently of its kinase activity in MDA-MB-231 breast cancer cells (Pyne et al., 2009). SK2 also possesses a non-catalytic (BH3-like) domain that in part mediates its pro-apoptotic function (Maceyka et al., 2005). Thus, SK1 and SK2 might function as adaptor proteins to modulate signalling cascades. Indeed, SK1 is involved in a number of protein-protein and lipid-protein interactions (Taha et al., 2006b). The use of pharmacological inhibitors of SK catalytic activity versus SK siRNA would assist in discriminating between catalytic and non-catalytic activities of these enzymes.

## 7.4 SK inhibitors: exploiting the addiction

The SK1/S1P pathway has crucial functions not only in diseased cells, but also in normal cells where it regulates many physiological processes (discussed in Chapter 1 and reviewed in Hannun and Obeid, 2008). Hence, it might be argued that the use of SK1 inhibitors as anticancer agents might result in severe adverse effects. However, it is important to appreciate that cancer cells that over-express SK1 appear to have a "non-oncogene addiction" for SK1, that is, an over-reliance on this enzyme for proliferation and survival compared with normal cells (Vadas et al., 2008).

SK1 and SK2 could have redundant functions in normal cells, as evidenced by the finding that selective knock-out of *Sk1* or *Sk2* does not result in major phenotypic abnormalities in mice whereas *Sk1*/*Sk2* double knock-out results in an embryonic lethal phenotype (Allende et al., 2004; Mizugishi et al., 2005). These studies indicate that in normal cells, SK1 and SK2 might compensate for a deficiency in each other activity. Conversely, the addiction of cancer cells to SK1 is indicative of a loss of redundancy for SK1 in cancer.

Hence, the "addiction" and lack of redundancy for SK1 in cancer cells might be exploited for therapeutic purposes, that is, the use of SK1 inhibitors to exert cancer cell-selective growth inhibition and apoptosis. This notion is supported by studies showing that FTY720 induces the loss of cell viability in cancer cell lines (including prostate cancer cells), while the normal counterparts of these cells are resistant to FTY720-induced apoptosis (Wang et al., 1999; Azuma et al., 2003; Ubai et al., 2007).

Interestingly, it has been recently shown that in some cancer cell lines, SK2-selective ablation is more effective than SK1 removal in inhibiting cell proliferation and migration, and combined deletion of both SK1 and SK2 does not further enhance the anti-tumour effects of SK2 knock-down alone (Van Brocklyn et al., 2005; Gao and Smith, 2011). These findings suggest that SK2-targeted therapy might be more efficacious for the treatment of some types of cancer. Conversely, in tumour cells where SK2 exerts a pro-apoptotic function, ablation of this enzyme by anti-cancer agents might be deleterious.

Thus, further investigation is required to establish whether SK1 and SK2 exert redundant, complementary or opposing activities in cancer cells and to define the exact roles of the two enzymes in different cancer types. This information is needed for developing effective SK-targeted therapeutic strategies. In particular, this would allow assessment of whether isoform-selective ablation is to be preferred to obtain the optimal anti-cancer activity or whether dual inhibition of both isoforms is required. In this regard, the data from knock-out mice (Allende et al., 2004; Mizugishi et al., 2005) underscore the need for caution in using SK1/SK2 dual inhibitors as anti-cancer agents, so that tumour-targeted delivery of these inhibitors would probably be the best option in this case.

## 7.5 Conclusions

The results presented in the current study provide important information about the function of SK in prostate cancer cells and provide further evidence that targeting SK1 might represent a successful therapeutic approach for the treatment of prostate cancer. Indeed, inhibition of this enzyme has multiple effects that are anticipated to reduce prostate cancer growth and progression.

In addition, the findings presented in this study could assist in the design of more effective SK1 inhibitors for treatment of cancer and help to overcome some of the major issues related to anti-cancer therapies (i.e. limited efficacy of current therapies and the development of resistance to therapy).

# CHAPTER 8:

# REFERENCES

## 8. REFERENCES


- Ader, I., Brizuela, L., Bouquerel, P., Malavaud, B., Cuvillier, O. (2008) Sphingosine kinase 1: a new modulator of hypoxia inducible factor 1alpha during hypoxia in human cancer cells. *Cancer Res.* 68, 8635-8642
- Ahmad, M., Long. J.S., Pyne, N.J., Pyne. S. (2006) The effect of hypoxia on lipid phosphate receptor and sphingosine kinase expression and mitogen activated protein kinase signalling in human pulmonary smooth muscle cells. *Prostaglandins Other Lipid Mediat.* 79, 278-286
- Akao, Y., Banno, Y., Nakagawa, Y., Hasegawa, N., Kim, T., Murate, T., Igarashi, Y., Nozawa, Y. (2006) High expression of sphingosine kinase 1 and S1P receptors in chemotherapy-resistant prostate cancer PC-3 cells and their camptothecin-induced up-regulation. *Biochem. Biophys. Res. Commun.* 342, 1284-1290
- 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, 413-428
- Alimirah, F., Panchanathan, R., Chen, J., Zhang, X., Ho, S.M., Choubey, D. (2007) Expression of androgen receptor is negatively regulated by p53. *Neoplasia* 9, 1152-1159
- Allende, M.L., Yamashita, T., Proia, R.L. (2003) G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood* 102, 3665-3667
- Allende, M.L., Sasaki, T., Kawai, H., Olivera, A., Mi, Y., van Echten-Deckert, G., Hajdu, R., Rosenbach, M., Keohane, C.A., Mandala, S., Spiegel, S., Proia, R.L. (2004) Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J. Biol. Chem.* 279, 52487-52492
- Alvarez, S.E., Milstien, S. and Spiegel, S. (2007) Autocrine and paracrine roles of sphingosine-1-phosphate. *Trends Endocrinol. Metab.* 18, 300-307
- 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, 1084-1088
- An, S., Bleu, T., Zheng, Y. (1999) Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. Mol. Pharmacol. 55, 787-794
- An, S., Zheng, Y. and Bleu, T. (2000) Sphingosine 1-phosphate-induced cell proliferation, survival and related events mediated by G protein-coupled receptors EDG3 and EDG5. *J. Biol. Chem.* 275, 288-296
- Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chac, S.S., Stefansson, S., Liau, G., Hla, T. (2002) Extracellular export of sphingosine kinase-1 enzyme: sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J. Biol. Chem.* 277, 6667-6675

- Anelli, V., Gault, C.R., Cheng, A.B., Obeid, L.M. (2008) Sphingosine kinase is up-regulated during hypoxia in U87MG glioma cells. Role of hypoxia-inducible factors 1 and 2. *J. Biol. Chem.* 283, 3365-3375
- Anelli, V., Gault, C.R., Snider, A.J., Obeid, L.M. (2010) Role of sphingosine kinase-1 in paracrine/transcellular angiogenesis and lymphangiogenesis in vitro. *FASEB J.* 24, 2727-2738
- Antoon, J.W., White, M.D., Meacham, W.D., Slaughter, E.M., Muir, S.E., Elliott, S., Rhodes, L.V., Ashe, H.B., Wiese, T.E., Smith, C.D., Burow, M.E., Beckman, B.S. (2010) Antiestrogenic effects of the novel sphingosine kinase-2 inhibitor ABC294640. *Endocrinology* 151, 5124-5135
- 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, 205-216
- Antoon, J.W., White, M.D., Burow, M.E., Beckman, B.S. (2012) Dual inhibition of sphingosine kinase isoforms ablates TNF-induced drug resistance. *Oncol. Rep.* 27, 1779-1786
- Avery, K., Avery, S., Shepherd, J., Heath, P. R., Moore, H. (2008) Sphingosine-1-phosphate mediates transcriptional regulation of key targets associated with survival, proliferation, and pluripotency in human embryonic stem cells. *Stem Cells Dev.* 17, 1195-1205
- Azuma, H., Takahara, S., Ichimaru, N., Wang, J.D., Itoh, Y., Otsuki, Y., et al. (2002) Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. *Cancer Res.* 62, 1410-1419
- Azuma, H., Takahara, S., Horie, S., Muto, S., Otsuki, Y., Katsuoka, Y. (2003) Induction of apoptosis in human bladder cancer cells in vitro and in vivo caused by FTY720 treatment. *J. Urol.* 169, 2372-2377
- Bakali, H.M., Herman, M.D., Johnson, K.A., Kelly, A.A., Wieslander, A., Hallberg, B.M., et al. (2007) Crystal structure of YegS, a homologue to the mammalian diacylglycerol kinases, reveals a novel regulatory metal binding site. *J. Biol. Chem.* 282, 19644-19652
- 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, 33697-33700
- Baran, Y., Salas, A., Senkal, C.E., Gunduz, U., Bielawski, J., Obeid, L.M., Ogretmen, B. (2007) Alterations of ceramide/sphingosine 1-phosphate rheostat involved in the regulation of resistance to imatinib-induced apoptosis in K562 human chronic myeloid leukemia cells. *J. Biol. Chem.* 282, 10922-10934
- Baron, S., Manin, M., Beaudoin, C., Leotoing, L., Communal, Y., et al. (2004) Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. *J. Biol. Chem.* 279, 14579-14586
- 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, 34994-35002

- Baudhuin, L.M., Jiang, Y., Zaslavsky, A., Ishii, I., Chun, J., Xu, Y. (2004) S1P3-mediated Akt activation and cross-talk with platelet-derived growth factor receptor (PDGFR). *FASEB J.* 18, 341-343
- Bayerl, M.G., Bruggeman, R.D., Conroy, E.J., et al. (2008) Sphingosine kinase 1 protein and mRNA are overexpressed in non-Hodgkin lymphomas and are attractive targets for novel pharmacological interventions. *Leuk. Lymphoma* 49, 948-954
- Beckham, T.H., Lu, P., Cheng, J.C., Zhao, D., Turner, L.S., Zhang, X., Hoffman, S., Armeson, K.E., Liu, A., Marrison, T., Hannun, Y.A., Liu, X. (2012) Acid ceramidase-mediated production of sphingosine 1-phosphate promotes prostate cancer invasion through upregulation of cathepsin B. *Int. J. Cancer* doi: 10.1002/ijc.27480
- Bektas, M., Jolly, P.S., Muller, C., Eberle, J., Spiegel, S., Geilen, C.C. (2005) Sphingosine kinase activity counteracts ceramide-mediated cell death in human melanoma cells: role of Bcl-2 expression. *Oncogene* 24, 178-187
- Beljanski, V., Knaak, C., Smith, C.D. (2010) A novel sphingosine kinase inhibitor induces autophagy in tumor cells. *J. Pharmacol. Exp. Ther.* 333, 454-464
- Beljanski, V., Knaak, C., Zhuang, Y., Smith, C.D. (2011) Combined anticancer effects of sphingosine kinase inhibitors and sorafenib. *Invest. New Drugs* 29, 1132-1142
- Bento, C.F., Fernandes, R., Ramalho, J., Marques, C., Shang, F., Taylor, A., Pereira, P. (2010) The chaperone-dependent ubiquitin ligase CHIP targets HIF-1α for degradation in the presence of methylglyoxal. *PLoS One* 5, e15062
- Berdyshev, E.V., Gorshkova, I.A., Usatyuk, P., Zhao, Y., Saatian, B., Hubbard, W., Natarajan, V. (2006) De novo biosynthesis of dihydrosphingosine-1-phosphate by sphingosine kinase 1 in mammalian cells. *Cell. Signal.* 18, 1779-1792
- 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, 5467-5477
- Berg, J.M., Tymoczko, J.L., Stryer, L. (2002) Biochemistry. 5th edition. New York: W.H. Freeman and Co
- Bergelin, N., Blom, T., Heikkilä, J., Löf, C., Alam, C., Balthasar, S., et al. (2009) Sphingosine kinase as an oncogene: autocrine sphingosine 1-phosphate modulates ML-1 thyroid carcinoma cell migration by a mechanism dependent on protein kinase C-alpha and ERK1/2. *Endocrinology* 150, 2055-2063
- Berridge, M.J., Lipp, P., Bootman, M.D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell. Biol.* 1, 11-21
- Betito, S. and Cuvillier, O. (2006) Regulation by sphingosine 1-phosphate of Bax and Bad activities during apoptosis in a MEK-dependent manner. *Biochem. Biophys. Res. Commun.* 340, 1273-1277
- Billich, A., Bornancin, F., Dévay, P., Mechtcheriakova, D., Urtz, N., Baumruker, T. (2003) Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J. Biol. Chem.* 278, 47408-47415
- 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, 95-102

- Bonhoure, E., Lauret, A., Barnes, D.J., Martin, C., Malavaud, B., Kohama, T., Melo, J.V., Cuvillier, O. (2008) Sphingosine kinase-1 is a downstream regulator of imatinib-induced apoptosis in chronic myeloid leukaemia cells. *Leukemia* 22, 971-979
- Borg, A., Tandon, A.K., Sigurdsson, H. (1990) HER2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res.* 50, 4332-4337
- Bouchier-Hayes L. (2010) The role of caspase-2 in stress-induced apoptosis. *J. Cell. Mol. Med.* 14, 1212-1224
- 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., 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, 21453-21457
- Brinkmann, V., Cyster, J.G., Hla, T. (2004) FTY720: sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. *Am. J. Transplant.* 4, 1019-1025
- Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., et al. (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat. Rev. Drug Discov.* 9, 883-897
- 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, 3882-3894
- Bubendorf, L., Kononen, J., Koivisto, P., Schraml, P., Moch, H., Gasser, T.C., et al. (1999) Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. *Cancer Res.* 59, 803-806
- Buchanan, G., Irvine, R.A., Coetzee, G.A., Tilley, W.D. (2001) Contribution of the androgen receptor to prostate cancer predisposition and progression. *Cancer Metastasis Rev.* 20, 207-223
- Caron, C., Boyault, C., Khochbin, S. (2005) Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. *Bioessays* 27, 408-415
- Calatayud, C.A., Pasquini, L.A., Pasquini, J.M., Soto, E.F. (2005) Involvement of the ubiquitin-mediated proteolytic system in the signaling pathway induced by ceramide in primary astrocyte cultures. *Dev. Neurosci.* 27, 397-407
- Ceraline, J., Cruchant, M.D., Erdmann, E., Erbs, P., Kurtz, J.E., et al. (2004) Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. *Int. J. Cancer* 108, 152-157
- 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, 1082-1089

