University of Strathclyde

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The Role of Sphingosine Kinases and Dihydroceramide Desaturase in Regulating Senescence in Prostate Cancer Cells

By

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

Signed:

Date:

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Abstract

Sphingosine kinase 1 and Sphingosine kinase 2 (SK1 and SK2) are lipid kinases which form the bioactive lipid sphingosine-1-phosphate (S1P). SKs have been widely implicated in human cancers and inflammation. This has been demonstrated to be a consequence of the actions of S1P, which acts as a ligand at five G protein-coupled receptors - termed $S1P_{1-5.}$ S1P is also an intracellular effector which binds to proteins such as HDAC1/2 and TRAF2 to elicit cell responses. Recently, other mediators in the pathway have also emerged as important mediators of autophagic, apoptotic, senescent and proliferative cell processes.

This study aims to investigate the molecular pathways which link SK1 and SK2 with senescence in order to identify novel signalling networks which could in turn lead to potential therapeutic approaches to cancer treatment. These aims were achieved by down-regulating SK1 and SK2 using a series of SK inhibitors in an advanced stage castrate resistant prostate cancer cell line (LNCaP AI cells). The current study demonstrated that dual inhibition of SK1 and SK2 as well as knockdown of an additional target up stream in the sphingolipid signalling pathway – dihydroceramide desaturase (Des1) – was responsible for promoting a senescent phenotype in LNCaP AI cells. As an adjunct to these findings it is reported here for the first time that the sphingosine kinase 2 inhibitor, ABC294640 (3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide), which is currently in phase 2 clinical trials for renal cancer and B-cell lymphoma, induces the proteasomal degradation of sphingosine kinase 1; therefore ABC294640 cannot be considered an SK2 selective inhibitor in a cellular context. Also as an adjunct to these findings, Des1 was discovered as a novel target for ABC294640.

Thus, this study highlights a role of SKs and the sphingolipid signalling pathway in mediating senescence in human cancer cells which could provide novel therapeutic approaches to cancer treatment. In addition, the study challenged a central dogma in the field and provided useful and previously unknown mechanistic information about a drug which is currently in phase 2 clinical trials. Finally, the study identified a common novel target (Des1) of two existing compounds which, in combination with SK1, is responsible for inducing senescence in LNCaP AI cells. Taken together these findings provide novel and useful information not only regarding the role of SKs in senescence but in a more general context with respect to the mechanisms of action of these inhibitors, which are commonly used as tools to investigate SK signalling *in vitro* and *in vivo*.

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Appendix: Supplementary Data

Publications

- **McNaughton, M.,** Pitman, M., Pitson, S. M., Pyne, N. J., & Pyne, S. (2016). Proteasomal degradation of sphingosine kinase 1 and inhibition of dihydroceramide desaturase by the sphingosine kinase inhibitors, SKi or ABC294640, induces growth arrest in androgen-independent LNCaP-AI prostate cancer cells. *Oncotarget*, *7*(13), 16663–16675.
- Pyne, N.J., **McNaughton, M.,** Boomkamp, S., MacRitchie, N., Evangelisti, C., Martelli, A.M., Jiang, H.R., Ubhi, S., & Pyne S. (2016) Role of sphingosine 1-phosphate receptors, sphingosine kinases and sphingosine in cancer and inflammation. *Adv Biol Regul*, 60, 151-159.
- Barbour, M., **McNaughton, M.,** Boomkamp, S. D., MacRitchie, N., Jiang, H., Pyne, N. J., & Pyne, S. (2017). Effect of sphingosine kinase modulators on interleukin-1β release, sphingosine 1-phosphate receptor 1 expression and experimental autoimmune encephalomyelitis. *British Journal of Pharmacology*, 174(2), 210–222.

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CHAPTER 1:

GENERAL INTRODUCTION

1. CHAPTER 1 - General introduction

1.1 Sphingolipid metabolism

Sphingolipids are a class of lipids which are commonly found in cell membranes. They were discovered in the 1870s and are named after the mythological creature the sphinx because of their mysterious and enigmatic nature. Like phospholipids, sphingolipids are composed of a polar head group and two non–polar tails. They also contain a sphingosine back bone. Historically, it was thought that sphingolipids existed merely as structural components of the cell membrane. In the past 20 years, however, this has drastically changed and it has become recognised that these lipids are physiologically active. Bioactive lipids are loosely defined as lipids which are able to cause functional changes either via alterations in their levels or interactions with proteins and other lipids alike (Hannun and Obeid, 2008). Currently, sphingolipids are widely accepted as important and ubiquitous mediators of metabolism at the cellular level and aberrations in sphingolipid signalling either due to altered quantity, quality or spatio-temporal location have been proven in numerous pathological states. In recent years, major advances have been made in discerning the complexities of the sphingolipid metabolism however there are many mechanisms and interactions which are still not fully understood (Hannun and Obeid, 2017).

Sphingolipid metabolism is dynamic, reactions are almost always reversible and there is often more than one route to the generation and breakdown of each molecule. Also, reactions occur in distinct cellular compartments and are temporally as well as spatially controlled. In spite of this, sphingolipids diverge from a unique metabolic entry point and converge on a sole exit point in the pathway (Gault et al., 2010).

Ceramide is at the centre of the sphingolipid metabolic network and has been described as the hub of sphingolipid metabolism. Ceramide is comprised of sphingosine connected to a fatty acid of variable chain length. It can be formed via three distinct metabolic pathways (Hannun and Obeid, 2010). The first of these pathways is the *de novo* synthesis pathway which forms new endogenous ceramide. This is located on the cytoplasmic face of the endoplasmic reticulum and involves the condensation of palmitoyl CoA and serine by the enzyme serine palmitoyl transferase to form 3-*keto*-dihydrosphingosine (Mandon et al., 1992), which is then reduced to dihydrosphingosine. Dihydrosphingosine is acylated by one of 6 ceramide synthase isoforms (which use different acyl CoA molecules) to generate multiple molecular species of dihydroceramides of differing chain lengths (Gault et al., 2010). Finally, these

dihydroceramides are converted to ceramides by the action of dihydroceramide desaturase (Des1) (Michel et al., 1997). Ceramides are transported from the endoplasmic reticulum (ER) to the golgi by ceramide transport protein (CERT) and can then be exported via vesicles (Hanada et al., 2003). Interestingly, CERT has also been shown to mediate the transfer of dihydroceramides and phyto-ceramides in a cell free assay system (Kumagai et al., 2005).

The second pathway, termed the 'salvage' pathway constitutes around 50-90% of sphingolipid synthesis and takes place in acidic lysosomes and late endosomes. This pathway involves the breakdown of complex sphingolipids and glycosphingolipids to form sphingomyelin. Sphingomyelin is then converted to ceramide by acidic sphingomyelinase (Hage-Sleiman et al., 2013). The third and final pathway (the sphingomyelin pathway) occurs at the plasma membrane and involves the hydrolysis of common membrane lipid sphingomyelin to form ceramide by one of three sphingomyelinase enzymes (neutral, acidic or alkali). This pathway has been implicated in more rapid formation of ceramide (Gault et al., 2010).

Ceramide has well established signalling roles but also functions as a precursor for other bioactive sphingolipids. Ceramide can be glycosylated by glucosylceramide synthase to form glucosylceramides which are then used to synthesise complex glycosphingolipids (Ichikawa et al., 1996). Alternatively, ceramide can be phosphorylated to form ceramide-1-phosphate (Kolesnick and Hemer, 1990) or back converted to sphingomyelin by sphingomyelin synthase (Tafesse et al., 2006). Importantly, ceramide can be deacylated by ceramidase to generate sphingosine (el Bawab et al., 2002). It is from sphingosine that sphingosine kinases (SKs) form sphingosine-1-phosphate (S1P), a bioactive lipid with an abundance of physiological roles (Hannun and Obeid, 2008). S1P can then be 'recycled' via dephosphorylation by S1P phosphatases (S1PP) or lipid phosphate phosphatases (LPPs). Alternatively, S1P is irreversibly cleaved by S1P lyase (S1PL) to form hexadecenal and phosphoethanolamine in a unique exit point from the pathway (Gault *et al*., 2010). Significantly, S1P can also be released from the cell via various mechanisms. In endothelial cells this is mediated by a transporter protein, SPNS2 (Nagahashi et al., 2014). In other cell types (e.g. erythrocytes) this is mediated by ABC transporters and ATP (Nishi et al., 2014).

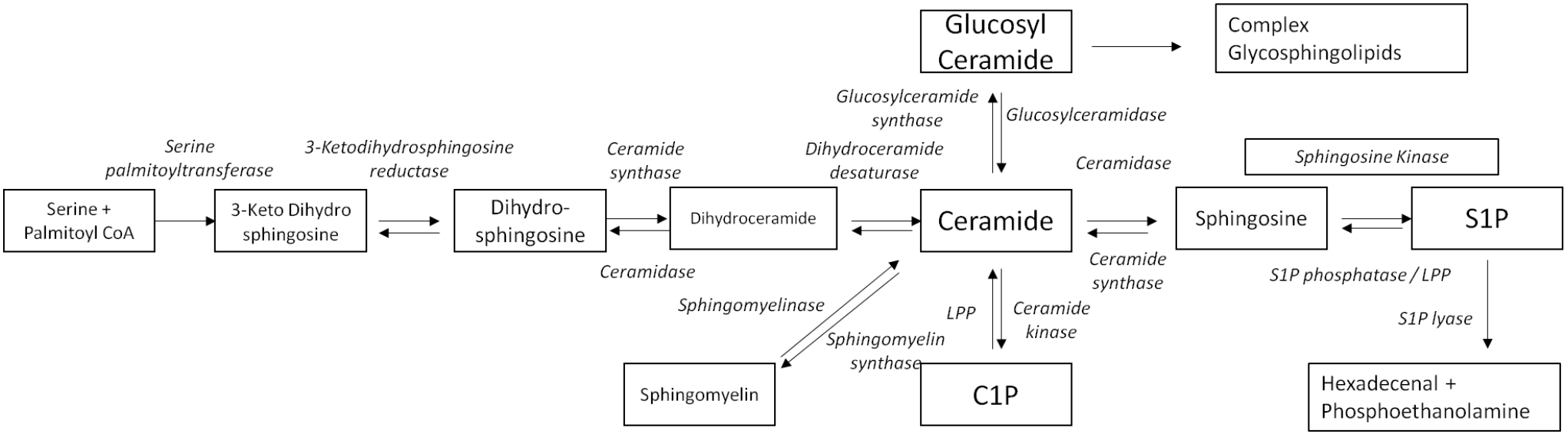


Figure 1.1 ***Sphingolipid metabolism (adapted from Pitson, 2011).*** *Enzymes which catalyse each reaction are in italics. S1P, sphingosine-1-phosphate; LPP, lipid phosphate phosphatase.*

1.2 The Sphingolipid Rheostat

Generally, ceramide is considered to be a pro-apoptotic lipid which elicits responses such as apoptosis, senescence and growth arrest. In contrast, S1P is considered to enhance pro-survival functions as well as proliferation and migration. This has lead to the concept of the 'sphingolipid rheostat' in which the inter-conversion and dynamic balance of ceramide and S1P controls cell fate (Cuvillier et al., 1996; Pyne et al., 1996, Pyne et al., 2010). In recent years, however, it has become apparent that other, previously uncharacterised, sphingolipids (such as dihydroceramide and *trans*-2-hexadecenal (Kumar et al., 2011)) may have diverse signalling functions of their own. Additionally, the discovery of novel isoforms of enzymes in the pathway which in turn form unique products (e.g. ceramides of different chain lengths) has highlighted the fact that the pathway is actually far more complex and that there are many things which remain to be discerned (Pyne et al.,2016).