- Chalfant, C.E., Kishikawa, K., Mumby, M.C., Kamibayashi, C., Bielawska, A., Hannun, Y.A. (1999) Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. *J. Biol. Chem.* 274, 20313-20317
- Chan, S.C., Li, Y., Dehm, S.M. (2012) Androgen receptor splice variants activate androgen receptor target genes and support aberrant prostate cancer cell growth independent of canonical androgen receptor nuclear localization signal. *J. Biol. Chem.* 287, 19736-19749
- Chang, C.L., Ho, M.C., Lee, P.H., Hsu, C.Y., Huang, W.P., Lee, H. (2009) S1P5 is required for sphingosine 1-phosphate-induced autophagy in human prostate cancer PC-3 cells. *Am. J. Physiol. Cell Physiol.* 297, C451-C458
- Chen, C.D., Welsbie, D.S., Tran, C., Baek, S.H., Chen, R., Vessella, R., Rosenfeld, M.G., Sawyers, C.L. (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* 10, 33-39
- Chen, S., Rehman, S.K., Zhang, W., Wen, A., Yao, L., Zhang, J. (2010) Autophagy is a therapeutic target in anticancer drug resistance. *Biochim. Biophys. Acta* 1806, 220-229
- Chen, Y., Sawyers, C.L., Scher, H.I. (2008) Targeting the androgen receptor pathway in prostate cancer. *Curr. Opin. Pharmacol.* 8, 440-448
- Chhipa, R.R., Wu, Y., Ip, C. (2011) AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia. *Cell. Signal.* 23, 1466-1472
- Chun, J., Goetzl, E.J., Hla, T., Igarashi, Y., Lynch, K.R., Moolenaar, W., Pyne, S., Tigyi, G. (2002) International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev.* 54, 265-269
- Chun, J., Brinkmann, V. (2011) A mechanistically novel, first oral therapy for multiple sclerosis: the development of fingolimod (FTY720, Gilenya). *Discov. Med.* 12, 213-228
- Clague, M.J., Urbé, S. (2010) Ubiquitin: same molecule, different degradation pathways. *Cell* 143, 682-685
- Colombel, M., Symmans, F., Gil, S., O'Toole, K.M., Chopin, D., Benson, M., et al. (1993) Detection of the apoptosis-suppressing oncoprotein Bcl2 in hormone-refractory human prostate cancers. *Am. J. Pathol.* 143, 390-400
- Coppé, J.P., Patil, C.K., Rodier, F., Sun, Y., Muñoz, D.P., Goldstein, J., et al. (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol.* 6, 2853-2868
- Craft, N., Shostak, Y., Carey, M., Sawyers, C.L. (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat. Med.* 5, 280-285
- Cross, T.G., Scheel-Toellner, D., Henriquez, N.V., Deacon, E., Salmon, M., Lord, J.M. (2000) Serine/threonine protein kinases and apoptosis. *Exp. Cell Res.* 256, 34-41
- Culig, Z., Hobisch, A., Cronauer, M.V., et al. (1994) Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res.* 54, 5474-5478

- 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, 800-803
- Cuvillier, O., Rosenthal, D.S., Smulson, M.E., Spiegel, S. (1998) Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J. Biol. Chem.* 273, 2910-2916
- Dai, J.L., Maiorino, C.A., Gkonos, P.J., Burnstein, K.L. (1996) Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells. *Steroids* 61, 531-539
- Dayon A., Brizuela, L., Martin, C., Mazerolles, C., Pirot, N., Doumerc, N., Nogueira, L., Golzio, M., Teissié, J., Serre, G., Rischmann, P., Malavaud, B., Cuvillier, O. (2009) Sphingosine kinase-1 is central to androgen-regulated prostate cancer growth and survival. *PloS ONE* 4, e8048
- de Boer, W., Bolt, J., Kuiper, G.G., Brinkmann, A.O., Mulder, E. (1987) Analysis of steroid- and DNA-binding domains of the calf uterine androgen receptor by limited proteolysis. *J. Steroid Biochem.* 28, 9-19
- Dehm, S.M., Tindall, D.J. (2006) Molecular regulation of androgen action in prostate cancer. *J. Cell. Biochem.* 99, 333-344
- Dehm, S.M., Schmidt, L.J., Heemers, H.V., Vessella, R.L., Tindall, D.J. (2008) Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res.* 68, 5469-5477
- 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, 44763-44774
- Denis, L.J., Griffiths, K. (2000) Endocrine treatment in prostate cancer. *Semin. Surg. Oncol.* 18, 52-74
- Denmeade, S.R., Lin, X.S., Isaacs, J.T. (1996) Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* 28, 251-265.
- Dhanasekaran, D.N., Reddy, E.P. (2008) JNK signaling in apoptosis. *Oncogene* 27, 6245-6251
- Dickson, M.A., Carvajal, R.D., Merrill, A.H. Jr, 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, 2484-2492
- Ding, G., Sonoda, H., Yu, H., Kajimoto, T., Goparaju, S.K., Jahangeer, S., et al. (2007) Protein kinase D-mediated phosphorylation and nuclear export of sphingosine kinase 2. *J. Biol. Chem.* 282, 27493-27502
- Dobrowsky, R.T., Kamibayashi, C., Mumby, M.C., Hannun, Y.A. (1993) Ceramide activates heterotrimeric protein phosphatase 2A. *J. Biol. Chem.* 268, 15523-15530
- Döll, F., Pfeilschifter, J., Huwiler, A. (2005) The epidermal growth factor stimulates sphingosine kinase-1 expression and activity in the human mammary carcinoma cell line MCF7. *Biochim. Biophys. Acta* 1738, 72-81
- Domingo-Domenech, J., Oliva, C., Rovira, A., Codony-Servat, J., Bosch, M., Filella, X., et al. (2006) Interleukin 6, a nuclear factor-kappaB target, predicts resistance to docetaxel in hormone-independent prostate cancer and nuclear

factor-kappaB inhibition by PS-1145 enhances docetaxel antitumor activity. Clin. Cancer Res. 12, 5578-5586

- Eder, I.E., Culig, Z., Ramoner, R., Thurnher, M., Putz, T., Nessler-Menardi, C., et al. (2000) Inhibition of LNCaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther.* 7, 997-1007
- Eder, I.E., Hoffmann, J., Rogatsch, H., Schäfer, G., Zopf, D., Bartsch, G., Klocker, H. (2002) Inhibition of LNCaP prostate tumor growth in vivo by an antisense oligonucleotide directed against the human androgen receptor. *Cancer Gene Ther.* 9, 117-125
- Edwards, J., Krishna, N.S., Grigor, K.M., Bartlett, J.M. (2003) Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. *Br. J. Cancer* 89, 552-556
- Edwards, J. and Bartlett, J.M. (2005) The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: Androgen-receptor cofactors and bypass pathways. *BJU Int.* 95, 1327-1335
- el Bawab, S., Mao, C., Obeid, L.M., Hannun, Y.A. (2002) Ceramidases in the regulation of ceramide levels and function. *Subcell. Biochem.*36, 187-205
- Elliott, M.R., Chekeni, F.B., Trampont, P.C., Lazarowski, E.R., Kadl, A., Walk, S.F., et al. (2009) Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 461, 282-286
- Endo, K., Igarashi, Y., Nisar, M., Zhou, Q.H., Hakomori, S. (1991) Cell membrane signalling as target in cancer therapy: inhibitory effect of N, N-dimethyl and N, N, N-trimethyl sphingosine derivatives on in vitro and in vivo growth of human tumor cells in nude mice. *Cancer Res.* 51, 1613-1618
- Endo, A., Nagashima, K.I., Kurose, H., Mochizuki, S., Matsuda, M., Mochizuki, N. (2002) Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells via vascular endothelial growth factor receptor and CrkII. *J. Biol. Chem.* 277, 23747-23754
- Fajardo, A.M., Mackenzie, D.A., Ji, M., Deck, L.M., Jagt, D.L., Thompson, T.A., Bisoffi, M. (2011) The curcumin analog ca27 down-regulates androgen receptor through an oxidative stress mediated mechanism in human prostate cancer cells. *Prostate* 72, 612-625
- Feldman, B.J., Feldman, D. (2001) The development of androgen independent prostate cancer. *Nat. Rev. Cancer* 1, 34-45
- Fisher, D.I., McLennan, A.G. (2008) Correlation of intracellular diadenosine triphosphate (Ap3A) with apoptosis in Fhit-positive HEK293 cells. *Cancer Lett.* 259, 186-191
- Ford, O.H. 3rd, Gregory, C.W., Kim, D., Smitherman, A.B., Mohler, J.L. (2003) Androgen receptor gene amplification and protein expression in recurrent prostate cancer. *J. Urol.* 170, 1817-1821
- Franklin, R.A., McCubrey, J.A. (2000) Kinases: positive and negative regulators of apoptosis. *Leukemia* 14, 2019-2034
- 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, 5962-5969
- French, K.J., Upson, J.J., Keller, S.N., Zhuang, Y., Yun, J.K., Smith, C.D. (2006) Antitumour activity of sphingosine kinase inhibitors. *J. Pharmacol. Exp. Ther.* 318, 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, 129-139
- Gao, P., Smith, C.D. (2011) Ablation of sphingosine kinase-2 inhibits tumor cell proliferation and migration. *Mol. Cancer Res.* 9, 1509-1519
- Garzotto, M., White-Jones, M., Jiang, Y., Ehleiter, D., Liao, W.C., Haimovitz-Friedman, A., Fuks, Z., Kolesnick, R. (1998) 12-O-tetradecanoylphorbol-13-acetate-induced apoptosis in LNCaP cells is mediated through ceramide synthase. *Cancer Res.* 58, 2260-2264
- Garzotto, M., Haimovitz-Friedman, A., Liao, W.C., White-Jones, M., Huryk, R., Heston, W.D., et al. (1999) Reversal of radiation resistance in LNCaP cells by targeting apoptosis through ceramide synthase. *Cancer Res.* 59, 5194-5201
- Gault, C.R., Obeid, L.M., Hannun, Y.A. (2010) An overview of sphingolipid metabolism: from synthesis to breakdown. *Adv. Exp. Med. Biol.* 688, 1-23
- Ghafar, M.A., Anastasiadis, A.G., Chen, M.W., Burchardt, M., Olsson, L.E., Xie, H., et al. (2003) Acute hypoxia increases the aggressive characteristics and survival properties of prostate cancer cells. *Prostate* 54, 58-67
- Ghosh, P.M., Malik, S., Bedolla, R., Kreisberg, J.I. (2003) Akt in prostate cancer: possible role in androgen-independence. *Curr. Drug Metab.* 4, 487-496
- Gibbs, T.C., Rubio, M.V., Zhang, Z., Xie, Y., Kipp, K.R., Meier, K.E. (2009) Signal transduction responses to lysophosphatidic acid and sphingosine 1-phosphate in human prostate cancer cells. *Prostate* 69, 1493-1506
- 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, 675-684
- Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E., Goldie, J. (1999) Progression to androgen independence is delayed by adjuvant treatment with antisense BCL-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. *Clin. Cancer Res.* 5, 2891-2898
- 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 S1P2 receptor negatively regulates platelet-derived growth factor-induced motility and proliferation. *Mol. Cell. Biol.* 25, 4237-4249
- Gräler, M.H., Goetzl, E.J. (2004) The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* 18, 551-553
- Gratschev, D., Löf, C., Heikkilä, J., Björkbom, A., Sukumaran, P., Hinkkanen, A., et al. (2009) Sphingosine kinase as a regulator of calcium entry through autocrine sphingosine 1-phosphate signaling in thyroid FRTL-5 cells. *Endocrinology* 150, 5125-5134
- Grossmann, M.E., Huang, H., Tindall, D.J. (2001) Androgen receptor signaling in androgen-refractory prostate cancer. *J. Natl. Cancer Inst.* 93, 1687-1697
- Guillermet-Guibert, J., Davenne, L., Pchejetski, D., Saint-Laurent, N., Brizuela, L., Guilbeau-Frugier, C., et al. (2009) Targeting the sphingolipid metabolism to defeat pancreatic cancer cell resistance to the chemotherapeutic gemcitabine drug. *Mol. Cancer Ther.* 8, 809-820

- Guo, Z., Yang, X., Sun, F., Jiang, R., Linn, D.E., et al. (2009) A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Res.* 69, 2305-2313
- Haag, P., Bektic, J., Bartsch, G., Klocker, H., Eder, I.E. (2005) Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. *J. Steroid Biochem. Mol. Biol.* 96, 251-258
- 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, 29462-29469
- 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, 12058-12065
- 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, 1254-1257
- Halkidou, K., Gnanapragasam, V.J., Mehta, P.B., Logan, I.R., Brady, M.E., Cook, S., Leung, H.Y., et al. (2003) Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene* 22, 2466-2477
- Hammad, S.M., Taha, T.A., Nareika, A., Johnson, K.R., Lopes-Virella, M.F., Obeid, L.M. (2006) Oxidized LDL immune complexes induce release of sphingosine kinase in human U937 monocytic cells. *Prostaglandins Other Lipid Mediat.* 79, 126-140
- Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M., Nishijima, M. (2003) Molecular machinery for non-vesicular trafficking of ceramide. *Nature* 426, 803-809
- Hanahan, D., Weinberg, R.A. (2000) The hallmarks of cancer. *Cell* 100, 57-70
- Heffernan-Stroud, L.A., Helke, K.L., Jenkins, R.W., De Costa, A.M., Hannun, Y.A., Obeid, L.M. (2012) Defining a role for sphingosine kinase 1 in p53-dependent tumors. *Oncogene* 31, 1166-1175
- Heinrich, M., Neumeyer, J., Jakob, M., Hallas, C., Tchikov, V., Winoto-Morbach, S., et al. (2004) Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. *Cell Death Differ.*11, 550-563
- 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, 62-73
- Hisano, Y., Kobayashi, N., Yamaguchi, A., Nishi, T. (2012) Mouse SPNS2 Functions as a Sphingosine-1-Phosphate Transporter in Vascular Endothelial Cells. *PLoS One* 7, e38941
- Hla, T., Lee, M.J., Ancellin, N., Paik, J.H., Kluk, M.J. (2001) Lysophospholipids--receptor revelations. *Science* 294, 1875-1878
- Ho, J.W., Man, K., Sun, C.K., Lee, T.K., Poon, R.T., Fan, S.T. (2005) Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma. *Mol. Cancer Ther.* 4, 1430-1438

- Hobisch, A., Culig, Z., Radmayr, C., Bartsch, G., Klocker, H., Hittmair, A. (1995) Distant metastases from prostatic carcinoma express androgen receptor protein. *Cancer Res.* 55, 3068-3072
- Hobson, J.P., Rosenfeldt, H.M., Barak, L.S., Olivera, A., Poulton, S., Caron, M.G., Milstien, S., Spiegel, S. (2001) Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science* 291, 1800-1803
- Hofmann, L.P., Ren, S., Schwalm, S., Pfeilschifter, J., Huwiler, A. (2008) Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. *Biol Chem.* 389, 1399-1407
- Hollstein, M., Sidransky, D., Vogelstein, B., Harris, C.C. (1991) p53 mutations in human cancers. *Science* 253, 49-53
- Holzbeierlein, J., Lal, P., LaTulippe, E., Smith, A., Satagopan, J., Zhang, L., et al. (2004) Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. *Am. J. Pathol.* 164, 217-227
- Hong, S.K., Kim, J.H., Lin, M.F., Park, J.I. (2011) The Raf/MEK/extracellular signal-regulated kinase 1/2 pathway can mediate growth inhibitory and differentiation signaling via androgen receptor downregulation in prostate cancer cells. *Exp. Cell Res.* 317, 2671-2682
- Horinaga, M., Okita, H., Nakashima, J., Kanao, K., Sakamoto, M., Murai, M. (2005) Clinical and pathologic significance of activation of signal transducer and activator of transcription 3 in prostate cancer. *Urology* 66, 671-675
- Horoszewicz, J.S., Leong, S.S., Chu, T.M., Wajsman, Z.L., Friedman, M., Papsidero, L., et al. (1980) The LNCaP cell line-a new model for studies on human prostatic carcinoma. *Prog. Clin. Biol. Res.* 37, 115-132
- Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, et al. (1983) LNCaP model of human prostatic carcinoma. *Cancer Res.* 43, 1809-1818
- Huang, Z.Q., Li, J.W., Wong, J.M. (2002) AR possesses an intrinsic hormone-independent transcriptional activity. *Mol. Endocrinol.* 16, 924-37
- Hung, J.H., Lu, Y.S., Wang, Y.C., Ma, Y.H., Wang, D.S., Kulp, S.K., et al. (2008) FTY720 induces apoptosis in hepatocellular carcinoma cells through activation of protein kinase C delta signaling. *Cancer Res.* 68, 1204-1212
- Huwiler, A., Döll, F., Ren, S., Klawitter, S., Greening, A., Römer, I., et al. (2006) Histamine increases sphingosine kinase-1 expression and activity in the human arterial endothelial cell line EA.hy 926 by a PKC-alpha-dependent mechanism. *Biochim. Biophys. Acta* 1761, 367-376
- Huwiler, A., Kotelevets, N., Xin, C., Pastukhov, O., Pfeilschifter, J., Zangemeister-Wittke, U. (2011) Loss of sphingosine kinase-1 in carcinoma cells increases formation of reactive oxygen species and sensitivity to doxorubicin-induced DNA damage. *Br. J. Pharmacol.* 162, 532-543
- Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K.I., Hirabayashi, Y. (1996) Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc. Natl. Acad. Sci. USA* 93, 4638-4643
- Idkowiak-Baldys, J., Apraiz, A., Li, L., Rahmaniyan, M., Clarke, C.J., Kraveka, J.M., Asumendi, A., Hannun, Y.A. (2010) Dihydroceramide desaturase activity is modulated by oxidative stress. *Biochem. J.* 427, 265-274

- 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, 46832-46839
- Illuzzi, G., Bernacchioni, C., Aureli, M., Prioni, S., Frera, G., Donati, C., et al. (2010) Sphingosine kinase mediates resistance to the synthetic retinoid N-(4-hydroxyphenyl)retinamide in human ovarian cancer cells. *J. Biol. Chem.* 285, 18594-18602
- 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, 483-492
- Jin, S., White, E. (2008) Tumor suppression by autophagy through the management of metabolic stress. *Autophagy* 4, 563-566
- 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 tumour lung tissue. *J. Histochem. Cytochem.* 53, 1159-1166
- Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., et al. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 19, 5720-5728
- Kai, L., Levenson, A.S. (2011) Combination of resveratrol and antiandrogen flutamide has synergistic effect on androgen receptor inhibition in prostate cancer cells. *Anticancer Res.* 31, 3323-3330
- Karan, D., Kelly, D.L., Rizzino, A., Lin, M.F., Batra, S.K. (2002) Expression profile of differentially-regulated genes during progression of androgen-independent growth in human prostate cancer cells. *Carcinogenesis* 23, 967-975
- Kawamori, T., Osta, W., Johnson, K.R., Pettus, B.J., Bielawski, J., Tanaka, T., Wargovich, M.J., Reddy, B.S., Hannun, Y.A., Obeid, L.M., Zhou D. (2006) Sphingosine kinase 1 is up-regulated in colon carcinogenesis. *FASEB J.* 20, 386-388
- 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, 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, 4532-4539
- Kim, E.S., Kim, J.S., Kim, S.G., Hwang, S., Lee, C.H., Moon, A. (2011) Sphingosine 1-phosphate regulates matrix metalloproteinase-9 expression and breast cell invasion through S1P3-Gαq coupling. *J. Cell. Sci.* 124, 2220-2230
- Klionsky, D.J., Emr, S.D. (2000) Autophagy as a regulated pathway of cellular degradation. Science 290, 1717-1721
- 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, 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, 23722-23728

- Kohno, M., Momoi, M., Oo, M.L., Paik, J.H., Lee, Y.M., Venkataraman, K., Ai, Y., Ristimaki, A. P., Fyrst, H., Sano, H., Rosenberg, D., Saba, J.D., Proia, R.L., Hla, T. (2006) Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation. *Mol. Cell. Biol.* 26, 7211-7223
- Koivisto, P., Kononen, J., Palmberg, C., Tammela, T., Hyytinen, E., Isola, J., et al. (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res.* 57, 314-319
- Kokontis, J., Takakura, K., Hay, N., Liao, S. (1994) Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. *Cancer Res.* 54, 1566-1573
- Kolesnick, R.N., Hemer, M.R. (1990) Characterization of a ceramide kinase activity from human leukemia (HL-60) cells. Separation from diacylglycerol kinase activity. *J. Biol. Chem.* 265, 18803-18808
- Kon, J., Sato, K., Watanabe, T., Tomura, H., Kuwabara, A., Kimura, T., et al. (1999) Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J. Biol. Chem.* 274, 23940-23947
- Kondoh, H. (2008) Cellular life span and the Warburg effect. *Exp. Cell. Res.* 314, 1923-1928
- Kreisberg, J.I., Malik, S.N., Prihoda, T.J., Bedolla, R.G., Troyer, D.A., Kreisberg, S., Ghosh, P.M. (2004) Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer Res.* 64, 5232-5236
- Kumar, A., Byun, H.S., Bittman, R., Saba, J.D. (2011) The sphingolipid degradation product trans-2-hexadecenal induces cytoskeletal reorganization and apoptosis in a JNK-dependent manner. *Cell. Signal.* 23, 1144-1152
- Lahiri, S., Park, H., Laviad, E.L., Bittman, R., Futerman, A.H. (2009) Ceramide synthesis is modulated by the sphingosine analogue FTY720 via a mixture of uncompetitive and noncompetitive inhibition of acyl-CoA chain length dependent manner. *J. Biol. Chem.* 284, 16090-16098
- LaMontagne, K., Littlewood-Evans, A., Schnell, C. et al. (2006) Antagonism of sphingosine 1-phosphate receptors by FTY720 inhibits angiogenesis and tumour vascularisation. *Cancer Res.* 66, 221-231
- Latil, A., Bièche, I., Vidaud, D., Lidereau, R., Berthon, P., Cussenot, O., Vidaud, M. (2001) Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. *Cancer Res.* 61, 1919-1926
- Laviad, E.L., Albee, L., Pankova-Kholmyansky, I., Epstein, S., Park, H., Merrill, A.H. Jr, Futerman, A.H. (2008) Characterization of ceramide synthase 2: tissue distribution, substrate specificity, and inhibition by sphingosine 1-phosphate. *J. Biol. Chem.* 283, 5677-5684
- 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, 8518-8527

- Lavieu, G., Scarlatti, F., Sala, G., Carpentier, S., Levade, T., Ghidoni, R., Botti, J., Codogno, P. (2008) Sphingolipids in macroautophagy. *Methods Mol. Biol.* 445, 159-173
- Leclercq, T.M., Moretti, P.A., Vadas, M.A., Pitson, S.M. (2008) Eukaryotic elongation factor 1A interacts with sphingosine kinase and directly enhances its catalytic activity. J. Biol. Chem. 283, 9606-9614
- Leclercq, T.M., Moretti, P.A., Pitson, S.M. (2011) Guanine nucleotides regulate sphingosine kinase 1 activation by eukaryotic elongation factor 1A and provide a mechanism for eEF1A-associated oncogenesis. *Oncogene* 30, 372-378
- Lee, H., Deng, J., Kujawski, M., Yang, C., Liu, Y., Herrmann, A., et al. (2010) STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. *Nat. Med.* 16, 1421-1428
- Lee, J.G., Zheng, R., McCafferty-Cepero, J.M., Burnstein, K.L., Nanus, D.M., Shen, R. (2009) Endothelin-1 enhances the expression of the androgen receptor via activation of the c-myc pathway in prostate cancer cells. *Mol. Carcinog.* 48, 141-149
- Lee, J.T., Lehmann, B.D., Terrian, D.M., Chappell, W.H., Stivala, F., Libra, M., et al. (2008) Targeting prostate cancer based on signal transduction and cell cycle pathways. *Cell Cycle* 7, 1745-1762
- 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, 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, 301-312
- Lepley, D., Paik, J.H., Hla, T., Ferrer, F. (2005) The G protein-coupled receptor S1P2 regulates Rho/Rho kinase pathway to inhibit tumor cell migration. *Cancer Res.* 65, 3788-3795
- Le Stunff, H., Giussani, P., Maceyka, M., Lépine, S., Milstien, S., Spiegel, S. (2007) Recycling of sphingosine is regulated by the concerted actions of sphingosine-1-phosphate phosphohydrolase 1 and sphingosine kinase 2. *J. Biol. Chem.* 282, 34372-34380
- Li, M., Jiang, X., Liu, D., Na, Y., Gao, G.F., Xi, Z. (2008a) Autophagy protects LNCaP cells under androgen deprivation conditions. *Autophagy* 4, 54-60
- Li, Q. F., Wu, C. T., Guo, Q., Wang, H., Wang, L. S. (2008b) Sphingosine 1-phosphate induces Mcl-1 upregulation and protects multiple myeloma cells against apoptosis. *Biochem. Biophys. Res. Commun.* 371, 159-162
- Li, W., Yu, C.P., Xia, J.T., Zhang, L., Weng, G.X., Zheng, H.Q., et al. (2009) Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. *Clin. Cancer Res.* 15, 1393-1399
- Li, X., Becker, K.A., Zhang, Y. (2010) Ceramide in redox signaling and cardiovascular diseases. *Cell Physiol Biochem.* 26, 41-48
- Liao, X., Tang, S., Thrasher, J.B., Griebling, T.L., Li, B. (2005) Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol. Cancer Ther.* 4, 505-515

- Libertini, S.J., Tepper, C.G., Rodriguez, V., Asmuth, D.M., Kung, H.J., Mudryj, M. (2007) Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence. *Cancer Res.* 67, 9001-9005
- 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, 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, 18633-18640
- Lim, K.G., Gray, A.I., Pyne, S., Pyne, N.J. (2012a) Resveratrol Dimers Are Novel Sphingosine Kinase 1 Inhibitors and Affect Sphingosine Kinase 1 Expression and Cancer Cell Growth and Survival. *Br. J. Pharmacol.* 166, 1605-1616
- Lim, K.G., Tonelli, F., Berdyshev, E., Gorshkova, I., Leclercq, T., Pitson, S.M., Bittman, R., Pyne, S., Pyne, N.J. (2012b) Inhibition kinetics and regulation of sphingosine kinase 1 expression in prostate cancer cells: Functional differences between sphingosine kinase 1a and 1b. *Int. J. Biochem. Cell. Biol.* 44, 1457-1464
- Limaye, V., Li, X., Hahn, C., Xia, P., Berndt, M.C., Vadas, M.A., Gamble, J.R. (2005) Sphingosine kinase-1 enhances endothelial cell survival through a PECAM-1-dependent activation of PI-3K/Akt and regulation of Bcl-2 family members. *Blood* 105, 3169-3177
- Lin, H.K., Yeh, S.Y., Kang, H.Y., Chang, C.S. (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc. Natl. Acad. Sci. USA* 98, 7200-7205
- Lin, H.K., Wang, L., Hu, Y.C., Altuwaijri, S., Chang, C. (2002) Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *EMBO J.* 21, 4037-4048
- Lin, H.K., Hu, Y.C., Yang, L., Altuwaijri, S., Chen, Y.T., Kang, H.Y., Chang, C. (2003) Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J. Biol. Chem.* 278, 50902-50907
- Lin, S.S., Bassik, M.C., Suh, H., Nishino, M., Arroyo, J.D., Hahn, W.C., et al. (2006) PP2A regulates BCL-2 phosphorylation and proteasome-mediated degradation at the endoplasmic reticulum. *J. Biol. Chem.* 281, 23003-23012
- Lindzey, J., Kumar, M.V., Grossmann, M., Young, C., Tindall, D.J. (1994) Molecular mechanisms of androgen action. *Vitam. Horm.* 49, 383-432
- Linja, M.J., Savinainen, K.J., Saramäki, O.R., Tammela, T.L., Vessella, R.L., Visakorpi, T. (2001) Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.* 61, 3550-3555
- Liu, B., Obeid, L., Hannun, Y. (1998) Sphingomyelinases in cell regulation. *Semin. Cell. Dev. Biol.* 8, 311-322
- Liu, B., Chen, Y., St Clair, D.K. (2008) ROS and p53: a versatile partnership. *Free Radic. Biol. Med.* 44, 1529-1535
- 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, 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, 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, 951-961
- Locke, J.A., Guns, E.S., Lubik, A.A., et al. (2008) Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration resistant prostate cancer. Cancer Res. 68, 6407-6415
- Long, J.S., Natarajan, V., Tigyi, G., Pyne, S., Pyne, N.J. (2006) The functional PDGFβ receptor-S1P1 receptor signalling complex is involved in regulating migration of mouse embryonic fibroblasts in response to platelet derived growth factor. *Prostaglandins Other Lipid Mediat.* 80, 1920-1929
- 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, 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, 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, 38841-38852
- Lu, S., Ren, C., Liu, Y., Epner, D.E. (2006) PI3K/Akt signaling is involved in the regulation of p21(WAF/CIP) expression and androgen-independent growth in prostate cancer cells. *Int. J. Oncol.* 28, 245-251
- 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, 3192-3195
- 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, 37118-37129
- Maceyka, M., Alvarez, S.E., Milstien, S., Spiegel, S. (2008) Filamin A links sphingosine kinase 1 and sphingosine-1-phosphate receptor 1 at lamellipodia to orchestrate cell migration. *Mol. Cell. Biol.* 28, 5687-5697
- MacLean, H.E., Warne, G.L., Zajac, J.D. (1997) Localization of functional domains in the androgen receptor. *J. Steroid Biochem. Mol. Biol.* 62, 233-242
- Maki, C.G., Huibregtse, J.M., Howley, P.M. (1996) In vivo ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res.* 56, 2649-2654

- Malavaud, B., Pchejetski, D., Mazerolles, C., de Paiva, G.R., Calvet, C., Doumerc, N., Pitson, S., Rischmann, P., Cuvillier, O. (2010) Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. *Eur. J. Cancer* 46, 3417-3424
- Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., et al. (2002) Alteration of lymphocyte trafficking by sphingosine 1-phosphate receptor agonists. *Science* 296, 346-349
- Mandon, E.C., Ehses, I., Rother, J., van Echten, G., Sandhoff, K. (1992) Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine N-acyltransferase in mouse liver. *J. Biol. Chem.* 267, 11144-11148
- Mangelsdorf, D.J., Thummel, G., Beato, M., et al. (1995) The nuclear receptor superfamily: the second decade. *Cell* 83, 835-839
- Marcelli, M., Ittmann, M., Mariani, S., Sutherland, R., Nigam, R., Murthy, L., et al. (2000) Androgen receptor mutations in prostate cancer. *Cancer Res.* 60, 944-949
- Marfe, G., Di Stefano, C., Gambacurta, A., Ottone, T., Martini, V., Abruzzese, E., et al. (2011) Sphingosine kinase 1 overexpression is regulated by signaling through PI3K, AKT2, and mTOR in imatinib-resistant chronic myeloid leukemia cells. *Exp. Hematol.* 39, 653-665
- 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, 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, 355-360
- Matsuoka, Y., Nagahara, Y., Ikekita, M., Shinomiya, T. (2003) A novel immunosuppressive agent FTY720 induced AKT dephosphorylation in leukemia cells. *Br. J. Pharmacol.* 138, 1303-1312
- Mattie, M., Brooker, G., Spiegel, S. (1994) Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. *J. Biol. Chem.* 269, 3181-3188
- McCall, P., Gemmell, L.K., Mukherjee, R., Bartlett, J.M., Edwards, J. (2008) Phosphorylation of the androgen receptor is associated with reduced survival in hormone-refractory prostate cancer patients. *Br. J. Cancer* 98, 1094-1101
- McDonnell, T.J., Troncoso, P., Brisbay, S.M., Logothetis, C., Chung, L.W., Hsieh, J.T., et al. (1992) Expression of the proto-oncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.* 52, 6940-6944.
- 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, 19-26
- Mellinghoff, I.K., Vivanco, I., Kwon, A., Tran, C., Wongvipat, J., Sawyers, C.L. (2004) HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell* 6, 517-527