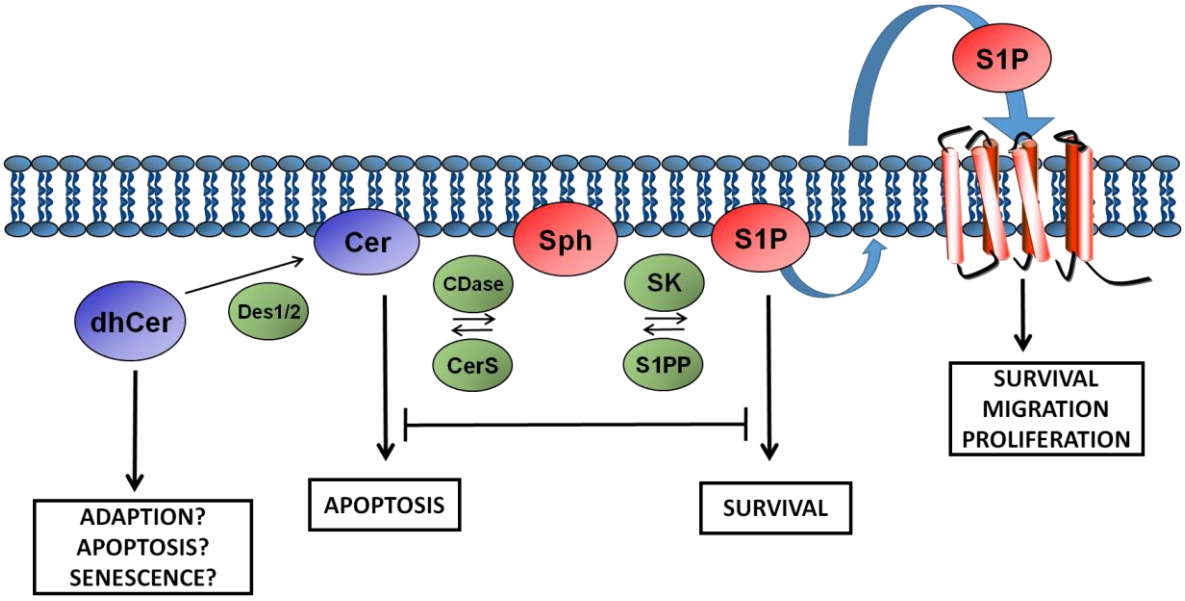


Figure 1.2 ***Sphingolipid signalling and the sphingolipid rheostat (Adapted from Pyne and Pyne, 2010).*** *Des1/2, dihydroceramide desaturase 1/2; dhCer, dihydroceramide; CerS ceramide synthase; CDase, Ceramidase; S1P, sphingosine-1-phosphate; SK, sphingosine kinase.*

1.3 S1P

S1P is a pleiotropic lipid which is present both intra and extra cellularly (Hannun and Obeid, 2008, Blaho and Hla, 2014). S1P has well established roles in both normal physiology and pathophysiology (Pyne et al., 2013). There are five G-protein coupled receptors through which S1P signals, termed $S1P_1$-$S1P_5$. Each receptor is differentially expressed in tissues and has specific physiological roles in modulating the cardiovascular, immune and nervous systems as well as embryonic development (Blaho and Hla, 2014). S1P receptors also regulate processes such as proliferation and survival, angiogenesis, cell motility and cell adhesion. Additionally, S1P regulates fundamental cellular processes such as cytoskeletal rearrangement and calcium homeostasis (Taha et al., 2006a).

Under normal basal conditions, S1P is maintained at a very low level (10-30 pmol/10^6 cells) and this is subject to tight regulation (Spiegel et al., 1998). Upon stimulation however, S1P levels can rapidly increase. This is due to increased formation by SKs which become activated in response to stimulation. When S1P becomes dysregulated it largely contributes to the pathophysiology of cancer and other proliferative diseases, such as inflammatory diseases (Pyne and Pyne, 2010).

1.3.1 S1P as a receptor agonist

S1P is an agonist at five G protein coupled receptors. S1P receptors were originally named endothelial differentiation gene (EDG) receptors. The first S1P receptor to be identified was $S1P_1$ (coined EDG1) at the time (Hla and Maciag, 1990). Next, $S1P_2$ and $S1P_3$ were discovered (Okazaki et al., 1993; MacLennan et al., 1994) followed by $S1P_4$ and $S1P_5$ (Graler et al., 1998; Im et al., 2000). Each of the S1PRs have since been characterised and the actions of S1P at each of its receptors are now well defined.

$S1P_1$-$S1P_3$ are ubiquitously expressed whereas $S1P_4$-$S1P_5$ are expressed mainly in the lymphoid system and central nervous system, respectively (Chun *et al*., 2002). Each S1P receptor is coupled to distinct G proteins which is in turn are coupled to a distinct downstream signalling network (see Figure 1.3). S1P acting on its GPCRs can activate a number of downstream signalling networks including extracellular regulated kinases 1/2 (ERK 1/2), small GTPases (Rac and Rho), the phosphoinositide 3-kinase (PI3K)/AKT and phospholipase C (PLC) to ultimately enhance cell proliferation, survival and migration (Meyer zu Heringdorf & Jakobs, 2007; Pyne and Pyne, 2010). This, taken together with the fact that cells and tissues express different S1P receptor profiles, leads to cell and

circumstance specific effects of S1P (Sanchez and Hla, 2004). Ultimately, S1P is able to exert an extremely diverse range of biological effects which vary greatly between cells and tissues.

S1P can bind to S1PRs in both a paracrine and autocrine manner in a process termed 'inside-out signalling'. This is where S1P is transported out of the cell to bind to and activate S1PRs (Alvarez *et al*., 2007). Several transporters of S1P have been discovered and it is now known that the S1P export proteins are expressed differentially in cells. At first, S1P was shown to be transported through the ATP binding ABC cassette (ABC) transporters ABCC1, ABCA1 and ABCG2 (Mitra et al., 2006; Sato et al., 2007; Takabe et al., 2008). Subsequently, a novel S1P transporter named spinster homologue protein, SPNS2, which was discovered in zebra fish (Hisano et al., 2011). Most recently, MFsd2b has been identified as an exporter of S1P in erythrocytes and platelets (Vu et a., 2017). This was confirmed when a vast intracellular accumulation of S1P in response to Mfsd2b knockout was observed in cells compared to wild type counterparts. It was concluded that this pathway contributes to around 50% of the S1P plasma pool and abrogation of this resulted in a change in red blood cell morphology and blood vessel leakiness (Vu et al., 2017). Interestingly, it has been suggested that S1P may diffuse laterally into $S1P_1$ from within the lipid bilayer, via a a gap between helices I and VI (Hanson et al., 2012). It has also recently has been proposed that S1PRs are able to laterally diffuse through the cell membrane in endosomes which can also contain S1P. This S1P is then able to mediate intracellular responses in an additional example of intracrine signalling (Adada et al., 2015). In support of this, an earlier report identified an intracellular role for $S1P_5$, which can localize to intracellular centrosomes, where it is proposed to participate in the regulation of mitosis (Gilles et al., 2009). Furthermore, SK1 and S1P have recently been shown to have a role in regulating endosomal processing. SK1 was found co-localised to early endosomes (attached via a hydrophobic patch) containing N-BAR proteins, which function as membrane anchors. Importantly, disruption of this interaction by site-directed mutagenesis (L194Q) of SK1 resulted in defects in endosome recycling (Shen et al., 2014).

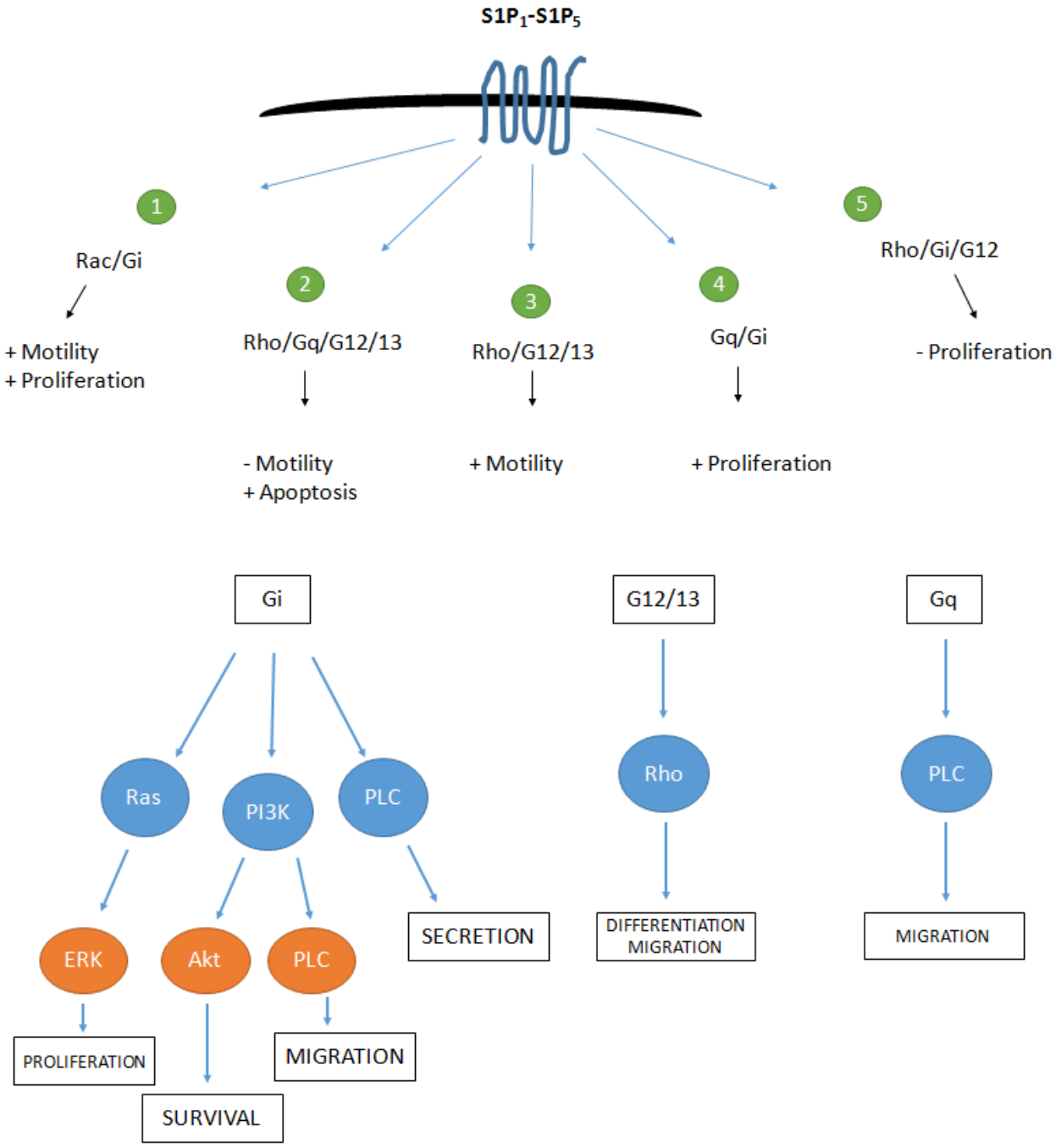


Figure 1.3 ***Extracellular S1P signalling (Adapted from Pyne and Pyne, 2010).*** *S1P receptor subtypes ($S1P_{1-5}$), GPCR coupling and downstream signalling networks.*