- Merrill, A.H. Jr, van Echten, G., Wang, E., Sandhoff, K. (1993) Fumonisin B1 inhibits sphingosine (sphinganine) N-acyltransferase and de novo sphingolipid biosynthesis in cultured neurons in situ. *J. Biol. Chem.* 268, 27299-27306
- Merrill, A.H. Jr, Sullards, M.C., Wang, E., Voss, K.A., Riley, R.T. (2001) Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environ. Health Perspect.* 2, 283-289
- Meyer zu Heringdorf, D., Lass, H., Kuchar, I., Lipinski, M., Alemany, R., Rümenapp, U., Jakobs, K.H. (2001) Stimulation of intracellular sphingosine-1-phosphate production by G-protein-coupled sphingosine-1-phosphate receptors. Eur. J. Pharmacol. 414, 145-154
- Meyer zu Heringdorf, D., Liliom, K., Schaefer, M., Danneberg, K., Jaggar, J.H., Tigyi, G., Jakobs, K.H. (2003) Photolysis of intracellular caged sphingosine-1-phosphate causes Ca2+ mobilization independently of G-protein-coupled receptors. *FEBS Lett.* 554, 443-449
- Meyer Zu Heringdorf, D. (2004) Lysophospholipid receptor-dependent and -independent calcium signaling. J. Cell. Biochem. 92, 937-948
- Michel, C., van Echten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., Merrill, A.H. Jr. (1997) Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide. *J. Biol. Chem.* 272, 22432-22437
- Miller, D.J., Jerga, A., Rock, C.O., White, S.W. (2008) Analysis if the Staphylococcus aureus DgKB structure reveals a common catalytic mechanism for the soluble diacylglycerol kinase. *Structure* 16, 1036-1046
- Mitchell, S.H., Zhu, W., Young, C.Y. (1999) Resveratrol inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. *Cancer Res.* 59, 5892-5895
- Mitra, P., Oskeritzian, C.A., Payne, S.G., Beaven, M.A., Milstien, S. and Spiegel, S. (2006) Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16394-16399
- Mizokami, A., Yeh, S.Y., Chang, C. (1994) Identification of 3',5'-cyclic adenosine monophosphate response element and other cis-acting elements in the human androgen receptor gene promoter. *Mol. Endocrinol.* 8, 77-88
- 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, 11113-11121
- Mizushima N. (2004) Methods for monitoring autophagy. *Int. J. Biochem. Cell. Biol.* 36, 2491-2502
- Mizushima, N., Yoshimori, T., Levine, B. (2010) Methods in mammalian autophagy research. *Cell* 140, 313-326
- Montgomery, R.B., Mostaghel, E.A., Vessella, R., Hess, D.L., Kalhorn, T.F., Higano, C.S., et al. (2008) Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. *Cancer Res.* 68, 4447-4454
- Mukherjee, R., McGuinness, D.H., McCall, P., Underwood, M.A., Seywright, M., Orange, C., Edwards, J. (2011) Upregulation of MAPK pathway is associated with survival in castrate-resistant prostate cancer. *Br. J. Cancer* 104, 1920-1928

- Mulders, A.C., Nau, S., Li, Y., Michel, M.C. (2007) Effects of sphingosine-1-phosphate and sphingosylphosphorylcholine on intracellular Ca2+ and cell death in prostate cancer cell lines. *Auton. Autacoid Pharmacol.* 27, 173-179
- Murillo, H., Huang, H., Schmidt, L.J., Smith, D.I., Tindall, D.J. (2001) Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* 142, 4795-4805
- Nagaoka, Y., Otsuki, K., Fujita, T., Uesato, S. (2008) Effects of phosphorylation of immunomodulatory agent FTY720 (fingolimod) on antiproliferative activity against breast cancer and colon cancer cells. *Biol. Pharm. Bull.* 31, 1177-1181
- Nakade, Y., Banno, Y., T-Koizumi, K., Hagiwara, K., Sobue, S., Koda, M., et al. (2003) Regulation of sphingosine kinase 1 gene expression by protein kinase C in a human leukemia cell line, MEG-O1. *Biochim. Biophys. Acta* 1635, 104-116
- Nava, V.E., Lacana, E., Poulton, S., Liu, H., Sugiura, M., Kono, K., Milstien, S., Kohama, T., Spiegel, S. (2000a) Functional characterization of human sphingosine kinase-1. *FEBS Lett.* 473, 81-84
- Nava, V.E., Cuvillier, O., Edsall, L.C., Kimura, K., Milstien, S., Gelmann, E.P., Spiegel, S. (2000b) Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer Res.* 60, 4468-4474
- Nava, V.E., Hobson, J.P., Murthy, S., Milstien, S., Spiegel, S. (2002) Sphingosine kinase type 1 promotes estrogen-dependent tumourigenesis of breast cancer MCF-7 cells. *Exp. Cell Res.* 281, 115-127
- Neviani, P., Santhanam, R., Oaks, J.J., et al. (2007) FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J. Clin. Invest.* 117, 2408-2421
- Nichols, C.E., Lamb, H.K., Lockyer, M., Charles, I.G., Pyne, S., Hawkins, A.R., et al. (2007) Characterisation of salmonella typhimurium YegS, a putative lipid kinase homologous to eukaryotic sphingosine and diacylglycerol kinase. *Proteins* 68, 13-25
- Obeng, E.A., Carlson, L.M., Gutman, D.M., Harrington, W.J. Jr, Lee, K.P., Boise, L.H. (2006) Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. *Blood* 107, 4907-4916
- Ogretmen, B., Hannun, Y.A. (2004) Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* 4, 604-616
- Ohotski, J., Long, J.S., Orange, C., Elsberger, B., Mallon, E., Doughty, J., Pyne, S., Pyne, N.J., Edwards, J. (2012) Expression of sphingosine 1-phosphate receptor 4 and sphingosine kinase 1 is associated with outcome in oestrogen receptor-negative breast cancer. *Br. J. Cancer.* 106, 1453-1459
- Okada, T., Ding, G., Sonoda, H., Kajimoto, T., Haga, Y., Khosrowbeygi, A., et al. (2005) Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. *J. Biol. Chem.* 280, 36318-36325
- Okamoto H, Takuwa N, Gonda K, Okazaki H, Chang K, Yatomi Y, et al. (1998) EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca2+ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* 273, 27104-27110

- 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, 9247-9261
- Okazaki, T., Bell, R.M., Hannun, Y.A. (1989) Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. Role in cell differentiation. *J. Biol. Chem.* 264, 19076-19080
- Olivera, A., Spiegel, S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* 365, 557-560
- Olivera, A., Kohama, T., Tu, Z., Milstien, S., Spiegel, S. (1998) Purification and characterization of rat kidney sphingosine kinase. *J. Biol. Chem.* 273, 12576-12583
- Olivera, A., Kohama, T., Edsall, L., Nava, V., Cuvillier, O., Poulton, S., Spiegel, S. (1999) Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J. Cell. Biol.* 147, 545-558
- Olivera, A., Rosenfeldt, H.M., Bektas, M., Wang, F., Ishii, I., Chun, J., Milstien, S., Spiegel, S. (2003) Sphingosine kinase type 1 induces G12/13-mediated stress fiber formation yet promotes growth and survival independent of G protein-coupled receptors. *J. Biol. Chem.* 278, 46452-46460
- Omar, H.A., Chou, C.C., Berman-Booty, L.D., Ma, Y., Hung, J.H., Wang, D., et al. (2011) Antitumor effects of OSU-2S, a nonimmunosuppressive analogue of FTY720, in hepatocellular carcinoma. *Hepatology* 53, 1943-1958
- Oskouian, B., Saba, J.D. (2010) Cancer treatment strategies targeting sphingolipid metabolism. *Adv. Exp. Med. Biol.* 688, 185-205
- Paik, J.H., Skoura, A., Chae, S.S., Cowan, A.E., Han, D.K., Proia, R.L., Hla, T. (2004) Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. *Genes Dev.* 18, 2392-2403
- Pappu, R., Schwab, S.R., Cornelissen, I., Pereira, J.P., Regard, J.B., Xu, Y., et al. (2007) Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* 316, 295-298
- Park, K.S., Kim, M.K., Lee, H.Y., Kim, S.D., Lee, S.Y., Kim, J.M., et al. (2007) S1P stimulates chemotactic migration and invasion in OVCAR3 ovarian cancer cells. *Biochem. Biophys. Res. Comm.* 356, 239-244
- Pastorino, J.G., Tafani, M., Rothman, R.J., Marcinkeviciute, A., Hoek, J.B., Farber, J.L. (1999) Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. *J. Biol. Chem.* 274, 31734-31739
- Paugh, S.W., Payne, S.G., Barbour, S.E., Milstien, S., Spiegel, S. (2003) The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS Lett.* 554, 189-193
- Payne, S.G., Oskeritzian, C.A., Griffiths, R., 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, 1077-1085
- Pchejetski, D., Golzio, M., Bonhoure, E., Calvet, C., Doumerc, N., Garcia, V., Mazerolles, C., Rischmann, P., Teissié, J., Malavaud, B. and Cuvillier, O.

(2005) Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models. *Cancer Res.* 65, 11667-11675

- Pchejetski, D., Doumerc, N., Golzio, M., Naymark, M., Teissié, J., Kohama, T., Waxman, J., Malavaud, J., Cuvillier, O. (2008) Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. *Mol. Cancer Ther.* 7, 1836-1845
- Pchejetski, D., Bohler, T., Brizuela, L., Sauer, L., Doumerc, N., Golzio, M., Salunkhe, V., Teissié, J., Malavaud, B., Waxman, J., Cuvillier, O. (2010) FTY720 (fingolimod) sensitizes prostate cancer cells to radiotherapy by inhibition of sphingosine kinase-1. *Cancer Res.* 70, 8651-8661
- Pébay, A., Toutant, M., Prémont, J., Calvo, C.F., Venance, L., Cordier, J., et al. (2001) Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. *Eur. J. Neurosci.* 13, 2067-2076
- Pelley, R.P., Chinnakannu, K., Murthy, S., Strickland, F.M., Menon, M., Dou, Q.P., et al. (2006) Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. *Cancer Res.* 66, 11754-11762
- Pernicová, Z., Slabáková, E., Kharaishvili, G., Bouchal, J., Král, M., Kunická, Z., et al. (2011) Androgen depletion induces senescence in prostate cancer cells through down-regulation of Skp2. *Neoplasia* 13, 526-536
- Pfeil, K., Eder, I.E., Putz, T., Ramoner, R., Culig, Z., et al. (2004) Long-term androgen-ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. *Prostate* 58, 259-268
- Pilorget, A., Demeule, M., Barakat, S., Marvaldi, J., Luis, J., Béliveau, R. (2007) Modulation of P-glycoprotein function by sphingosine kinase-1 in brain endothelial cells. J. Neurochem. 100, 1203-1210
- Pitman, M.R., Barr, R.K., Gliddon, B.L., Magarey, A.M., Moretti, P.A., Pitson, S.M. (2011) A critical role for the protein phosphatase 2A B'α regulatory subunit in dephosphorylation of sphingosine kinase 1. *Int. J. Biochem. Cell. Biol.* 43, 342-347
- 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,, 429-441
- 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, 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, 49545-49553
- 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, 5491-5500
- 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, 49-54
- Pitson, S.M. (2011) Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem. Sci.* 36, 97-107
- Pyne, N.J., Pyne, S. (2010) Sphingosine 1-phosphate and cancer. *Nat. Rev. Cancer* 10, 489-503
- Pyne, S., Kong, K.C., Darroch, P.I. (2004) Lysophosphatidic acid and sphingosine 1-phosphate biology: the role of lipid phosphate phosphatases. *Semin. Cell Dev. Biol.* 15, 491-501
- Pyne, S., Long, J.S., Ktistakis, N.T., Pyne, N.J. (2005) Lipid phosphate phosphatases and lipid phosphate signalling. Biochem. Soc. Trans. 33, 1370-1374
- 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, 14-21
- Rakhit, S., Conway, A.-M., Tate, R., Bower, T., Pyne, N.J., Pyne, S. (1999) Sphingosine-1-phosphate stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle. *Biochem. J.* 338, 643-649
- Radeff-Huang, J., Seasholtz, T.M., Matteo, R.G., Brown, J.H. (2004) G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J. Cell. Biochem.* 92, 949-966
- Raffo, A.J., Perlman, H., Chen, M.W., Day, M.L., Streitman, J.S., Buttyan, R. (1995) Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Res.* 55, 4438-4445
- Ren, S., Xin, C., Pfeilschifter, J., Huwiler, A. (2010) A novel mode of action of the putative sphingosine kinase inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI II): induction of lysosomal sphingosine kinase 1 degradation. *Cell. Physiol. Biochem.* 26, 97-104
- Rigogliuso, S., Donati, C., Cassarà, D., Taverna, S., Salamone, M., Bruni, P., Vittorelli, M.L. (2010) An active form of sphingosine kinase-1 is released in the extracellular medium as component of membrane vesicles shed by two human tumor cell lines. *J. Oncol.* 2010:509329
- Romero Rosales, K., Singh, G., Wu, K., Chen, J., Janes, M.R., Lilly, M.B., et al. (2011) Sphingolipid-based drugs selectively kill cancer cells by down-regulating nutrient transporter proteins. *Biochem J.* 439, 299-311
- Roy, V., Perez, E.A. (2009) Beyond trastuzumab: small molecule tyrosine kinase inhibitors in HER-2-positive breast cancer. *Oncologist* 14, 1061-1069
- Ruckhäberle, E., Rody, A., Engels, K., Gaetje, R., von Minckwitz, G., Schiffmann, S., et al. (2008) Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. *Breast Cancer Res. Treat.* 112, 41-52
- Ruizeveld de Winter, J.A., Janssen, P.J., Sleddens, H.M., Verleun-Mooijman, M.C., Trapman, J., Brinkmann, A.O., et al. (1994) Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. *Am. J. Pathol.* 144, 735-746