1.3.2 S1P as an intracellular effector

Although less well characterised than the actions of S1P at S1PRs, several intracellular targets of S1P have emerged which confer extremely important functions. One of these targets is ceramide synthase 2 (Cers2), which is thought to be negatively regulated by S1P, in an example of negative feedback between ceramide and S1P (Laviad et al., 2008). In the nucleus, SK2 derived S1P inhibits histone deacetylases 1 and 2 (HDAC 1/2) which in turn enhances transcription of senescence marker p21 and transcription factor c-fos (Hait et al., 2009). In the cytosol, S1P binds to and activates TRAF2, an E3 ubiquitin ligase responsible for the polyubiquitination of RIP1, a scaffold protein for the IKK complex which activates the important pro-inflammatory transcription factor NF_KB (Alvarez *et al*., 2010). In the mitochondria, SK2 derived S1P inhibits BAK translocation and hexadecenal inhibits BAX translocation, which in turn inhibits cytochrome c release from mitochondria leading to apoptotic outer membrane permeabilisation (Chipuk *et al*., 2012; Gulbins, 2013). Furthermore, S1P has been shown to stimulate phospholipase D (PLD) via activation of PKCḍ in human lung adenocarcinoma cells (Meacci et al., 2003).

It should be noted that actions of S1P have been reported which are not obviously attributable to its activation of S1PRs or action at any of the above intracellular targets. Therefore it is highly likely that additional intracellular targets of S1P exist which have yet to be reported.

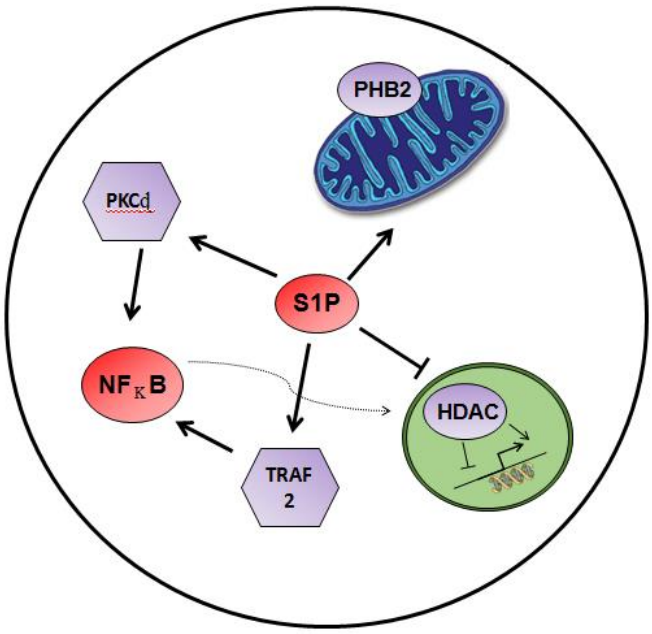


Figure 1.4 ***Intracellular S1P signalling.*** *An overview of intracellular targets of S1P.*

1.4 S1P in cancer

There is abundant evidence that S1P has a role in cancer. S1P has been implicated in regulating several cellular processes which are directly linked to progression of cancer (Pyne and Pyne, 2010). Firstly, S1P promotes neovascularisation of tumours (LaMontagne et al., 2006). Furthermore, S1P drives cancer progression by activating inflammation via a positive feedback loop involving SK1, $S1P_1$, NF_kB, STAT3 and IL-6 whereby STAT3 up regulates the expression of $S1P_1$ which in turn activates STAT3 and up regulates IL-6. This ultimately promotes tumour growth and metastasis by activating IL-6/JAK2 signalling (Lee e*t al*., 2010). Another example is in cervical cancer (HeLa) cells, where S1P activates NF_kB via $S1P_2$. This protects cells from FasL induced apoptosis via a mechanism involving NF_kB which in turn further enhances S1P secretion in an amplification loop (Blom et al., 2010). Importantly, high expression of $S1P_1$ and $S1P_3$ in oestrogen receptor (ER) positive breast cancer tumours is associated with poor prognosis, demonstrating that the oncogenic actions of S1P in cells can be correlated in clinical samples (Watson et al., 2010). S1P, also via S1PRs, activates the anti-apoptotic PI3K/AKT pathway which protects gastric endothelial cells against radiation induced apoptosis (Baudhuin *et al*., 2002; Bonnaud *et al*., 2010). Related to this, PI3K and AKT were shown to inhibit radiation induced apoptosis of stem cells via suppression of protein p53 up regulated modulator of apoptosis (PUMA) (Qiu *et al*., 2010). Also, via $S1P_1$, S1P mediates migration and angiogenesis via activation of a cascade of phospholipase D2 (PLD), protein kinase C (PKC) and Rac1 in human lung endothelial cells (Gorshkova et al., 2008).

Recently, S1P has also been shown to have a systemic role in tumour migration and metastasis via $S1P_2$ dependant suppression of breast tumour metastasis suppressor 1 (Brms1) (Ponnusamy et al., 2012). Furthermore, S1P acting at $S1P_3$ receptors stimulates the translocation of SK1 into membrane ruffles/lamellipodia, which in turn promotes migration of MCF-7 breast cancer cells (Long et al., 2010a). In addition, siRNA knockdown of SK1 in these cells reduced $S1P_3$ expression and ERK1/2 activation in response to S1P, demonstrating that SK1 and $S1P_3$ function in an amplification loop to promote cancer progression (Long et al., 2010b). In ML-1 human follicular carcinoma cells, S1P induces migration via activation of $S1P_1$ and $S1P_3$ and downstream Gi and PI3K-Akt activation. Interestingly, over expression of SK1 enhanced secretion of S1P and migration of ML-1 cells and this was blocked by SK1 inhibition or ATP binding cassette transporters, suggesting a role of 'inside-out' or autocrine S1P signalling in this process (Bergelin et al., 2009). Interestingly, it has also been

demonstrated that S1P inhibits protein phosphatase 2A (PP2A) via $S1P_2$. In this study, SK1/S1P/$S1P_2$ signalling was shown to modulate oncoprotein Bcr-Abl stability by affecting its proteasomal degradation. Furthermore, PP2A is an important downstream mediator of ceramides apoptotic action. This is therefore another example of S1P counteracting the action of ceramide (Salas et al., 2011).

$S1P_4$ has also been directly linked to cancer. In MDA-MB-453 breast cancer cells, S1P acting on $S1P_4$ stimulated the tyrosine phosphorylation of oncogenic HER2. This enhanced ERK1/2 signalling in these cells, which has been linked to promotion of metastasis (Long et al., 2010b). Indeed, high tumour expression of $S1P_4$ and SK1 are associated with shorter disease recurrence times and overall survival times in ER negative breast cancer (Ohotski et al., 2012). Importantly, high $S1P_4$ expression in tumours of ER negative breast cancer patients is correlated with node positive status, affirming a role for $S1P_4$ in cancer in clinical patient samples (Ohotski et al., 2012; Pyne et al., 2012).

1.5 Sphingosine kinases

Two sphingosine kinases exist in mammalian cells (SK1 and SK2) which are transcribed from two distinct genes, *SPHK1* (17q25.2) and *SPHK2* (19q13.2). Both catalyse the ATP dependant phosphorylation of Sph to generate S1P. Structurally, they share 80% similarity and 45% overall sequence identity (Liu et al., 2000). Three splice variants of SK1 (SK1a, SK1b and SK1c) and three splice variants of SK2 (SK2a, SK2b, SK2c) have been discovered in cells and each differ by N- terminal extensions (Pitson, et al., 2011). SK1 and SK2 are considerably different in size (314aa for SK1a versus 618aa for SK2a). SK2 has an additional central region and an additional N-terminal extension (Liu et al., 2000).

SK1 and SK2 contain five conserved domains (C1-C5) with the ATP binding domain present in C2 and the catalytic domain present in C1-C3 (Pitson et al., 2003). SK2, however, has a transmembrane domain which SK1 lacks (Liu *et al*, 2000). Although both enzymes are ubiquitously expressed in human tissue, SK1 is more highly expressed in the lung, spleen and immune system whereas SK2 is more highly expressed in the kidney and liver (Neubauer and Pitson, 2013). SK1 and SK2 also exhibit different sub-cellular distribution patterns (see below). Such compartmentalization allows S1P to exert different signalling roles with multiple down-stream signalling targets. This is widely accepted as being important in dictating overall function of SKs and may account for the different physiological roles of SK1 and SK2 (Wattenberg, 2010; Siow and Wattenberg, 2011).

Substrate specificity is different for both SK isoforms. SK2 is able to phosphorylate a wider range of substrates than SK1; including D,L-*threo*-dihydrosphingosine, phytosphingosine and interestingly *threo*-DHS, which is a potent inhibitor of SK1 . More recently, SK2 was shown to bind and phosphorylate the pro drug FTY720, which perhaps binds better due to interaction with the central region or N terminal extension of SK2 which SK1 lacks (Siow et al., 2010).

It has been shown that SK1 or SK2 can take on a compensatory role if the one SK isoform is down regulated. Indeed, it has been shown that SK2 knockdown by siRNA results in enhanced SK1 expression and activity (Gao and Smith, 2011). In support of this functional redundancy, $SK1^{-/-}$ or $SK2^{-/-}$ mice are phenotypically normal whereas $SK1^{-/-}/SK2^{-/-}$ double knockout mice die *in utero* due to developmental defects in the cardiovascular system and CNS (Mizugishi et al., 2005).

The activity of both SK isoforms is enhanced by various external stimuli such as cytokines such as TNFα and IL-1β (Mastrandrea et al., 2005), growth factors such as EGF, mediated by the activation of intracellular kinases such as ERK1/2 and protein kinase c (PKC) (Hait et al., 2005). Both SK enzymes have basal catalytic activity in the absence of stimulation and this has been proposed to form 'housekeeping' S1P to maintain physiological ceramide and sphingosine levels (Chan and Pitson, 2013).