- Sadi, M.V., Walsh, P.C., Barrack, E.R. (1991) Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy. *Cancer* 67, 3057-3064
- Salas, A., Ponnusamy, S., Senkal, C.E., Meyers-Needham, M., Selvam, S.P., Saddoughi, S.A., Apohan, E., Sentelle, R.D., Smith, C., Gault, C.R., Obeid, L.M., El-Shewy, H.M., Oaks, J., Santhanam, R., Marcucci, G., Baran, Y., Mahajan, S., Fernandes, D., Stuart, R., Perrotti, D., Ogretmen, B. (2011) Sphingosine kinase-1 and sphingosine 1-phosphate receptor 2 mediate Bcr-Abl1 stability and drug resistance by modulation of protein phosphatase 2A. *Blood* 117, 5941-5952
- Sanchez, T., Estrada-Hernandez, T., Paik, J.H., Wu, M.T., Venkataraman, K., Brinkmann, V., Claffey, K., Hla, T. (2003) Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J. Biol. Chem.* 278, 47281-47290
- Sankala, H.M., Hait, N.C., Paugh, S.W., Shida, D., Lepine, S., Elmore, L., Dent, W., Milstien, S., Spiegel, S. (2007) Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. *Cancer Res.* 67, 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, 5313-5317
- Sato, K., Malchinkhuu, E., Horiuchi, Y., Mogi, C., Tomura, H., Tosaka, M., et al. (2007) Critical role of ABCA1 transporter in sphingosine 1-phosphate release from astrocytes. *J. Neurochem.* 103, 2610-2619
- Sauer, B., Gonska, H., Manggau, M., Kim, D.S., Schraut, C., Schäfer-Korting, M., Kleuser, B. (2005) Sphingosine 1-phosphate is involved in cytoprotective actions of calcitriol in human fibroblasts and enhances the intracellular Bcl-2/Bax rheostat. Pharmazie 60, 298-304
- Sauer, L., Nunes, J., Salunkhe, V., Skalska, L., Kohama, T., Cuvillier, O., Waxman, J., Pchejetski, D. (2009) Sphingosine kinase 1 inhibition sensitizes hormone-resistant prostate cancer to docetaxel. *Int. J. Cancer* 125, 2728-2736
- Scarlatti, F., Bauvy, C., Ventruti, A., Sala, G., Cluzeaud, F., Vandewalle, A., et al. (2004) Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. *J. Biol. Chem.* 279, 18384-18391
- Scarlatti, F., Sala, G., Ricci, C., Maioli, C., Milani, F., Minella, M., et al. (2007) Resveratrol sensitization of DU145 prostate cancer cells to ionizing radiation is associated to ceramide increase. *Cancer Lett.* 253, 124-130
- Schefe, J.H., Lehmann, K.E., Buschmann, I.R., Unger, T., Funke-Kaiser, H. (2006) Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. *J. Mol. Med.* 84, 901-910
- Schnitzer, S.E., Weigert, A., Zhou, J., Brüne, B. (2009) Hypoxia enhances sphingosine kinase 2 activity and provokes sphingosine 1-phosphate-mediated chemoresistance in A549 lung cancer cells. *Mol. Cancer Res.* 7, 393-401
- Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., Cyster, J.G. (2005) Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* 309, 1735-1739

- Sekine, Y., Suzuki, K., Remaley, A.T. (2011) HDL and sphingosine-1-phosphate activate stat3 in prostate cancer DU145 cells via ERK1/2 and S1P receptors, and promote cell migration and invasion. *Prostate* 71, 690-699
- Shang, Y., Myers, M., Brown, M. (2002) Formation of the androgen receptor transcription complex. *Mol. Cell.* 9, 601-610
- Sheflin, L., Keegan, B., Zhang, W., Spaulding, S.W. (2000) Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. *Biochem. Biophys. Res. Commun.* 276, 144-150
- Shi, D.Y., Xie, F.Z., Zhai, C., Stern, J.S., Liu, Y., Liu, S.L. (2009) The role of cellular oxidative stress in regulating glycolysis energy metabolism in hepatoma cells. *Mol. Cancer* 8:32
- Shi, X.B., Ma, A.H., Tepper, C.G., Xia, L., Gregg, J.P., Gandour-Edwards, R., et al. (2004) Molecular alterations associated with LNCaP cell progression to androgen independence. *Prostate* 60, 257-271
- Shida, D., Kitayama, J., Yamaguchi, H., Yamashita, H., Mori, K., Watanabe, T., et al. (2004) Sphingosine 1-phosphate transactivates c-Met as well as epidermal growth factor receptor (EGFR) in human gastric cancer cells. *FEBS Lett.* 577, 333-338
- Shida, D., Takabe, K., Kapitonov, D., Milstien, S., Spiegel, S. (2008) Targeting SphK1 as a new strategy against cancer. *Curr. Drug Targets* 9, 662-673
- Shirahama, T., Sweeney, E.A., Sakakura, C., Singhal, A.K., Nishiyama, K., Akiyama, S., et al. (1997) In vitro and in vivo induction of apoptosis by sphingosine and N,N-dimethylsphingosine in human epidermoid carcinoma KB-3-1 and its multidrug-resistant cells. *Clin. Cancer Res.* 3, 257-264
- 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, 7758-7768
- Siegel, R., Naishadham, D., Jemal, A. (2012) Cancer statistics, 2012. *CA Cancer J. Clin.* 2012 62, 10-29
- Signorelli, P., Munoz-Olaya, J.M., Gagliostro, V., Casas, J., Ghidoni, R., Fabriàs, G. (2009) Dihydroceramide intracellular increase in response to resveratrol treatment mediates autophagy in gastric cancer cells. *Cancer Lett.* 282, 238-243
- Signoretti, S., Montironi, R., Manola, J., Altimari, A., Tam, C., Bubley, G., et al. (2000) HER-2-neu expression and progression toward androgen independence in human prostate cancer. *J. Natl. Cancer Inst.* 92, 1918-1925
- Siow, D.L., Anderson, C.D., Berdyshev, E.V., Skobeleva, A., Pitson, S.M., Wattenberg, B.W. (2010) Intracellular localization of sphingosine kinase 1 alters access to substrate pools but does not affect the degradative fate of sphingosine-1-phosphate. *J. Lipid Res.* 51, 2546-2559
- Slife, C.W., Wang, E., Hunter, R., Wang, S., Burgess, C., Liotta, D.C., Merrill, A.H. Jr. (1989) Free sphingosine formation from endogenous substrates by a liver plasma membrane system with a divalent cation dependence and a neutral pH optimum. *J. Biol. Chem.* 264, 10371-10377
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., et al. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85

- Smith, C.D., French, K.J., and Zhuang, Y. (2008) inventors; Apogee Biotechnology Corporation, assignee. Sphingosine kinase inhibitors. U.S. Patent 7,338,961. 2008 Mar 4
- Snoek, R., Cheng, H., Margiotti, K., Wafa, L.A., Wong, C.A., Wong, E.C., et al. (2009) *In vivo* knockdown of the androgen receptor results in growth inhibition and regression of well-established, castration-resistant prostate tumors. *Clin. Cancer Res.* 15, 39-47
- Sobue, S., Iwasaki, T., Sugisaki, C., et al. (2006) Quantitative RT-PCR analysis of sphingolipid metabolic enzymes in acute leukemia and myelodysplastic syndromes. *Leukemia* 20, 2042-2046
- Sobue, S., Nemoto, S., Murakami, M., Ito, H., Kimura, A., Gao, S., et al. (2008) Implications of sphingosine kinase 1 expression level for the cellular sphingolipid rheostat: relevance as a marker for daunorubicin sensitivity of leukaemia cells. *Int. J. Haematol.* 87, 266-275
- Soldi, R., Mandinova, A., Venkataraman, K., Hla, T., Vadas, M., Pitson, S., et al. (2007) Sphingosine kinase 1 is a critical component of the copper-dependent FGF1 export pathway. *Exp. Cell. Res.*313, 3308-3318
- Spiegel, S., Cuvillier, O., Edsall, L., Kohama, T., Menzeleev, R., Olivera, A., Thomas, D., Tu, Z., Van Brocklyn, J., Wang, F. (1998) Roles of sphingosine-1-phosphate in cell growth, differentiation, and death. *Biochemistry (Mosc)* 63, 69-73
- Spiegel, S., Milstien, S. (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell. Biol.* 4, 397-407
- 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, 43030-43038
- Stanbrough, M., Bubley, G.J., Ross, K., Golub, T.R., Rubin, M.A., Penning, T.M., et al. (2006) Increased expression of genes converting adrenal androgens to testosterone in androgen independent prostate cancer. *Cancer Res.* 66, 2815-2825
- 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, 600-612
- Sugiura, M., Kono, K., Liu, H., Shimizugawa, T., Minekura, H., Spiegel, S., Kohama, T. (2002) Ceramide kinase, a novel lipid kinase. Molecular cloning and functional characterization. *J. Biol. Chem.* 277, 23294-23300
- 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, 2002-2012
- 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, 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 signalling pathway. *Endocrinology* 150, 4484-4492

- Sun, M., Yang, L., Feldman, R.I., Sun, X.M., Bhalla, K.N., et al. (2003) Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. *J. Biol. Chem.* 278, 42992-43000
- Sun, S., Sprenger, C.C., Vessella, R.L., Haugk, K., Soriano, K., et al. (2010) Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *J. Clin. Invest.* 120, 2715-2730
- Sweeney, E.A., Sakakura, C., Shirahama, T., Masamune, A., Ohta, H., Hakomori, S., Igarashi, Y. (1996) Sphingosine and its methylated derivative N, N-dimethylsphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. *Int. J. Cancer* 66, 358-366
- Tafesse, F.G., Ternes, P., Holthuis, J.C. (2006) The multigenic sphingomyelin synthase family. *J. Biol. Chem.* 281, 29421-29425
- Taha, T.A., Osta, W., Kozhaya, L., Bielawski, J., Johnson, K.R., Gillanders, W.E., Dbaibo, G.S., Hannun, Y.A., Obeid, L.M. (2004) Down-regulation of sphingosine kinase-1 by DNA damage: dependence on proteases and p53. *J. Biol. Chem.* 279, 20546-20554
- Taha, T.A., Kitatani, K., El-Alwani, M., Bielawski, J., Hannun, Y.A., Obeid, L.M. (2006a) 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, 482-484
- Taha, T.A., Hannun, Y.A., Obeid, L.M. (2006b) Sphingosine kinase: biochemical and cellular regulation and role in disease. *J. Biochem. Mol. Biol.* 39, 113-131
- Takabe, K., Paugh, S.W., Milstien, S., Spiegel, S. (2008) "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. *Pharmacol. Rev.* 60, 181-195
- 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, 10477-10486
- Tani, M., Ito, M., Igarashi, Y. (2007) Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. Cell. Signal. 19, 229-237
- Taplin, M.E., Bubley, G.J., Shuster, T.D., Frantz, M.E., Spooner, A.E., Ogata, G.K., et al. (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.* 332, 1393-1398
- Thornton, S., Anand, N., Purcell, D., Lee, J. (2003) Not just for housekeeping: protein initiation and elongation factors in cell growth and tumorigenesis. *J. Mol. Med. (Berl)* 81, 536-548
- Tilley, W.D., Wilson, C.M., Marcelli, M., McPhaul, M.J. (1990) Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res.* 50, 5382-5386
- Tilley, W.D., Lim-Tio, S.S., Horsfall, D.J., Aspinall, J.O., Marshall, V.R., Skinner, J.M. (1994) Detection of discrete androgen receptor epitopes in prostate cancer by immunostaining: measurement by color video image analysis. *Cancer Res.* 54, 4096-4102

- Tilley, W.D., Buchanan, G., Hickey, T.E., Bentel, J.M. (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin. Cancer Res.* 2, 277-285
- 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, 1536-1542
- Trapman, J., Ris-Stalpers, C., van der Korput, J.A., Kuiper, G.G., Faber, P.W., Romijn, J.C., Mulder, E., Brinkmann, A.O. (1990) The androgen receptor: functional structure and expression in transplanted human prostate tumors and prostate tumor cell lines. *J. Steroid Biochem. Mol. Biol.* 37, 837-842
- Ubai, T., Azuma, H., Kotake, Y., Inamoto, T., Takahara, K., Ito, Y., et al. (2007) FTY720 induced Bcl-associated and Fas-independent apoptosis in human renal cancer cells in vitro and significantly reduced in vivo tumour growth in mouse xenograft. *Anticancer Res.* 27, 75-88
- Unni, E., Sun, S., Nan, B., McPhaul, M.J., Cheskis, B., Mancini, M.A., Marcelli, M. (2004) Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res.* 64, 7156-7168
- 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, 442-447
- Valentine, W.J., Kiss, G.N., Liu, J., 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, 1543-1553
- Van Brocklyn, J.R., Lee, M.J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D.M., Coopman, P.J., Thangada, S., Liu, C.H., Hla, T., Spiegel, S. (1998) Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J. Cell. Biol.* 142, 229-240
- Van Brocklyn, J.R., Young, N., Roof, R. (2003) Sphingosine-1-phosphate stimulates motility and invasiveness of human glioblastoma multiforme cells. Cancer Lett. 199, 53-60
- 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, 695-705
- van der Kwast, T.H., Schalken, J., Ruizeveld de Winter, J.A., van Vroonhoven, C.C., Mulder, E., Boersma, W., Trapman, J. (1991) Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int. J. Cancer* 48, 189-193
- van der Kwast, T.H., Tetu, B. (1996) Androgen receptors in untreated and treated prostatic intraepithelial neoplasia. *Eur. Urol.* 30, 265-268
- van Echten-Deckert, G., Zschoche, A., Bär, T., Schmidt, R.R., Raths, A., Heinemann, T., Sandhoff, K. (1997) cis-4-Methylsphingosine decreases

sphingolipid biosynthesis by specifically interfering with serine palmitoyltransferase activity in primary cultured neurons. *J. Biol. Chem.* 272, 15825-15833

- Van Veldhoven, P.P, Mannaerts, G.P. (1993) Sphingosine-phosphate lyase. *Adv. Lipid Res.* 26, 69-98
- Vaupel, P., Mayer, A. (2007) Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev.* 26, 225-239
- Veldscholte, J., Berrevoets, C.A., Ris-Stalpers, C., Kuiper, G.G., Jenster, G., Trapman, J., et al. (1992) The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J. Steroid Biochem. Mol. Biol.* 41, 665-669
- 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, 461-471
- Venkataraman, K., Lee, Y.M., Michaud, J., Thangada, S., Ai, Y., Bonkovsky, H.L., Parikh, N.S., Habrukowich, C., Hla, T. (2008) Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circ. Res. 102, 669-676
- Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., et al. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* 380, 75-79
- 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, 273-279
- Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinänen, R., Palmberg, C., et al. (1995) In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat. Genet.* 9, 401-406
- Visentin, B., Vekich, J.A., Sibbald, B.J., Cavalli, A.L., Moreno, K.M., Matteo, R.G., et al. (2006) Validation of an anti-sphingosine 1-phosphate antibody as a potential therapeutic in reducing growth, invasion and angiogenesis in multiple tumour lineages. *Cancer Cell* 9, 225-238
- Wallington-Beddoe, C.T., Hewson, J., Bradstock, K.F., Bendall, L.J. (2011) FTY720 produces caspase-independent cell death of acute lymphoblastic leukemia cells. *Autophagy* 7, 707-715
- Wang, J.D., Takahara, S., Nonomura, N., Ichimaru, N., Toki, K., Azuma, H., et al. (1999) Early induction of apoptosis in androgen-independent prostate cancer cell line by FTY720 requires caspase-3 activation. *Prostate* 40, 50-55
- Waters, C., Sambi, B., Kong, K.C., Thompson, D., Pitson, S.M., Pyne, S., Pyne, N.J. (2003) Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. J. Biol. Chem. 278, 6282-6290
- 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, 509-511