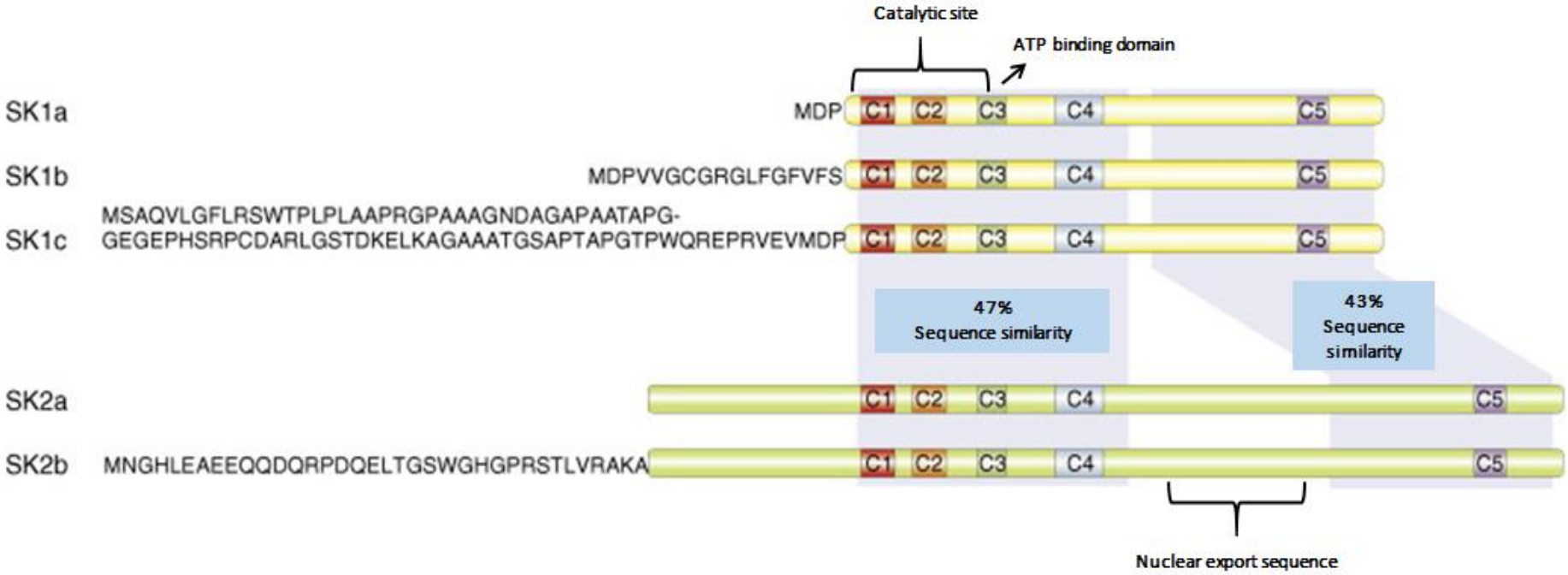


Figure 1.5 ***Human SK1 and SK2 (adapted from Pitson, 2011)*** *Sequence homology of SK1 and SK2. SK2 has an additonal central region containing a nuclear export sequence (NES) which SK1 lacks. SK2 also has an N terminal extension and putative transmembrane domain.*

1.5.1 SK1

SK1 has been extensively studied and is widely accepted as a ubiquitous mediator of cell survival and is expressed in human cells and tissues. SK1 is implicated not only in cancer but in all hyper-proliferative diseases. Human SK1 was first cloned in 2000 (Nava et al., 2000). Since then, there has been an overwhelming amount of literature which has established SK1 as a signalling mediator that universally promotes survival and proliferation in cells.

Under normal physiological circumstances (in the absence of stimulation), endogenous SK1 has high basal activity (Pitson et al., 2000). However, it can be rapidly and transiently activated in response to a wide range of external factors including growth factors, hormones and inflammatory mediators such as VEGF, IGF-1, TNF-α, EGF (Kim et al., 2011) and also Interleukins and Ca2+ mobilising agents (Alemany et al., 2007). This switches the function of SK1 from housekeeping to survival. Upon stimulation, SK1 is phosphorylated on Ser225 by ERK1/2 and in turn translocates to the plasma membrane. This increases SK1 activity around 14 fold (Pitson et al., 2003). It was initially thought that calmodulin was the key binding protein involved in SK1 plasma membrane translocation which facilitated the interaction between the plasma membrane and SK1. However, this has been superseded identification of calcium and integrin-binding protein 1 (CIB1) as the key binding partner of SK1 (Siow and Wattenberg, 2012). Interestingly, calcium and integrin-binding protein 2 (CIB2) has more recently also been shown to modulate SK1 plasma membrane redistribution but negatively. CIB2 binds SK1 at the same site as CIB1 but lacks the $Ca2^{+}$-myristoyl switch function and as a result inhibits the SK1 interaction at the plasma membrane, perhaps by acting in a dominant negative fashion. This inhibited SK1 oncogenic signalling in ovarian cancer cells *in vitro* and reduced neoplastic tumour growth *in vivo* (Zhu et al., 2017). This provides evidence that SK1 redistribution rather than phosphorylation is responsible for increasing SK activity. Indeed, artificially targeting a non-phosphorylatable mutant SK1 to the plasma membrane mimics the mitogenic and pro-survival functions of wild type SK1. Conversely, blocking SK1 translocation to the plasma membrane but not SK1 phosphorylation blocks SK1 induced cell growth (Pitson et al., 2005; Hengst et al., 2009; Jarman et al., 2010).

The aforementioned studies clearly demonstrate that SK1 sub-cellular localisation is critical for its activation and subsequent oncogenic actions. This is a result of the fact that SK1 substrate Sph is most abundant at the plasma membrane. Additionally, S1P produced by SK1

at the plasma membrane is in a more favourable location for export and subsequent action at S1PRs. Furthermore, S1P stimulates ERK 1/2, which phosphorylates SK1, leading to propagation of translocation of SK1 in a positive feedback loop. Lastly, S1P formed at the plasma membrane is less subject to breakdown or de-phosphorylation by ER bound SPL or S1PP (Gault *et al*., 2010).

SK1 is deactivated via de-phosphorylation on Ser225 by the tumour suppressor protein phosphatase A2 (PPA2), an effector of ceramide (Barr et al., 2008). PP2A belongs to serine/threonine phosphatases and also de-phosphorylates other pro survival targets such as pAKT, β-catenin, pERK-1/2, BAD and Bcl-2 (Kuo et al., 2008, Li et al., 2001). SK1 is also negatively regulated by degradation through the ubiquitin proteasomal system and lysosomal system (Ren et al., 2010). Interestingly, it is a common function of SK1 inhibitors to induce the proteasomal degradation of SK1. This occurs in a plethora of solid cancer cell lines (Loveridge et al., 2010; Tonelli et al., 2010; Lim et al., 2011) as well as in proliferating vascular smooth muscle cells (Loveridge et al., 2010; Baek et al., 2013; Byun et al., 2013). The degradation of SK1 in response to these SK1 inhibitors is correlated with either binding and/or inhibition of SK1 catalytic activity, as it correlates with inhibitor potency. Degradation of SK1 represents an additional mode of action of SK inhibitors as it results in an inhibition of DNA synthesis (Byun et al., 2013).

Insights into the structure and function of SK1 have been aided by the recent publication of the crystal structure of human SK1a (Wang et al., 2013) and subsequent structure of SK1 in the presence of a moderately potent inhibitor SKi or as it is alternatively known SKI-II (1-(p-hydroxyaniline)-4-(p-chlorophenyl)) thiazole) (Gustin et al., 2013). More recently, the structure of SK1 bound to nanomolar potency inhibitor, PF543 (1-[[4-[[3-methyl-5-[(phenylsulfonyl)methyl]phenoxy]methyl]phenyl]methyl]-2R-pyrrolidinemethanol) has been published (Wang et al., 2014). This has provided new insight into the mechanistic functioning and structural features of SK1. It is now proposed that SK1 exhibits a dimeric quaternary structure (Pyne et al., 2016). Indeed, earlier studies support this, which found that differentially-tagged recombinant SK1 forms co-immunoprecipitate when over-expressed in cells (Lim et al., 2011).

Early over-expression studies on SKs highlighted a proliferative role of SK1 (Oliviera et al., 1999). A pro-oncogenic role of SK1 was discovered soon after human SK1 was cloned. This hallmark study was conducted in stably transfected NIH3T3 fibroblasts, which were

converted from fibroblasts to fibrosarcoma. This study demonstrated that over expression of SK1 enhanced cell proliferation, oncogenic transformation and tumourigenicity. Moreover, SK1-3T3 cells were tumourigenic when injected into NOD/SCID mice and SK1 enhanced their survival. Importantly, this study demonstrated that these actions were dependant on S1P generation by SK1 rather than SK1 abundance, as these actions of SK1 were not recapitulated by over-expressing catalytically inactive SK1 mutant (G82D) (Xia et al., 2000).

Since these early studies, an overwhelming amount of evidence has been published which has affirmed a role for SK1 in cancer. This evidence ranges all the way from studies conducted in cell and tissues, to mice and finally to the clinic. For example, SK1 is highly expressed in PC3 prostate cancer cells, and this confers chemotherapeutic resistance (Akao et al., 2006). SK1 expression is also increased in lung cancer. Immunohistochemical staining of lung cancer tissue for SK1 showed strikingly increased levels in 25 patient samples comprising of carcinoid, squamous or adenocarcinoma tumours versus patient matched normal tissue (Johnson et al., 2005). Furthermore, in a murine colon cancer study, SK1 was significantly elevated in cancer tissues. 89% of colon cancer tissues stained positively for SK1 versus very weak staining in non cancer counterparts. Indeed, colon adenomas had higher expression of SK1 than normal mucosa. Furthermore, $SK1^{-/-}$ mice had reduced colon cancer development than wild type counterparts (Kawamori et al., 2009). Most importantly, SK1 expression has been linked to clinical outcome in cancer patients. High expression of SK1 correlated with shorter patient survival in glioblastoma patients. Patients whose tumours had low SK1 expression lived for 357 days (median) versus patients whose tumours expressed high SK1 who lived for 102 days (median) (Van Brocklyn et al., 2005). Similar findings have been reported in both astrocytoma patients (Li et al., 2008) and gastric cancer patients (Li et al., 2009). Furthermore, SK1 and S1P have been suggested as novel biomarkers for clinical prognosis in breast, prostate and haematological cancers (Pyne et al., 2012). Finally, a meta analysis revealed that SK1 positivity and expression were significantly associated with cancer and shorter 5 year survival, although this varied greatly between cancer types (Zhang et al., 2014).

Links have also been established between SK1 and oxidative stress. In U87MG glioma cells, SK1 expression and activity was increased in response to hypoxia. Interestingly, this was reported to be dependent on HIF2α and not HIF1α and was reversed using HIF2α siRNA. Importantly, this demonstrated a role of SK1 derived S1P in promoting angiogenesis in

hypoxic tumours and also suggests a role of SK1 as an oxidative stress biosensor (Anelli et al., 2008).

1.5.2 SK2

SK2 was first identified in 2000 (Liu et al., 2000). Of the two SK isoforms, SK2 is least characterised. In addition to this relative shortage of information, there are often conflicting reports on its physiological and pathophysiological role. Like SK1, SK2 is activated by phosphorylation by ERK 1/2 (Hait et al., 2007). In cells, stimulation of SK2 activation increases its activity around 6 fold, which is comparable with SK1. However, in a non cell based experiment, phosphorylation of SK2 by ERK 2 increased SK2 activity only 2 fold compared to the 14 fold increase observed for SK1 under identical conditions, suggesting that each isoform responds differently to stimulation (Pitson et al., 2003). Moreover, SK2 activity was enhanced following interaction with eukaryotic elongation factor 1A, confirming that alternative mechanisms exist to enhance SK2 activation other than phosphorylation (Leclercq et al., 2008). The sub-cellular localisation of SK2, however, appears to be far more complex than that of SK1. As previously touched upon, SK2 contains a nuclear localization sequence (NLS) and a nuclear export sequence (NES) in a region which is not present in SK1 (Ding et al., 2007). SK2 sub cellular distribution is cell type specific: SK2 is primarily cytoplasmic in HEK293 cells whereas SK2 is mostly found in the nucleus in HeLa cells (Igarashi et al., 2003). Interestingly, it has been demonstrated in COS-7 fibroblasts that confluence can also influence SK2 localisation. As cells reached higher confluence there was a proportionate increase in SK2 which localised to the nucleus. This supports a role of nuclear SK2 in inhibition of DNA synthesis and suggests nuclear SK2 may be involved in contact inhibition (Igarashi et al., 2007). It has also been demonstrated that SK2 can localise to the ER under conditions of cell stress. This can produce S1P which drives the salvage pathway via S1PPs and ultimately leads to generation of ceramide. Interestingly, falsely targeting SK1 to the ER or nucleus converted it from an anti-apoptotic to a pro-apoptotic protein (Maceyka et al., 2005).

Many studies exist which suggest that, like SK1, SK2 promotes survival and proliferation, particularly in the context of cancer. In support of an oncogenic role of SK2, it has been shown in various human cancers such as kidney and breast cancers that knockdown of SK2 exerts stronger anti-cancer properties (e.g. by reducing migration and proliferation) than removal of SK1 (Gao and Smith, 2011). The same has been reported in glioblastoma cells,

where genetic ablation of SK2 had higher anti-proliferative effects than SK1 down regulation (Van Brocklyn et al., 2005). Furthermore, in MCF7 breast cancer cells removal of SK2 resulted in reduced growth rates and a pro-inflammatory phenotype which halted disease progression (Weigert et al., 2009). Interestingly, SK2 and not SK1 was found to positively regulate invasion of HeLa cells via formed S1P acting on $S1P_2$. Surprisingly, this action was independent of extracellular S1P and not only highlights a role of SK2 in cancer progression but is another example of 'intracrine' signalling (Adada et al., 2015). In actue lymphoblastic leukemia (ALL), SK2 has also been suggested as a therapeutic target. Genetic ablation of SK2 resulted in impaired leukaemia development in a murine ALL model and treatment with the SK2 selective inhibitor, ABC294640, increased survival in mouse xenografts ALL models. These changes were associated with reduced cMyc protein expression and consequential down regulation of pro-survival cMyc target genes (Wallington-Bedoe et al., 2014). Interestingly, S1P derived from nuclear SK2 was recently shown to antagonise the retinoic acid receptor β, which resulted in abrogation of its tumour suppressor effects in HT-29 human colon carcinoma cells (Sun et al., 2012). This is contrary to all other reports of S1P derived from nuclear SK2, which assign SK2 a pro-apoptotic role.

Contrary to the above, many studies conducted examining SK2 have concluded that its over expression induces cell cycle arrest and apoptosis (Igarashi et al., 2003; Okada et al., 2005; Maceyka et al., 2005). In support of this, SK2 contains BH3 motif (usually found in pro-apoptotic members of the Bcl-2 family) which interacts with Bcl-$_{XL}$. Furthermore, over-expression of SK1 and SK2 had opposing effects on cell survival in NIH 3T3 fibroblasts, with SK1 over expression promoting survival and SK2 over expression promoting apoptosis. Moreover, SK2 induced cytochrome c release and activation of caspase 3. However, mutating the BH3 domain of SK2 only partially abrogated SK2 generated apoptosis suggesting additional mechanisms are involved in the pro-apoptotic function of SK2 (Liu et al., 2003). More recently, mitochondrial S1P generated by SK2 was shown to permeabilise the mitochondrial membranes which lead to cytochrome c release via modulation of BAX and BAK proteins (Chipuk et al., 2012). Several studies which knock down endogenous SK2 have also concluded a pro-apoptotic role for endogenous SK2. In HEK293T cells and mouse embryonic fibroblasts (MEFs), siRNA knockdown of SK2 prevented apoptosis in response to TNFα (Okada et al., 2005; Chipuk et al., 2012). Furthermore, mesanginal cells extracted from $SK2^{-/-}$ mice were more resistant to apoptosis in response to staurosporine than wild type or

$SK1^{-/-}$ counterparts (Hofmann et al., 2008). Thus, there is compelling evidence which suggests a role for SK2 in promotion of both ends of the cell fate spectrum.

Differences in SK2 sub cellular localisation are thought to be responsible for differences in signalling functions. Some evidence suggests that nuclear SK2 is pro-apoptotic whereas cytoplasmic SK2 has pro-survival functions. Indeed, when localised to the nucleus, SK2 inhibited DNA synthesis and this function was abrogated in an NLS mutant of SK2 (Igarashi et al., 2003). Also, as previously mentioned, artificial targeting of SK1 to the ER shifts its function from pro-survival to pro-apoptotic (Maceyka et al., 2005). In addition to being dictated by its sub cellular distribution, the role of SK2 is likely to be cell type and context specific, as it appears that most studies in support of an apoptotic role of SK2 are under physiological circumstances and those which demonstrate a pro-survival role are predominantly in cancer.

SK2 has also been linked to senescence. It was demonstrated that nuclear S1P formed by SK2 inhibited HDAC1/2 activity, leading to enhanced transcription of p21 and transcription factor c-fos (Hait et al., 2008). Moreover, SK2 derived S1P was shown to stabilise the catalytic sub unit of telomerase (hTERT) which in turn enhanced telomere maintenance and stability. Disruption of this process either via SK2 inhibition or mutagenesis of essential residue D684 reduced hTERT stability, telomere integrity and resulted in premature senescence (Panneer Selvam et al., 2015). These findings not only highlight an oncogenic role of SK2 but also link SK2 and senescence. Thus, SK2 inhibition could present a promising opportunity for inducing senescence in immortal cancer cells.

To date, two isoforms of SK2 have been identified and characterised – SK2a (Liu et al., 2000) and SK2b (Okada et al., 2005) and a third splice variant existed which is not well studied (Pitson, 2011). Unless explicitly specified, it is SK2a which is referred to in the literature as SK2. SK2a is 36aa shorter than SK2b. The N terminal extension present in SK2b which is thought to enhance catalytic activity. Indeed, SK2b phosphorylates its substrates at a higher rate than SK2a (Neubauer and Pitson, 2013). Furthermore, SK2b is more highly expressed than SK2a in a variety of human cell lines, suggesting it may be the most physiologically important isoform (Okada et al., 2005). It is certain that SK2 plays different role under different circumstances. The precise reason for these differences has yet to be elucidated. It is possible that the different SK2 isoforms localise to different areas of the cell

or are more active at different areas of the cell due to conformational changes and resultant differences in regulation and activity. This however requires further investigation.

1.6 Ceramide

As well as being a precursor molecule for many other sphingolipids, ceramide is an important signalling molecule in its own right. The actions of ceramide are largely thought to oppose those of S1P. Ceramide was first shown to cause apoptosis in 1993 when it was demonstrated that addition of C2 ceramide to U937 cells resulted in DNA fragmentation and apoptosis (Obeid et al., 1993). Since then, an extensive body of research has been published which has implicated ceramide in apoptosis and in regulating cell fate.

Ceramide accumulation occurs in response to a wide variety of extracellular agents and stress stimuli such as cytokines, environmental stresses, cytotoxic compounds (e.g. chemotherapeutic agents) and irradiation. In turn, ceramide modulates responses to these stresses by inducing fate decisions such as apoptosis, cell cycle arrest and senescence (Hannun and Luberto, 2000). To induce apoptosis, ceramide can activate both extrinsic and intrinsic signalling pathways (See Figure 1.6) (Morad and Cabot, 2013).

Exposure of cells to stress stimuli results in ceramide accumulation which can occur via two routes. Chronic stress leads to up-regulation of *de novo* ceramide synthesis whereas acute stress induces the formation of ceramide from sphingomyelin via rapid hydrolysis (Okousian and Saba, 2010). Ceramide then acts on intracellular target proteins to elicit apoptosis. Members of the Bcl-2 protein family (which govern mitochondrial outer membrane permeabilisation) are particularly important downstream mediators of ceramide action. For example, ceramide modulates PP2A, which in turn shifts the ratio of pro-apoptotic Bax and anti-apoptotic Bcl-2 activation (von Haefen et al., 2002). Additionally, ceramide activates cathepsin D which in turn cleaves Bcl-2 family member Bid, allowing its translocation to the mitochondrial outer membrane where it is active and can initiate apoptosis (Heinrich et al., 2004).

Via the extrinsic pathway, ceramide modulates ‘death receptor’ Fas signalling at the cell surface by modulating Fas clustering to enhance Fas ligand binding, which in turn initiates the Fas mediated ‘extrinsic’ apoptosis cascade (Park et al.,2008). The physiological importance of this is evident in the fact that tumour cells down regulate Fas expression as a means of resistance to apoptosis as a means of cancer progression (Paschall et al., 2014).

Ceramide is also an inducer of cellular senescence. Exogenous addition of ceramide to young WI-38 fibroblasts resulted in induction of a senescent phenotype (inhibition of mitogenesis and DNA synthesis) and endogenous ceramide levels increased 4 fold in WI-38 human fibroblasts as they aged (approached replicative senescence) (Venable et al, 1995). This suggests that ceramide may be a general mediator of senescence with modulates both premature and replicative senescence. This was also reported more recently in endothelial cells (Venable and Yin, 2009). Ceramide has also been linked to the senescence mediator p53. Ceramide elevation in response to cell stress was reported to be dependent upon p53 accumulation (Dbaibo et al., 1998). This was shown to be a result of CerS5 up regulation by p53 and consequential C16 ceramide formation (Panjarian et al., 2008). However, several other reports also suggest that ceramide is an important downstream mediator of the p53 response (Kim et al., 2002; Villani et al., 2006). In SKN-SH cells, ceramide induced apoptosis via alteration of the Bcl-2/Bax ratio and activation caspases and this was reversed using p53 antisense oligonucleotides (Kim et al., 2002). Conflictingly, other studies have shown that p53 and ceramide are concomitantly activated in response to cell stress and that ceramide accumulation can occur in cells irrespective of p53 status (Villani et al., 2006; Nasr et al., 2005; Deng et al., 2009).

Interestingly, it is now known that there are multiple intracellular pools of ceramides with unique chain length composition. Studies have also indicated that certain functions of ceramide are chain length specific and can differentially influence cell fate (Grosch et al., 2012).