- Watson, C., 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, S1P1 and S1P3, 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, 2205-2215
- Watson, P.A., Chen, Y.F., Balbas, M.D., Wongvipat, J., Socci, N.D., Viale, A., Kim, K., Sawyers, C.L. (2010) Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. *Proc. Natl. Acad. Sci. USA* 107, 16759-16765
- 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, 1128-1139
- Weigert, A., Schiffmann, S., Sekar, D., Ley, S., Menrad, H., Werno, C., et al. (2009) Sphingosine kinase 2 deficient tumor xenografts show impaired growth and fail to polarize macrophages towards an anti-inflammatory phenotype. *Int. J. Cancer* 125, 2114-2121
- Weigert, A., Cremer, S., Schmidt, M.V., von Knethen, A., Angioni, C., Geisslinger, G., Brüne, B. (2010) Cleavage of sphingosine kinase 2 by caspase-1 provokes its release from apoptotic cells. *Blood* 115, 3531-3540
- Wen, Y., Hu, M.C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D.H., Hung, M.C. (2000) HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res.* 60, 6841-6845
- Westwick, J.K., Bielawska, A.E., Dbaibo, G., Hannun, Y.A., Brenner, D.A. (1995) Ceramide activates the stress-activated protein kinases. J. Biol. Chem. 270, 22689-22692
- Wu, J., Spiegel, S., Sturgill, T.W. (1995) Sphingosine 1-phosphate rapidly activates the mitogen-activated protein kinase pathway by a G protein-dependent mechanism. *J. Biol. Chem.* 270, 11484-11488
- Wu, W., Shu, X., Hovsepyan, H., Mosteller, R.D., Broek, D. (2003) VEGF receptor expression and signaling in human bladder tumors. *Oncogene* 22 3361-3370
- Xia, P., Wang, L., Gamble, J.R., Vadas, M.A. (1999) Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells. *J. Biol. Chem.* 274, 34499-34505
- 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, 1527-1530
- Xin, M., Deng, X. (2006) Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation. *J. Biol. Chem.* 281, 18859-18867
- Yamaguchi, H., Kitayama, J., Takuwa, N., Arikawa, K., Inoki, I., Takehara, K., et al. (2003) Sphingosine-1-phosphate receptor subtype-specific positive and negative regulation of Rac and haematogenous metastasis of melanoma cells. *Biochem. J.* 374, 715-722
- Yamashita, H., Kitayama, J., Shida, D., Yamaguchi, H., Mori, K., Osada, M., et al. (2006) Sphingosine 1-phosphate receptor expression profile in human gastric

cancer cells: differential regulation on the migration and proliferation. *J. Surg. Res.* 130, 80-87

- Yang, H., Murthy, S., Sarkar, F.H., Sheng, S., Reddy, G.P., Dou, Q.P. (2008) Calpain-mediated androgen receptor breakdown in apoptotic prostate cancer cells. *J. Cell. Physiol.* 217, 569-576
- Yeh, S., Lin, H.K., Kang, H.Y., Thin, T.H., Lin, M.F., Chang, C. (1999a) From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc. Natl. Acad. Sci. USA* 96, 5458-5463
- Yeh, S., Chang, H.C., Miyamoto, H., Takatera, H., Rahman, M., Kang, H.Y., et al. (1999b) Differential induction of the androgen receptor transcriptional activity by selective androgen receptor coactivators. *Keio J. Med.* 48, 87-92
- Yeh, S., Tsai, M.Y., Xu, Q., Mu, X.M., Lardy, H., Huang, K.E., et al. (2002) Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc. Natl. Acad. Sci. USA* 99, 13498-13503
- 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, 106-110
- Young, N., Pearl, D.K., Van Brocklyn, J.R. (2009) Sphingosine-1-phosphate regulates glioblastoma cell invasiveness through the urokinase plasminogen activator system and CCN1/Cyr61. *Mol. Cancer Res.* 7, 23-32
- Yuan, X., Li, T., Wang, H., Zhang, T., Barua, M., Borgesi, R.A., et al. (2006) Androgen receptor remains critical for cell cycle progression in androgen-independent CWR22 prostate cancer cells. *Am. J. Pathol.* 169, 682-696
- Zegarra-Moro, O.L., Schmidt, L.J., Huang, H., Tindall, D.J. (2002) Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res.* 62, 1008-1013
- Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G., Spiegel, S. (1991) Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell. Biol.* 114, 155-167
- Zhao, S., Xu, W., Jiang, W., et al. (2010) Regulation of cellular metabolism by protein lysine acetylation. *Science* 327, 1000-1004
- Zhao, Y., Kalari, S.K., Usatyuk, P.V., Gorshkova, I., He, D., Watkins, T., Brindley, D.N., Sun, C., Bittman, R., Garcia, J.G., Berdyshev, E.V., Natarajan, V. (2007) Intracellular generation of sphingosine 1-phosphate in human lung endothelial cells: role of lipid phosphate phosphatase-1 and sphingosine kinase 1. J. Biol. Chem. 282, 14165-14177
- Zhou, C., Ling, M.T., Kin-Wah Lee, T., Man, K., Wang, X., Wong, Y.C. (2006) FTY720, a fungus metabolite, inhibits invasion ability of androgen-independent prostate cancer cells through inactivation of RhoA-GTPase. *Cancer Lett.* 233, 36-47

# APPENDIX:

# SUPPLEMENTARY DATA

# APPENDIX: SUPPLEMENTARY DATA

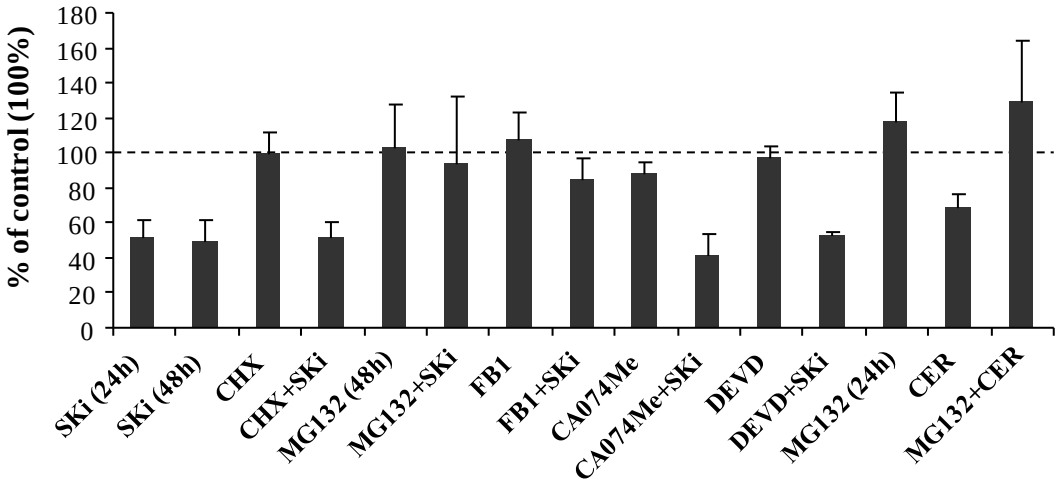



**Figure S1** ***Densitometric quantification of SK1b immunoreactivity in LNCaP cells.*** *Immunoreactivity of western blots for SK1b (total cell lysate protein equalised/sample using the BCA protein assay) was quantified by densitometry and expressed as a percentage of the control (DMSO, 24h or 48h) (control=100%). p values were as follows: SKi (24h) vs control, <0.001; SKi (48h) vs control, <0.001; cycloheximide (CHX) vs control, N.S.; cycloheximide+SKi vs SKi (24h), N.S.; MG132 (48h) vs control, N.S.; MG132+SKi vs SKi (48h), <0.01; fumonisin vs control, N.S.; fumonisin+SKi vs SKi (24h), <0.01; CA074Me vs control, <0.05; CA074Me+SKi vs SKi (48h), N.S.; Ac-DEVD-CHO vs control, N.S.; Ac-DEVD-CHO+SKi vs SKi (48 h), N.S.; C2-ceramide vs control, <0.001; MG132 (24h) vs control, N.S.; MG132+C2-ceramide vs C2-ceramide, <0.01. Data are presented as mean with standard deviation. Values of n were from at least three experiments. N.S. denotes not statistically significant (p value >0.05). The data refer to western blots in Chapter 3.*

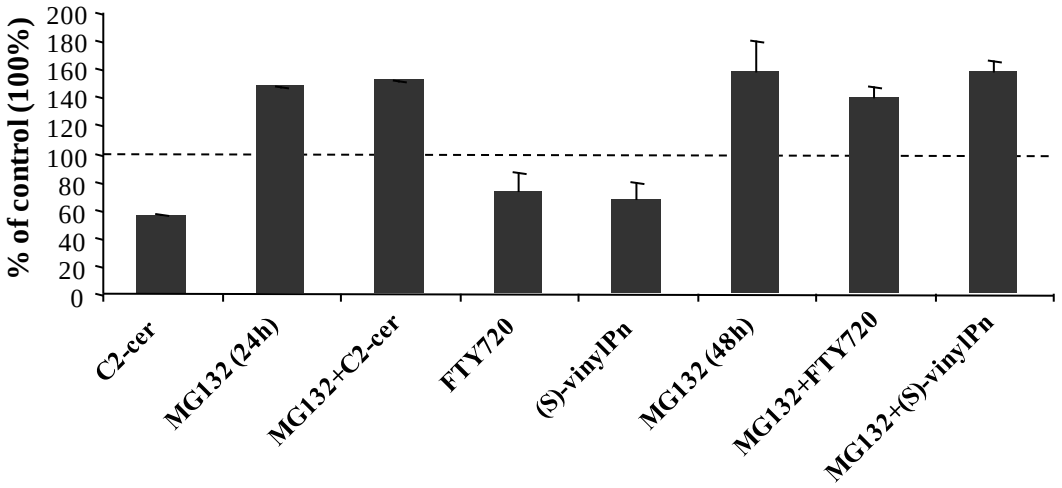



**Figure S2** ***Densitometric quantification of SK1a immunoreactivity in LNCaP-AI cells.*** *Immunoreactivity of western blots for SK1a (total cell lysate protein equalised/sample using the BCA protein assay) was quantified by densitometry and expressed as a percentage of the control (DMSO, 24h or 48h) (control=100%). p values were as follows: C2-ceramide vs control, <0.001; MG132 (24h) vs control, <0.0001; MG132+C2-ceramide vs C2-ceramide, <0.0001; FTY720 vs control, <0.01; (S)-FTY720-vinylphosphonate vs control, <0.001; MG132 (48h) vs control, <0.001; MG132+FTY720 vs FTY720, <0.05; MG132+(S)-FTY720-vinylphosphonate vs (S)-FTY720-vinylphosphonate, <0.01. Data are presented as mean with standard deviation. Values of n were from at least three experiments. The data refer to western blots in Chapter 3 and Chapter 4.*

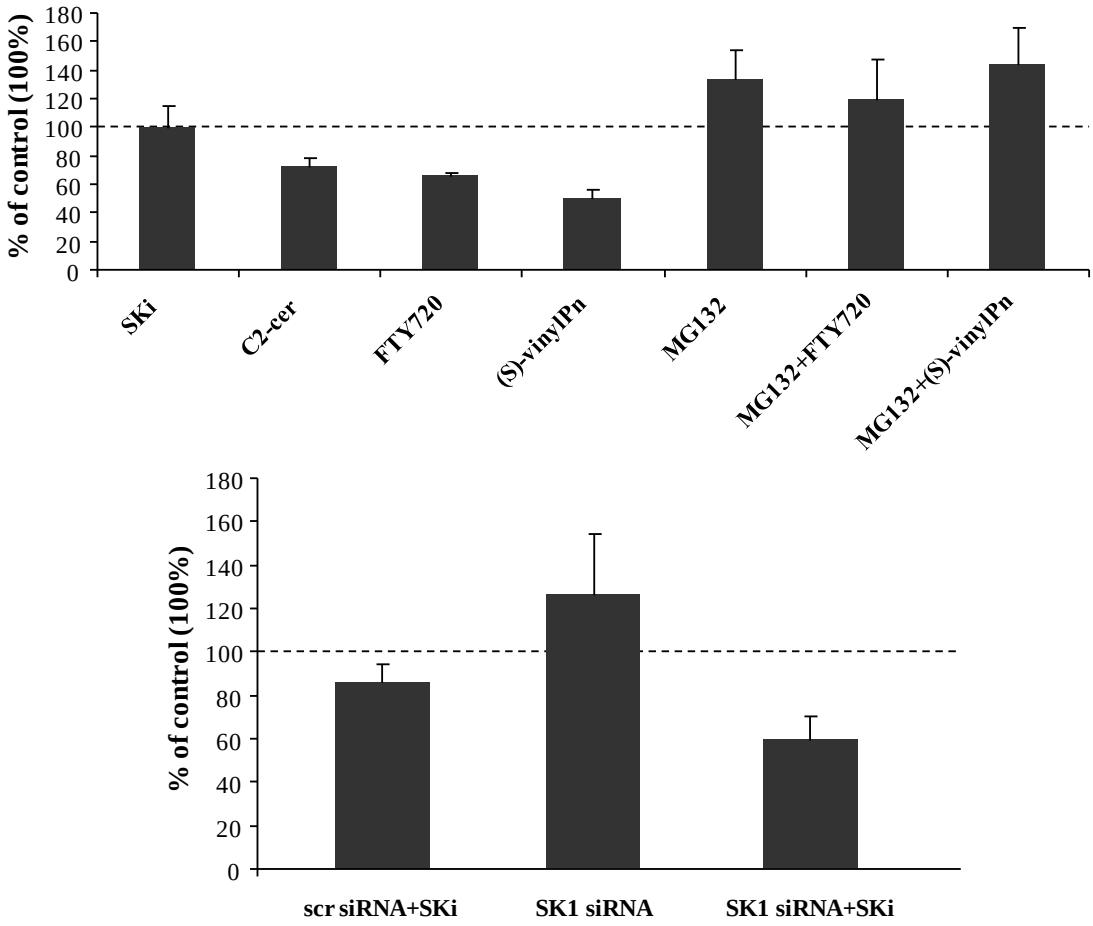



**Figure S3** ***Densitometric quantification of SK1b immunoreactivity in LNCaP-AI cells.*** *Immunoreactivity of western blots for SK1b (total cell lysate protein equalised/sample using the BCA protein assay) was quantified by densitometry and expressed as a percentage of the control (DMSO, 24h or 48h, unless otherwise specified) (control=100%). p values were as follows: SKi (48h) vs control, N.S.; C2-ceramide vs control, <0.01; FTY720 vs control, <0.001; (S)-FTY720-vinylphosphonate vs control, <0.001; MG132 (48h) vs control, <0.01; MG132+FTY720 vs FTY720, <0.05; MG132+(S)-FTY720-vinylphosphonate vs (S)-FTY720-vinylphosphonate, <0.01; FTY720 vs (S)-FTY720-vinylphosphonate, <0.05; SK1 siRNA vs control (scrambled siRNA), N.S.; scrambled siRNA+SKi vs control, N.S.; SK1 siRNA+SKi vs SK1 siRNA, <0.05; SK1 siRNA+SKi vs scrambled siRNA+SKi, <0.05. Data are presented as mean with standard deviation. Values of n were from at least three experiments. N.S. denotes not statistically significant (p >0.05). The data refer to western blots in Chapter 3 and Chapter 4.*