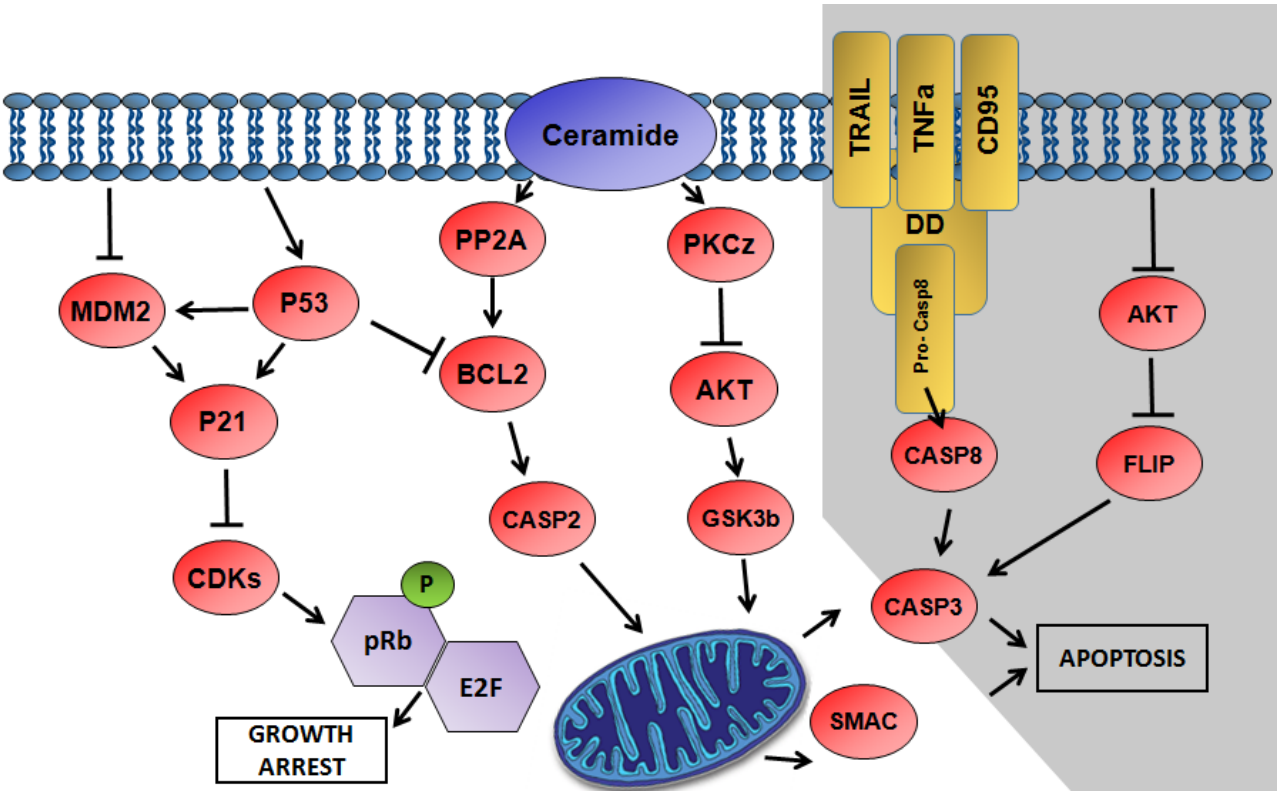


Figure 1.6 ***Ceramide downstream signalling pathways (adapted from Morad and Cabot 2013).*** *Extrinsic (grey background) and intrinsic (white background) pathways activated in response to ceramide accumulation are illustrated above. Caspase 3 is common to both pathways.*

1.7 Dihydroceramides and dihydroceramide desaturase 1

There has been a recent surge of interest in dihydroceramide (dhCer). This has in turn generated interest in the enzyme involved in its synthesis - Dihydroceramide desaturase 1 (Des1). Des1 is a relatively 'new player' in the sphingolipid signalling field and both Des1 and dhCer are now emerging as bioactive molecules which control important functions such as autophagy, hypoxia and proliferation (Siddique et al., 2015).

DhCers (and dihydro sphingolipids in general) traditionally received little attention due to an incorrect dogma in the field: they were considered to act solely as an intermediate in the synthesis of ceramide. Thus, it was assumed that conversion of dhCer to ceramide was necessary to elicit any biological responses. This was due to several studies which showed that addition of exogenous short chain dhCers did not induce apoptosis or growth arrest (Bielawska et al., 1993; Sugiki et al., 2000; Ahn et al., 2000). This idea was challenged when a study used mass spectrometry to profile the sphingolipiddome in response to fenretinide, which found that dihydroceramide species were extensively induced and resulted in the induction of autophagy (Zheng et al., 2006).

This idea that dhCers were biologically inactive was challenged when it was shown that dhCer mediated effects which did not overlap with the functions of ceramide (Rodriguez Cuenca et al., 2015). In 2007, it was shown in glioblastoma cells that knockdown of Des1 using siRNA resulted in growth cell cycle arrest at G0/G1 (Kraveka et al., 2007). Since then, addition of exogenous short chain dhCers to cells as well as the pharmacological and genetic ablation of Des1 has uncovered a plethora of signalling functions mediated by Des1 and dhCer (Rodriguez Cuenca et al., 2015). For example, the specific Des1 inhibitor XM462 induced dhCer accumulation in HCG27 gastric carcinoma cells. This resulted in delayed G1/S transition of the cell cycle, ER stress and induction of autophagy. Importantly, these effects were recapitulated by addition of short chain dhCers (Gagliostro et al., 2012).

Des1 catalyses the final step in the *de novo* pathway of ceramide synthesis by converting dihydroceramide to ceramide. This was first demonstrated in rat liver microsomes using ^{14}C labelled C_2 ceramide as a substrate. The reaction required NADH or NADPH as a co-substrate. Molecular oxygen was also required for enzymatic activity. Interestingly, the Des1 catalytic reaction was inhibited by dithiothrietol (DTT) and N-acetyl cysteine (NAC), suggesting Des1 is sensitive to thiol status (as addition of NAC would result in glutathione

(GSH) accumulation). The inhibition of Des1 by NAC is paradoxical as NAC is an antioxidant molecule, and Des1 is inhibited by ROS, which will be discussed at length later (see chapter 5). Other factors which influenced Des1 enzymatic activity were alkyl chain length of both the sphingoid base (C_{18} > C_{12} > C_8) and the fatty acid (C_8 > C_{18}) and also stereochemistry of the sphingoid base (D-*erythro*- > L-*threo*-dihydroceramides) (Michel et al., 1997).

Taking current knowledge into account, it remains unclear whether Des1 is a pro-survival or a pro-apoptotic enzyme or if the function of Des1 is cell type/context specific. Studies suggest that it acts as a stress sensor to mediate protection and adaption against intermediate level stresses by initiating protective mechanisms like autophagy or growth arrest/senescence in order to salvage the cell. Perhaps, when the stress reaches a certain threshold, the balance of dhCer to ceramide is altered and this is what determines whether the cells adapt or apoptose. Supporting this, Des1 is sensitive to thiol status. Thiols (e.g. GSH) usually function to protect against cell injury and stress. Thus, upon thiol accumulation, Des1 inhibition would result in dhCer accumulation and activation of downstream adaptive or protective mechanisms. Des1 inhibition by GSH could possibly be because Des1 contains disulfide bonds which are necessary for its catalytic efficiency. Furthermore, inhibition of Des1 and consequent dhCer increases induced autophagy and delayed the cell cycle in gastric cancer cells (Gagliostro et al., 2012). This was recapitulated in mouse embryonic fibroblasts where Des1 ablation resulted in induction of autophagy and inhibition of proliferation (Siddique et al., 2013) as well as resistance to chemotherapeutic agents via activation of protective pro-survival pathways (Siddique et al., 2012). Interestingly, recent studies have suggested that Des1 inhibition and subsequent dhCer accumulation could be primarily responsible for the anticancer properties of some SK inhibitors (Aurelio et al., 2016).

Des1 also functions as an oxidative stress biosensor in cells. Des1 requires O_2 and NADPH for its enzymatic function, and redox balance and Des1 activity can reciprocally influence one another (Fabrias et al., 2012). Indeed, Des1 activity was inhibited by oxidative stress in HEK293, MCF7, A549 and SMS-KCNR cells which resulted an accumulation of dhCers (Idkowiak-Baldys et al., 2010). Moreover, Des1 has been proposed to act as an oxygen biosensor in lung and colon cancer cell lines (Devlin et al., 2011). Furthermore, Des1 mediated adaption to chronic hypoxia in mouse cardiomyocytes. In this model, dhCer accumulated with hypoxia whilst ceramide and Des1 mRNA levels decreased in response to hypoxia. This suggests a role of Des1 as a regulatory checkpoint in this system (Azzam et al.,

2013). Also, all dhCer species are increased in response to hypoxia via Des1 down regulation. In normoxia, siRNA knockdown of Des1 recapitulated the effects of hypoxia on cell proliferation. This suggests that Des1 may control processes which drive the cellular response to hypoxia. Conversely, Des1 or Des2 over expression increased proliferation MCF7 cells in hypoxia (Devlin et al., 2011). There is currently very little published information available surrounding Des2. Genome wide association studies (GWAS) have shown a role for the Des2 gene loci in cognition in schizophrenia (Ohi et al., 2015) and sudden cardiac arrest in coronary artery disease patients (Aouizerat et al., 2011).

To date, two sphingosine-like inhibitors of Des1 have been used as study tools to aid understanding of Des1 function. GT11 (N-[(1R,2S)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octanamide) was the first of these and has a Ki of 6μM (Triola et al., 2003). In primary cultured cerebellar neurons, this inhibited Des1 with an IC_{50} of 23nM (Triola et al., 2004). At higher concentrations (>5μM), GT11 also inhibits S1PL and consequently *de novo* sphingolipid synthesis (Triola et al., 2004). XM642 was designed later and has an IC_{50} of 8.42μM in rat liver microsomes and an IC_{50} of 0.43μM in Jurkat cells (Munoz-Olaya et al., 2008). At concentrations which do not affect cell viability, XM642 was shown to induce an accumulation of dhCer which was followed by induction of autophagy, ER stress and delayed cell cycle transition at G1/S phase (Gagliostro et al., 2012; Signorelli et al., 2009). In addition to these inhibitors which have been specifically synthesised to act on Des1, several other compounds exist which are known to inhibit Des1 activity. These include fenretinide, resveratrol, celecoxib and tetrahydrocannabinol (THC) (Casasampere et al., 2015). Interestingly, the SK1/SK2 inhibitor SKi (see section 3.8.2) has also been shown to inhibit Des1 in cells (Cingolani et al., 2014). Furthermore, the SK2 selective inhibitor ABC294640 (see section 3.8.1) has also been demonstrated to affect Des1 in cells (Venant et al., 2015; McNaughton et al., 2016; Chapter 5).

Like ceramide, there are multiple molecular species of dhCer which are likely confer chain length specific properties. The physiological or clinical relevance of this is not yet fully understood. Furthermore, intracellular targets of dhCer are still being discovered. Thus, it is sensible to assume that as more targets become elucidated the clinical utility of exploiting dhCer and Des1 will become more apparent (Siddique et al., 2015). The molecular determinant that swings the balance between ceramide and dhCer generation also remains unknown. However, dhCer been suggested as an attenuator of the apoptotic action of ceramide via direct ceramide reduction. This is likely tightly regulated and it also must be

considered that this will not only affect the balance of dhCer to ceramide but the abundance of both molecule. The role of dhCer in ceramide induced apoptosis remains controversial and it ultimately remains unknown whether dhCer is a pro-survival or pro-apoptotic molecule and this could indeed vary under different circumstances. dhCer appears to elicit a pro-survival programme under situations of intermediate stress. If this is true, it is possible that like dhCer increases in response to manageable stress to drive an adaptive response. This could in turn mean that ceramide sits in the centre of two rheostat models (with S1P/Cer controlling the survival/apoptosis axis and dhCer/Cer controlling the adaption/apoptosis axis).

1.8 SK Inhibitors

There have been intense efforts to target the sphingolipid signalling pathway in order generate novel therapeutics, particularly for cancer treatment but also for treatment of other diseases such as atherosclerosis, sepsis and asthma (Kunkel et al., 2013; Santos and Lynch, 2015). Recent years have seen increasing interest in the development of novel compounds which are more potent and selective for either SK1 or SK2. These compounds have been important not only in the pursuit of novel therapeutics but in providing more specific tools to discern the sphingolipid network and associated pathways. Importantly, some sphingolipid-targeted molecules are beginning to progress towards the clinic. For example, the S1P receptor modulator and SK2 substrate FTY720 (Fingolimod) is licensed for the treatment of multiple sclerosis (Chun and Hartung., 2010) whereas the SK2 inhibitor ABC294640, which will be discussed in detail later, is in phase two clinical trials for renal cancer and β cell lymphoma (Thomas *et al*., 2013). For the purposes of this thesis, only inhibitors which have been used experimentally will be introduced at length. The chemical structures for these are detailed below (Figure 1.7). An extensive review of SK inhibitors including classical non-isoform selective 'pan' SK inhibitors is available (Gandy and Obeid, 2010). A updated review including novel inhibitors is also available (Pitman et al., 2016).

1.8.1 ABC294640

Aryladamantane compound ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide] is currently in phase 1 clinical trials (**YELIVA™**) for pancreatic cancer and solid tumours and phase 2 clinical trials for renal cancer and β cell lymphoma (Thomas *et al*., 2013). It has good bio-availability and is a non-lipid small molecule competitive inhibitor of SK2 activity with respect to sphingosine (Ki 9.8μM) (French et al., 2010). Although ABC294640 is catalytically selective for SK2 at concentrations up to

100μM, it is a relatively poor inhibitor of SK2 with an IC_{50} reported to be 60μM for recombinant SK2. However, it reduces S1P in cells with an IC_{50} of 26μM, which may be an indicator that it is more potent in cells than in an *in vitro* assay (French et al., 2010). In plasma as well as in cells and tumours, ABC294640 reduces SIP levels and inhibits proliferation (Beljanski et al., 2011). Furthermore, it exhibits dose dependant anti tumour activity when administered to mice *in vivo.* Importantly. ABC294640 has a favourable therapeutic index of >7 in mice (250 mg/kg nontoxic dose / 35 mg/kg antitumor activity) (French *et al*., 2010). When characterised in A498 kidney adenocarcinoma cells, ABC294640 was reported to reduce migration, invasion and proliferation and pronouncedly increase both SK1 and SK2 mRNA levels (>300% and >700%, respectively) (Gao *et al*., 2012). Recently it was shown that ABC294640 induced the proteasomal degradation of cMyc and myeloid cell leukaemia 1 (Mcl-1) in myeloma cells (Venkata et al., 2014). In addition to having anti-cancer properties, ABC294640 has shown efficacy in an array of additional diseases, mainly inflammatory diseases such rheumatoid arthritis (Fitzpatrick et al., 2011) and Crohn's disease (Maines et al., 2008). ABC204640 may also promote autophagic cell death as it increases autophagic markers LC3-II and Beclin-1 (Beljanski et al., 2010). Recently, the therapeutic potential of ABC294640 has been assessed in castrate sensitive (androgen receptor positive) prostate cancer. In LNCaP cells, ABC294640 inhibited cell growth (IC_{50} 12μM) and this was associated with a down regulation of several onco-proteins such as cMyc, androgen receptor and pAKT (Schrecengost et al., 2015). Furthermore, ABC294640 has also shown efficacy in other cancers, for example non small cell lung cancer (NSCLC), where it enhanced apoptosis in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Yang et al., 2015). Recently, ABC294640 has also shown efficacy in cholangiocarcinoma (Ding et al., 2016). Since its discovery several additional mechanisms of action have been reported for ABC294640, notably that it also inhibits Des1 (Venant et al., 2015; McNaughton et al., 2016; see Chapter 5) and that it induces the proteasomal degradation of SK1 in LNCaP AI prostate cancer cells (McNaughton et al., 2016; see Chapter 3).

1.8.2 SKI-II (SKi)

The thiazole compound SKi (1-(p-hydroxyaniline)-4-(p-chlorophenyl)) thiazole) (also known as SKI-II) was first identified by screening a library of synthetic compounds (French *et al*., 2003). It is a dual selective mixed inhibitor with a competitive inhibition constant of 17 μM

towards sphingosine and an uncompetitive inhibition constant of 48 μM towards ATP with respect to SK1 (Lim et al., 2012). SKi reduces S1P levels and also inhibits proliferation and induces apoptosis of several cancer cell lines and murine mammary adenocarcinoma (French *et al*., 2006). SKi promotes the degradation of SK1 by activation of the ubiquitin proteasomal pathway thus reducing its expression in cells (Tonelli *et al*., 2010). Indeed, a common trait of SK1 inhibitors is to induce proteasomal degradation of the enzyme in addition to their catalytic inhibition; this augments efficacy and is arguably an additional mode of action of these compounds. SKi is reported to promote formation of reactive oxygen species (ROS) in three human carcinoma cell lines (Huwiler *et al*., 2011) and is reported to reduce androgen receptor expression in an oxidative stress dependant manner in LNCaP AI prostate cancer cells (Tonelli *et al*., 2013). SKi also reduces S1P levels and increases Sph levels, concomitant with an increase in C22:0 ceramide in LNCaP AI cells, which resulted in their apoptosis. In this study, it was also reported that the master transcription factor cMyc was degraded by the proteasome in response to SKi in a manner comparable to SK1, resulting in an indirect antagonism of the Warburg effect (Watson *et al*., 2013). Furthermore, In glioblastoma cells SKi was tested in combination with the chemotherapeutic drug temozolomide (TMZ). Combination treatment of SKi and TMZ resulted in cell death associated with induction of oxidative stress, endoplasmic reticulum stress and autophagy. Importantly, the cell death response was due to a build up of ceramide 'intermediate' lipids (dihydrosphingosine and dhCer) as opposed to ceramide itself (Noack et al., 2014). This again highlights the importance of dhCer as a signalling molecule but raises the question of the true mechanism behind SKi's efficacy as an anti-cancer compound. Interestingly, SKi was recently reported to inhibit Des1 (Cingolani et al., 2014). However, the mechanism by which SKi inhibits Des1 has yet to be elucidated. As Des1 requires O2 and NADPH to function (Fabrias et al., 2012) and SKi is a known inducer of oxidative stress, it is possible that SKi indirectly inhibits Des1 activity via alteration of cellular redox balance. Indeed, Cingolani and colleagues concluded that inhibition of Des1 by SKi could be a result of NADH-cytochrome b5 reductase inhibition by Ski (Cingolani et al., 2014). Moreover, the current study (chapter 5) demonstrates that SKi removes Des1 from cells via the proteasome which accounts for its apparent reduction in activity. Indeed, it was shown by the Pitson group that Des1 inhibition was primarily responsible for the anticancer activity of SKi and several analogues which inhibited combinations of SK1, SK2 and Des1 (Aurelio et al., 2016). There are currently no non-cell based assays for Des1 available which would allow this theory to be tested.

1.8.3 PF543

PF543 is a competitive inhibitor with a with respect to sphingosine (Ki 3.6nM) and the first of its kind to exhibit nano-molar potency towards SK1. It is 100 fold more selective for SK1 than SK2 making it the most selective inhibitor commercially available for SK1. It reduces cellular S1P levels and also reduces S1P levels in blood (Schnute *et al*., 2012). Despite its profile as a highly selective and potent SK1 inhibitor it does not affect cell viability or proliferation (Byun et al., 2013). Of note however, is the fact that although PF543 is able to reduce S1P, it does not induce ceramide accumulation until concentrations of >3μM, therefore does not shift the 'sphingolipid rheostat' and this may account for the lack of effect on cell viability. PF543 has however been demonstrated to prevent sickling of cells due to high S1P in sickle cell disease suggesting that this compound may be therapeutically efficacious in this disease due to its ability to reduce S1P (Zhang et al., 2014). Furthermore, PF543 has demonstrated *in vivo* efficacy in several other studies. In a mouse model of pulmonary hypertension, PF543 reduced right ventricular hypertrophy and cardiomyocyte death. This was associated with an increase in transcription factor Nrf2 and a decrease in p53 (MacRitchie *et al*., 2016). Similarly, inhibition of SK1 using PF543 ameliorated cardiac dysfunction and remodelling in a mouse model of myocardial infarction (Zhang et al., 2015). These findings demonstrate clear *in vivo* efficacy of PF543 in the cardiovascular system which have not been demonstrated in cancer models, perhaps due to aforementioned reasons regarding a lack of ceramide accumulation in these cells in response to PF543. Indeed, in the MacRitchie study the effects of PF543 were not recapitulated by RB005, an SK1 inhibitor which has a secondary effect of ceramide synthase inhibition. Lack of ceramide accumulation in this system could account for this lack of consistency between the two compounds, however ceramide measurements would have to be made in order to confirm this (MacRitchie et al., 2016).

1.8.4 ROMe

The FTY720 analogue (2R)-2-Amino-3-(O-methyl)-(2-(4′-n-octylphenyl)ethyl)propanol ((R)-FTY720-OMe or ROMe)) is a SK2 selective sphingosine competitive inhibitor (Ki 16.5μM) which also substantially reduces SK2 expression in cells (Lim *et al*., 2011). It reduced S1P mediated rearrangement of actin filaments and also inhibited DNA synthesis in MCF-7 breast cancer cells. Importantly, the induction of SK2 degradation by ROMe augments its efficacy and could enhance potency *in vivo* (Lim *et al*., 2011). In LNCaP AI

cells, ROMe reduced S1P levels and increased Sph levels, but unlike SKi failed to increase C22:0 ceramide levels therefore did not cause apoptosis (Watson *et al*., 2013). In T-ALL cell lines and patient lymphoblasts, ROMe induces autophagic cell death. Furthermore, ROMe reduced prognostic markers of T-ALL such as cMyc and phospho-AKT (Evangelisti et al., 2014). Recently, ROMe also demonstrated anti-inflammatory action in a mouse model of experimental autoimmune encephalomyelitis. In this model, ROMe induced a reduction in $CD4^+$ and $CD8^+$ T-cells and reduced infiltration of inflammatory cells into the spinal cord which resulted in a reduction of disease progression. In the corresponding *in vitro* experiments, ROMe reduced expression of $S1P_1$ at the cell surface (Barbour *et al.*, 2017).

1.8.5 RB-005

RB-005 is a potent (IC_{50} = 3.6 µM) SK1 inhibitor which was synthesised from the FTY720 scaffold. It is highly selective for SK1 (15 times more selective for SK1 than SK2). Like many other SK1 inhibitors, it reduces SK1 expression and in pulmonary artery smooth muscle cells (PASMCs) and this is reversed by the proteasome inhibitor MG132 (Baek *et al*., 2013). In an *in vivo* mouse model of hypoxic pulmonary arterial hypertension, RB-005 was shown to also inhibit ceramide synthase. In this model, RB-005 and the nanomolar potent inhibitor of SK1, PF543, exhibited different effects on arterial remodelling and right ventricular hypertrophy. PF543 reduced right ventricular hypertrophy but did not induce vascular remodelling whereas RB-005 lacked effects on right ventricular hypertrophy. This was attributed to the combined inhibition of SK1 and ceramide synthase (MacRitchie et al, 2016).