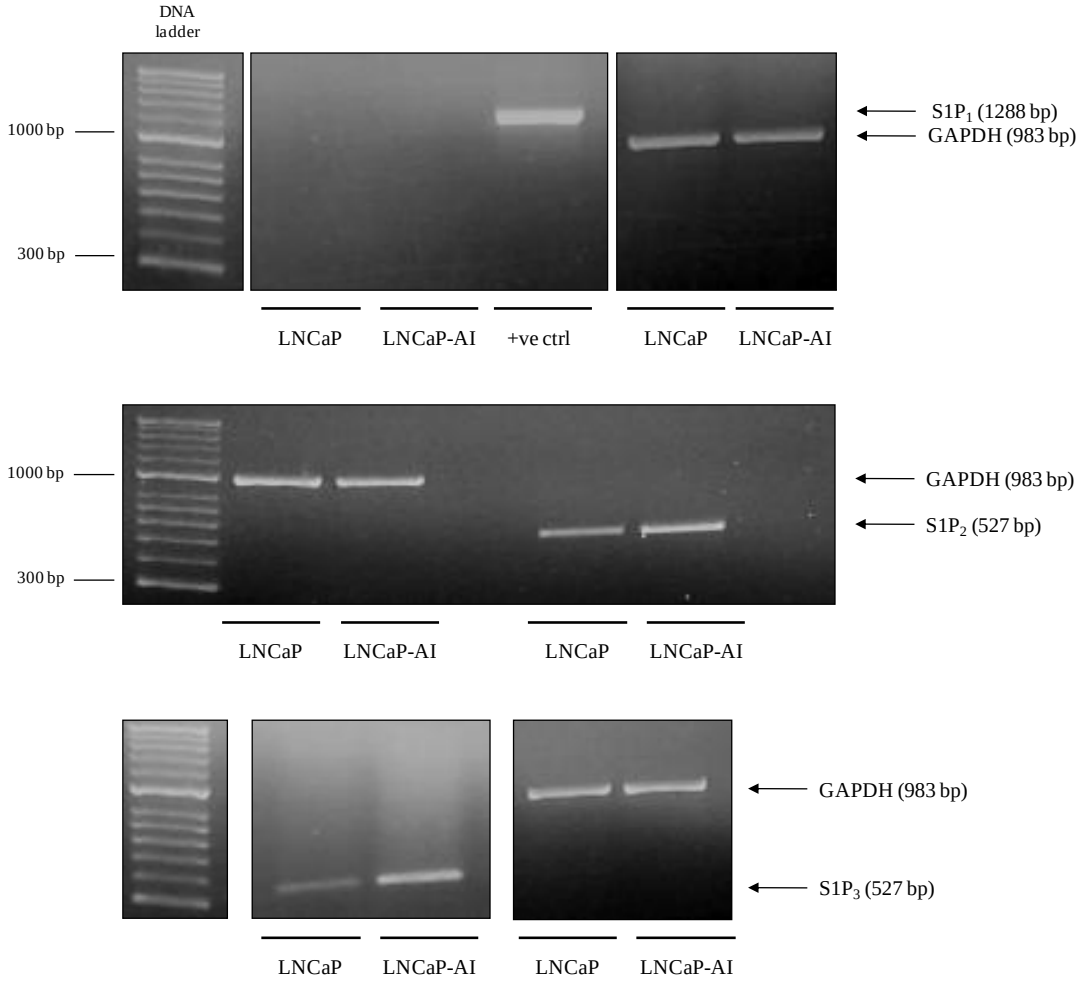



**Figure S4** ***RT-PCR analysis of mRNA transcript levels of S1P receptors in LNCaP and LNCaP-AI cells.*** *$S1P_1$, $S1P_2$ and $S1P_3$ mRNA levels were examined by RT-PCR using gene-specific primers, followed by agarose gel electrophoresis of the amplification products. The expression of the housekeeping gene GAPDH was analysed using gene-specific primers to ensure that comparable amounts of cDNA template were used for the two cell lines. When conducting PCR analysis for $S1P_1$, a control reaction was also set up in parallel using an $S1P_1$ plasmid as a template, which gave an amplification product of the predicted size (+ve ctrl). In each case, the results shown are representative of three independent experiments. The optical density of each band (amplification product) was quantified by densitometry. p values for LNCaP cells vs LNCaP-AI cells were as follows: $S1P_2$, <0.01; $S1P_3$, <0.05.*

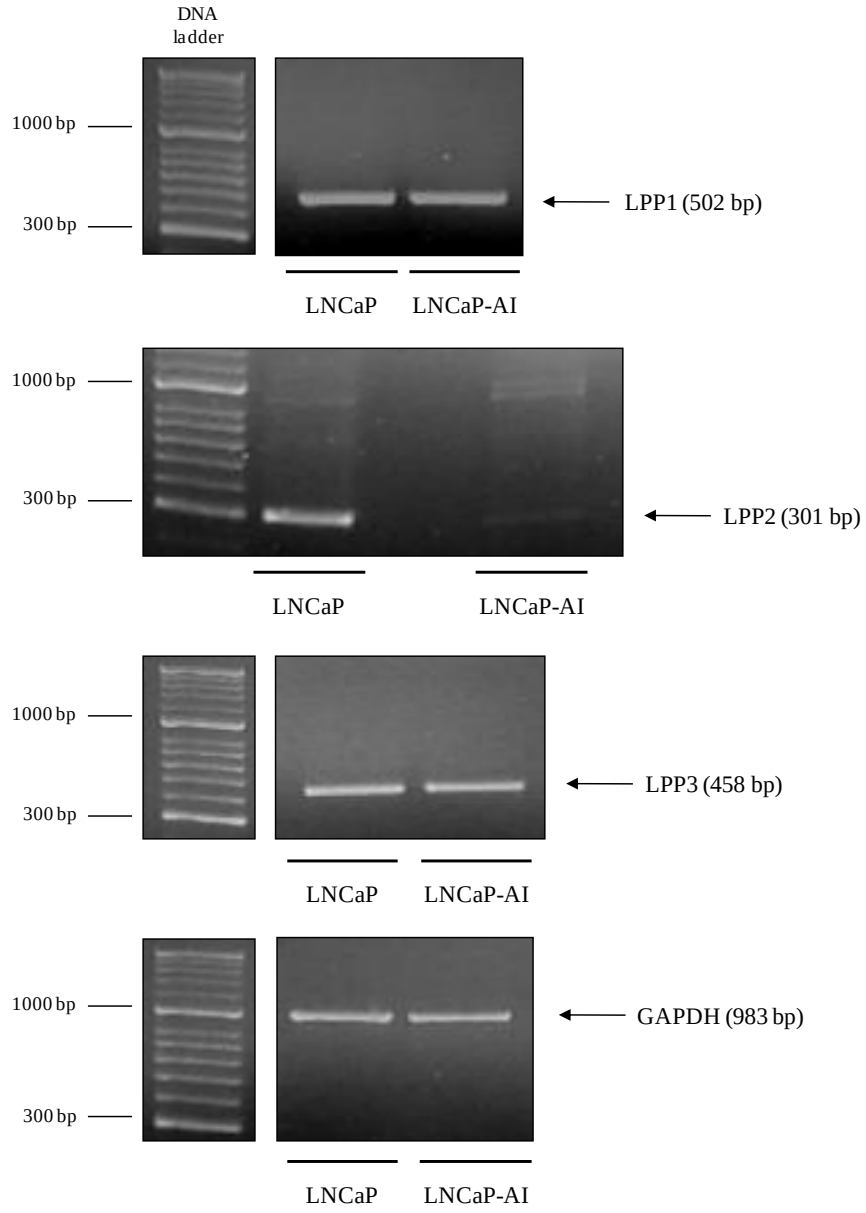



**Figure S5** ***RT-PCR analysis of mRNA transcript levels of LPPs in LNCaP and LNCaP-AI cells.*** *$LPP_1$, $LPP_2$ and $LPP_3$ mRNA levels were examined by RT-PCR using gene-specific primers, followed by agarose gel electrophoresis of the amplification products. The expression of the housekeeping gene GAPDH was analysed using gene-specific primers to ensure that comparable amounts of cDNA template were used for the two cell lines. In each case, the results shown are representative of three independent experiments. The optical density of each band (amplification product) was quantified by densitometry. p values for LNCaP cells vs LNCaP-AI cells were as follows: LPP1, N.S.; LPP2, <0.05; LPP3, N.S.. N.S. denotes not statistically significant (p value >0.05).*

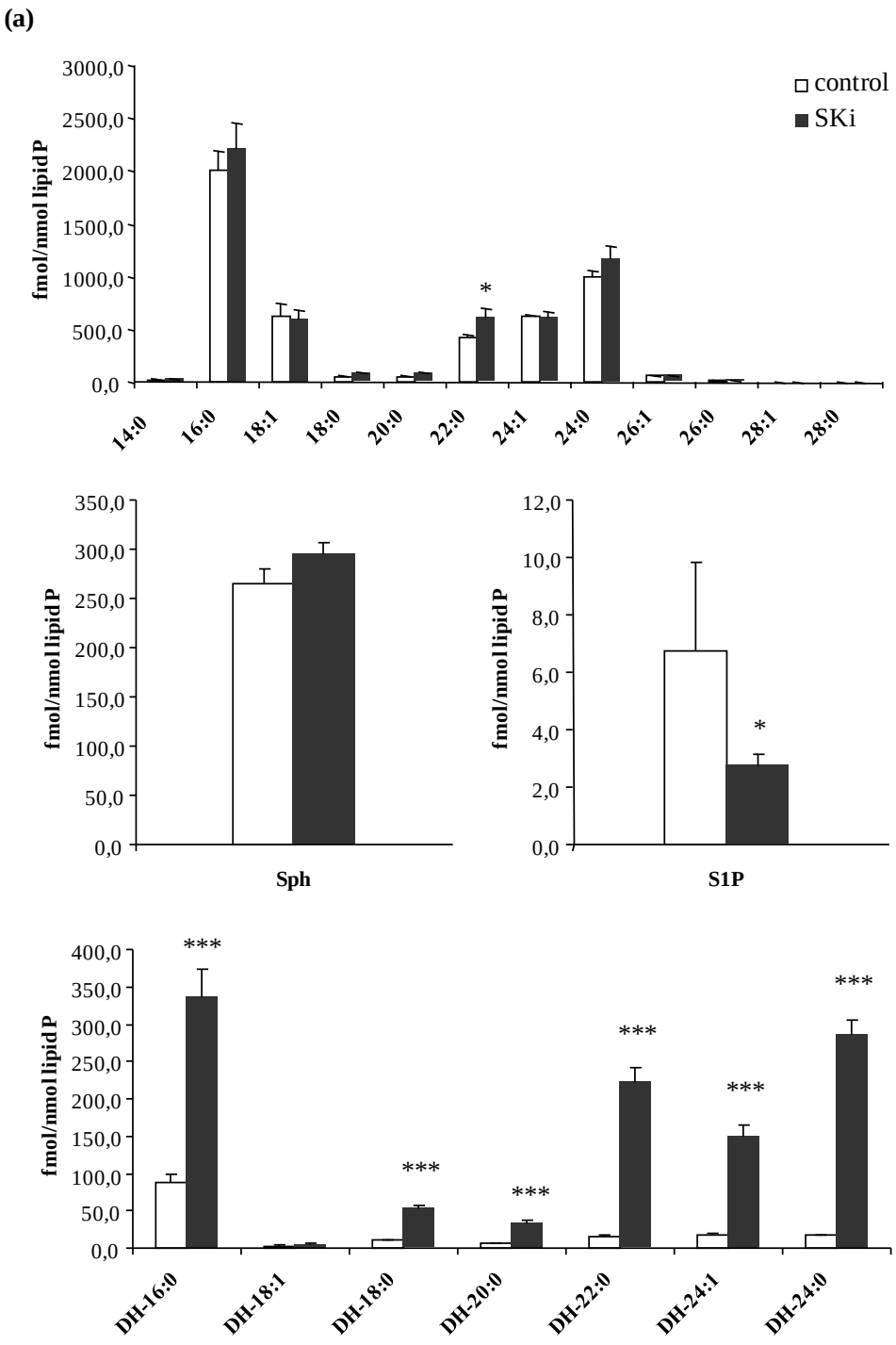



**Figure S6** ***MS analysis of sphingolipid levels in LNCaP and LNCaP-AI cells treated with SKi.*** *Bar charts showing the effect of SKi (10 µM, 24 hours) on sphingolipid levels in (a) LNCaP cells and (b) LNCaP-AI cells. Data are presented as mean with standard deviation of triplicate assays. * p value <0.05, ** p value <0.01, *** p value <0.001 for control vs SKi-treated cells. The sphingolipids analysed were: ceramide (C14:0-C28:0), dihydroceramide (DH-16:0-DH-24:0), sphingosine (Sph) and sphingosine 1-phosphate (S1P).*

**(b)**

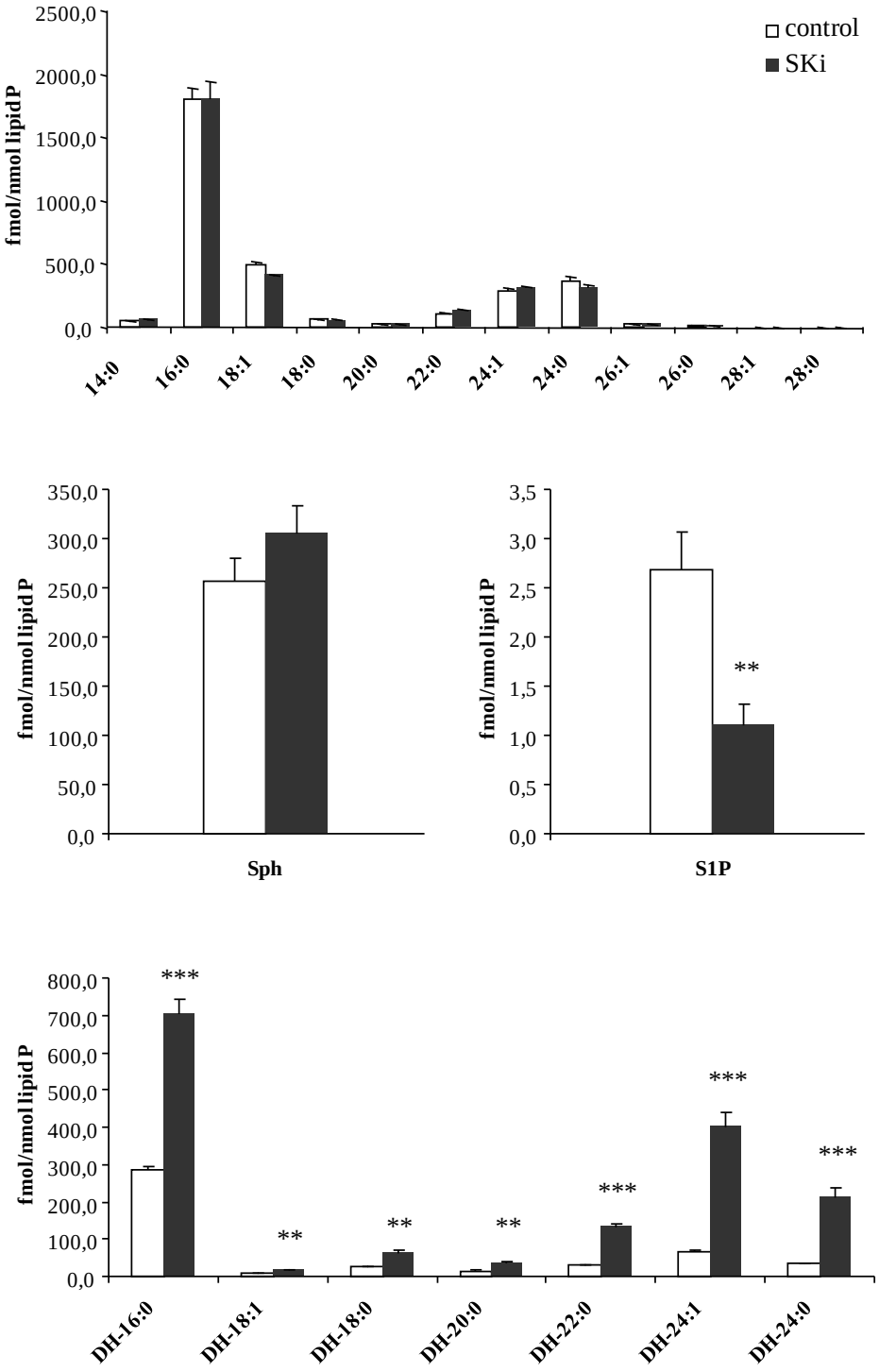



**Figure S6** ***MS analysis of sphingolipid levels in LNCaP and LNCaP-AI cells treated with SKi (continued).***

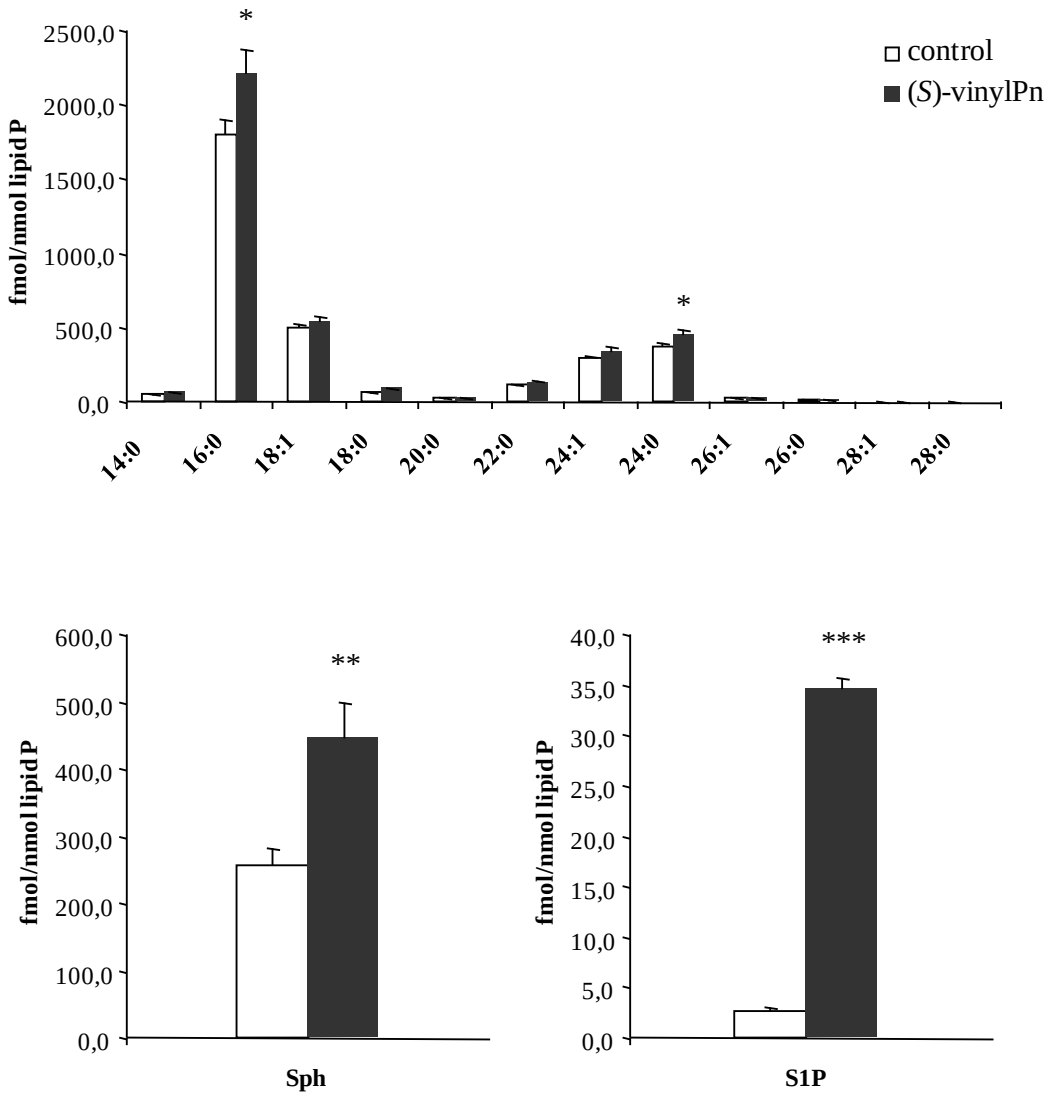



**Figure S7** ***MS analysis of sphingolipid levels in LNCaP-AI cells treated with (S)-FTY720 vinylphosphonate.*** *Bar charts showing the effect of (S)-FTY720 vinylphosphonate ((S)-vinylPn) (10 µM, 24 hours) on sphingolipid levels in LNCaP-AI cells. Data are presented as mean with standard deviation of triplicate assays. * p value <0.05, ** p value <0.01, *** p value <0.001 for control vs (S)-FTY720 vinylphosphonate-treated cells. The sphingolipids analysed were: ceramide (C14:0-C28:0), sphingosine (Sph) and sphingosine 1-phosphate (S1P).*

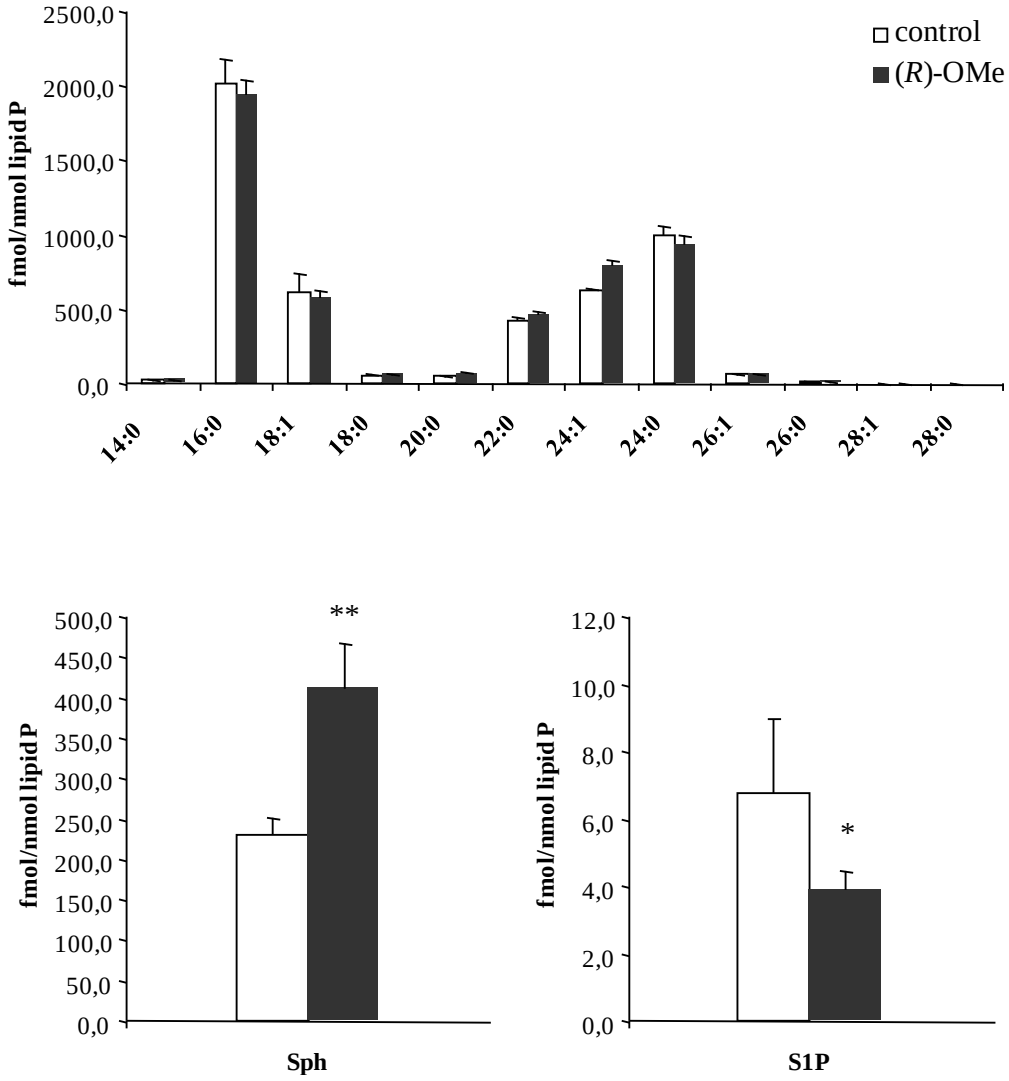



**Figure S8** ***MS analysis of sphingolipid levels in LNCaP cells treated with (R)-FTY720 methyl ether.*** *Bar charts showing the effect of (R)-FTY720-methyl ether ((R)-OMe) (10 µM, 24 hours) on sphingolipid levels in LNCaP cells. Data are presented as mean with standard deviation of triplicate assays. * p value <0.05, ** p value <0.01 for control vs (R)-FTY720-OMe-treated cells. The sphingolipids analysed were: ceramide (C14:0-C28:0), sphingosine (Sph) and sphingosine 1-phosphate (S1P).*

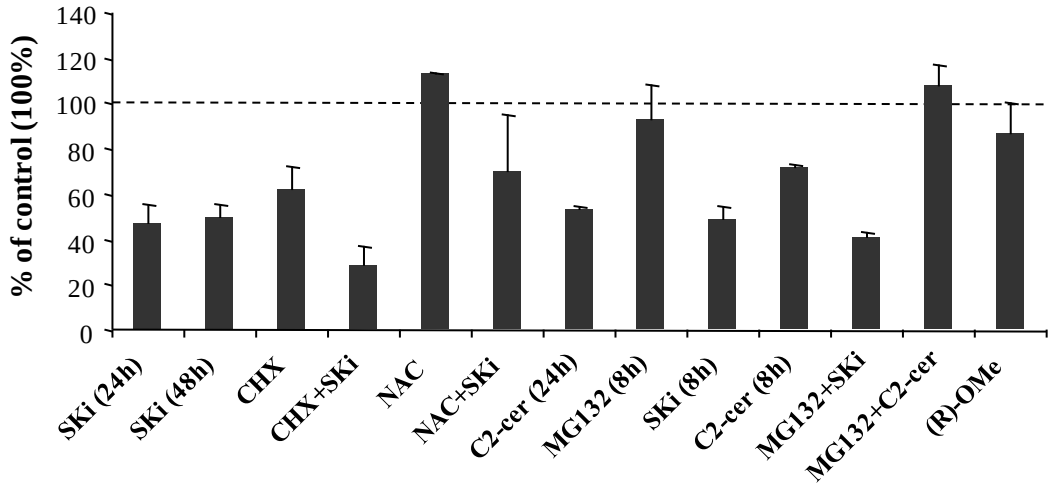



**Figure S9** ***Densitometric quantification of AR immunoreactivity in LNCaP cells.*** *Immunoreactivity of western blots for AR (total cell lysate protein equalised/sample using the BCA protein assay) was quantified by densitometry and expressed as a percentage of the control (DMSO, 24h or 48h) (control=100%). p values were as follows: SKi (24h) vs control, <0.001; SKi (48h) vs control, <0.001; cycloheximide vs control, <0.01; cycloheximide+SKi vs SKi (24h), N.S.; NAC vs control, N.S.; NAC+SKi vs SKi (24h), <0.05; C2-ceramide (24h) vs control, <0.001; MG132 (8h) vs control, N.S.; SKi (8h) vs control, <0.001; C2-ceramide (8h) vs control, <0.001; MG132+SKi vs SKi, N.S.; MG132+C2-ceramide vs C2-ceramide, <0.01; (R)-FTY720-OMe vs control, N.S.. Data are presented as mean with standard deviation. Values of n were from at least three experiments. N.S. denotes not statistically significant (p value >0.05). The data refer to western blots in Chapter 6.*

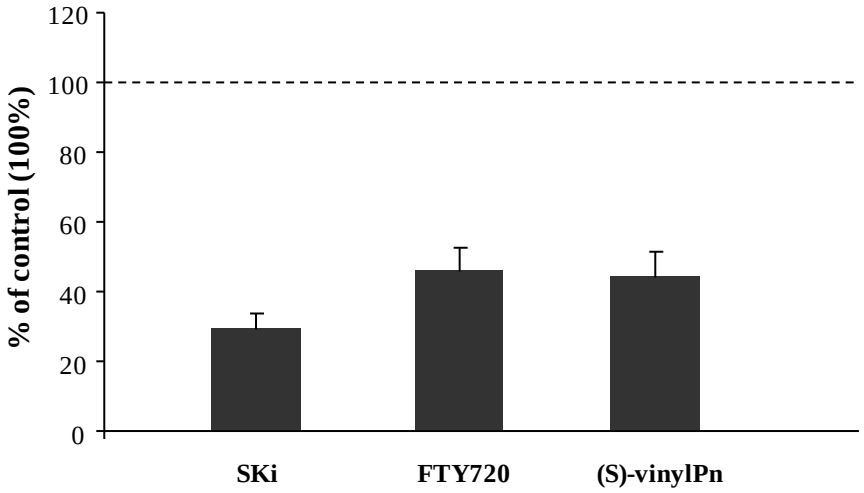



**Figure S10** ***Densitometric quantification of AR immunoreactivity in LNCaP-AI cells***. *Immunoreactivity of western blots for AR (total cell lysate protein equalised/sample using the BCA protein assay) was quantified by densitometry and expressed as a percentage of the control (DMSO, 48h) (control=100%). p values were as follows: SKi vs control, <0.001; FTY720 vs control, <0.001; (S)-FTY720-vinylphosphonate vs control, <0.001. Data are presented as mean with standard deviation. Values of n were from at least three experiments. These data refer to western blots in Chapter 6.*

| | LNCaP (1) | | LNCaP-AI (1) | | LNCaP (2) | | LNCaP-AI (2) | |
|---|---|---|---|---|---|---|---|---|
| **Metabolite** | **SKi/C** | **p value** | **SKi/C** | **p value** | **SKi/C** | **p value** | **SKi/C** | **p value** |
| **GSH** | 1.1 | 0.5 | 2.0 | <0.05 | 1.9 | <0.01 | 2.1 | <0.01 |
| **GSSG** | 67.6 | 0.9 | 310.0 | <0.05 | ND in C | | ND in C | |
| **NADPH** | 0.11 | <0.01 | 0.16 | <0.001 | 0.21 | <0.01 | 0.21 | <0.01 |
| **Phosphogluconate** | 5.1 | <0.01 | 5.0 | 0.7 | 3.5 | <0.05 | 3.5 | <0.05 |
| **Ribulose 5-P** | 2.7 | <0.05 | 4.2 | <0.01 | 3.7 | <0.001 | 2.9 | <0.05 |
| **Fructose 1,6-bisP** | 7.5 | <0.001 | 5.9 | <0.01 | 28.8 | <0.05 | 5.1 | <0.001 |
| **Glyceraldehyde 3-P** | 6.7 | <0.05 | 10.6 | <0.05 | 16.3 | <0.05 | 6.0 | <0.05 |
| **S-Lactoyl-GSH** | ND | | ND | | 13.4 | <0.05 | 21.6 | <0.05 |

**Table S1** ***MS analysis of the metabolome in LNCaP and LNCaP-AI cells treated with SKi****. Cells were treated for 24 hours with SKi (10 μM) or with the vehicle alone (DMSO, 0.1%). The levels of glutathione (GSH), oxidized glutathione (GSSG), NADPH, phosphogluconate, ribulose 5-phosphate (ribulose 5-P), fructose 1,6-bisphosphate (fructose 1,6-bisP), glyceraldehyde 3-phosphate (glyceraldehyde 3-P) and S-lactoylglutathione (S-lactoyl-GSH) were determined by MS analysis. Changes in metabolite levels are means for n=3 samples, two batches analysed six weeks apart. p values are also reported in the table. C: control (DMSO); ND: not detected.*