1.8.6 K145

3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) is a sphingosine competitive inhibitor (Ki 6.4µM) with respect to SK2. It has an IC_{50} of 4.30µM and is selective for SK2 only at concentrations up to 10µM. In U937 cells, it reduces S1P levels and inhibits proliferation in a dose dependant manner. However, it reduces S1P at concentrations much lower than its IC_{50} suggesting an additional mode of action. *In vivo*, K145 inhibited tumour cell growth of both U937 tumours in nude mice and JC mammary adenocarcinoma cells injected into immunocompetent BALB/c mice (Liu *et al*., 2013). Furthermore, K145 abolished the cardioprotective effects of hypoxic preconditioning on

hypoxia/re-oxygenation injury in primary rat neonatal cardiomyocytes. This was reproduced by SK2 knockdown (Zhang *et al*., 2016).

1.8.7 F-02

The sphinganine thiourea derivative F-02 is a moderately potent, moderately selective SK2 inhibitor with an IC_{50} of 21.8μM with respect to SK2 and 69μM with respect to SK1. It has yet to be characterised in cells (Byun *et al*., 2013).

There is continuing effort to synthesise increasingly potent isoform specific SK inhibitors. These efforts have been aided by the publication of the crystal structure of SK1 (Wang et al., 2013) and subsequently the structure of SK1 bound to PF-543 (Wang et al., 2014). This data is now being analysed to identify residues which could be exploited to make novel inhibitors therapeutic compounds (Santos and Lynch, 2015). An example of such an inhibitor is Amgen 82 (Gustin et al., 2013). Amgen 82 inhibits both isoforms of SK with nanomolar potency and was synthesised following analysis of the crystal structure of SK1 bound to SKi (Wang et al., 2013). Amgen 82 failed to reduce tumour volume *in vivo* despite reducing circulating S1P levels by 70% and also had no effect on tumour cell viability at concentrations which inhibit the enzyme (Rex et al., 2013). However, there is no published information on whether Amgen 82 is able to increase ceramide or whether it has off target effects on other enzymes in the pathway which would in turn affect the levels of other sphingolipids. In addition, several nano molar potency inhibitors of Des1 have been synthesised by the Pitson group which could be of potential chemotherapeutic benefit. They explored the structure activity relationships between SKi and Des1, SK1 and SK2 to synthesise a panel of inhibitors bearing higher specificity for Des1 alone or in combination with SK1 or SK2 and went on to characterise these in PC3 prostate cancer cells (Aurelio et al., 2016). This study delineated SK1 degradation and inhibition by reporting that compounds give rise to a mixture of SK1 inhibition, SK1 degradation or both. The study also concludes that PC3 cell growth is not inhibited by targeting SK1 and that Des1 is most likely to be the mediator of the anticancer activity of SKi (Aurelio et al., 2016).

The aforementioned study makes a step towards delineating the actions of SK inhibitors which have multiple targets whilst also synthesising higher potency inhibitors. This is most certainly the way the field must progress to achieve definitive answers which can in turn aid identification of therapeutic targets for drug discovery.

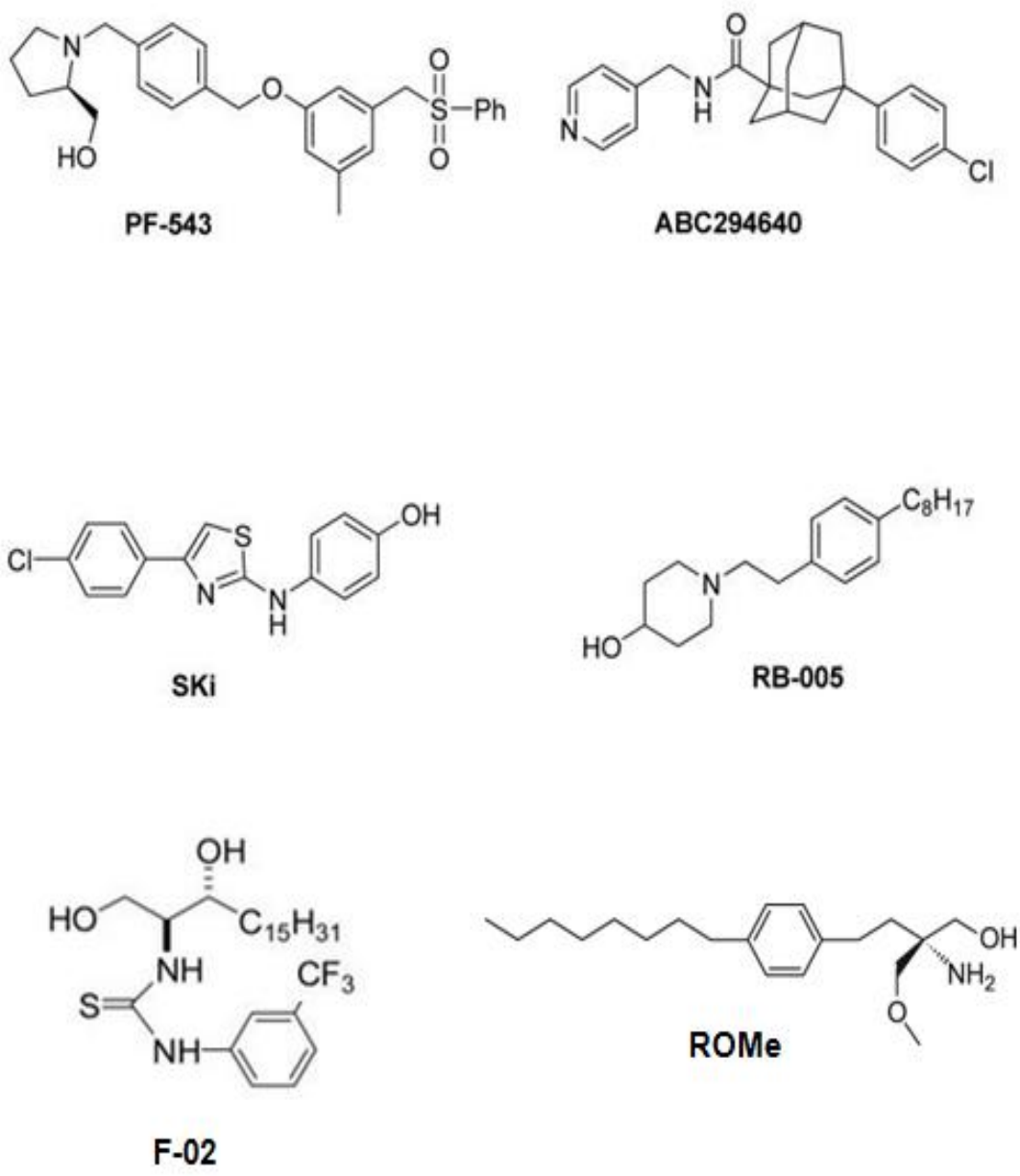


Figure 1.7 ***Chemical structures of SK inhibitors*** *Chemical structures of SK inhibitors utilised in this study. There include SK1 selective inhibitors PF543 (Schnute et al., 2012) and RB005 (Baek et al., 2013); SK2 selective inhibitors ROMe (Lim et al., 2011) F-02 (Byun et al., 2013) and ABC294640 (French et al., 2010); SK1 and SK2 inhibitor SKi (French et al., 2003).*

1.9 Senescence

Senescence (from the Latin word *senescere*: to grow old) is the term used to describe biological ageing and the resultant deterioration of function of an organism. Senescence can describe ageing of the whole organism or cells. It is thought that cellular senescence cumulatively leads to whole organism senescence. For the purposes of this thesis, any use of the word senescence will refer to cellular senescence.

Cellular senescence is defined as the permanent exit from the cell cycle, when a cell no longer grows or divides but remain metabolically active. There are two types of cellular senescence. The first - replicative senescence (RS) - occurs naturally due to attrition. This is characterised by repeated passage and telomere shortening. Telomeres function to protect chromosomal DNA from damage and when they are shortened beyond a certain threshold, chromosomal DNA is damaged and a DNA damage response occurs (DDR). Cells are protected from DDR by telomerase, which lengthens telomeres. This type of senescence was first observed in 1961 by Dr Leonard Hayflick when it was realised that human fibroblasts could undergo a limited amount of passages before ceasing to divide. This lead to the coining of the term 'the Hayflick limit' which refers to the maximum number of passages a cell can undergo before reaching senescence (Hayflick and Moorhead, 1961).

The second type of senescence – premature senescence – is senescence which occurs independently of telomere shortening. This type of senescence reflects a change in cell state rather than cell age. The resultant phenotype is complex and only partially understood. Paradoxically, senescent cells adopt a pro-survival phenotype but also limit tumourigenesis. This is because they are recognised and processed by the immune system (Burton and Krizhanovsky, 2014). While natural senescence is considered to be deleterious as an organism loses its ability to repair and regenerate, pathological senescence in the context of cancer is seen as a beneficial and as a potential means of tumour cell suppression (Fabrizio and Fagagna, 2007). Again, for the purposes of this thesis, any use of the word senescence will refer to premature senescence in the context of cancer suppression.

There is substantial evidence that senescence is a potent tumour-suppressor mechanism, as it stops cancer cells from proliferating which, as well as preventing tumour growth, halts accumulation of additional somatic mutations. Senescence can be triggered by a variety of

stimuli, which engage two main molecular pathways. As well as telomere dysfunction, known triggers of senescence include (but are not limited to) oncogene activation, DNA replication stress and oxidative stress (Burton and Krizhanovsky, 2014). Oncogene induced senescence (OIS), which is a paradoxical growth arrest triggered in response to chronic activation of proliferative genes such as RAS and RAF, is a testament to the tumour suppressive nature of senescence and is a natural protective mechanism against oncogenic transformation. One of the main differences between the DDR in RS and OIS appears to be the degree of oxidative stress, as OIS is often associated with accumulation of reactive oxygen species (ROS) and mitochondrial dysfunction (Mallette et al., 2007; Moiseeva et al., 2009).

Interestingly, ROS alone can activate a senescence programme in cells. Extracellular and intracellular ROS, as well as ionising radiation and some chemotherapeutic drugs can also induce a DDR which results in senescence. This type of senescence is often referred to as stress-induced premature senescence (SIPS). Various forms of ROS can induce this type of senescence including hydrogen peroxide, superoxide, and hydroxyl radicals (Toussaint et al., 2000). Importantly, mitochondrial dysfunction and impairment of antioxidant enzymes can also result in SIPS (Wei and Lee, 2002). In response to mild DNA damage, transient growth arrest occurs whereas in response to chronic DNA damage, a programme of senescence is activated. When a DDR is extensive and beyond a certain threshold, apoptosis occurs (Burton and Krizhanovsky, 2014). The molecular determinants that regulate the switch between temporary growth arrest, senescence and apoptosis are not yet fully understood.

Two main molecular pathways are activated to induce senescence, named after two paradigmatic tumour suppressor proteins – p53 and pRB (Figure 1.8). Like the sphingolipid signalling network, the pathways which govern senescence are complex. This thesis focussed primarily on the p53 pathway; therefore the pRb pathway will be discussed only briefly herein.

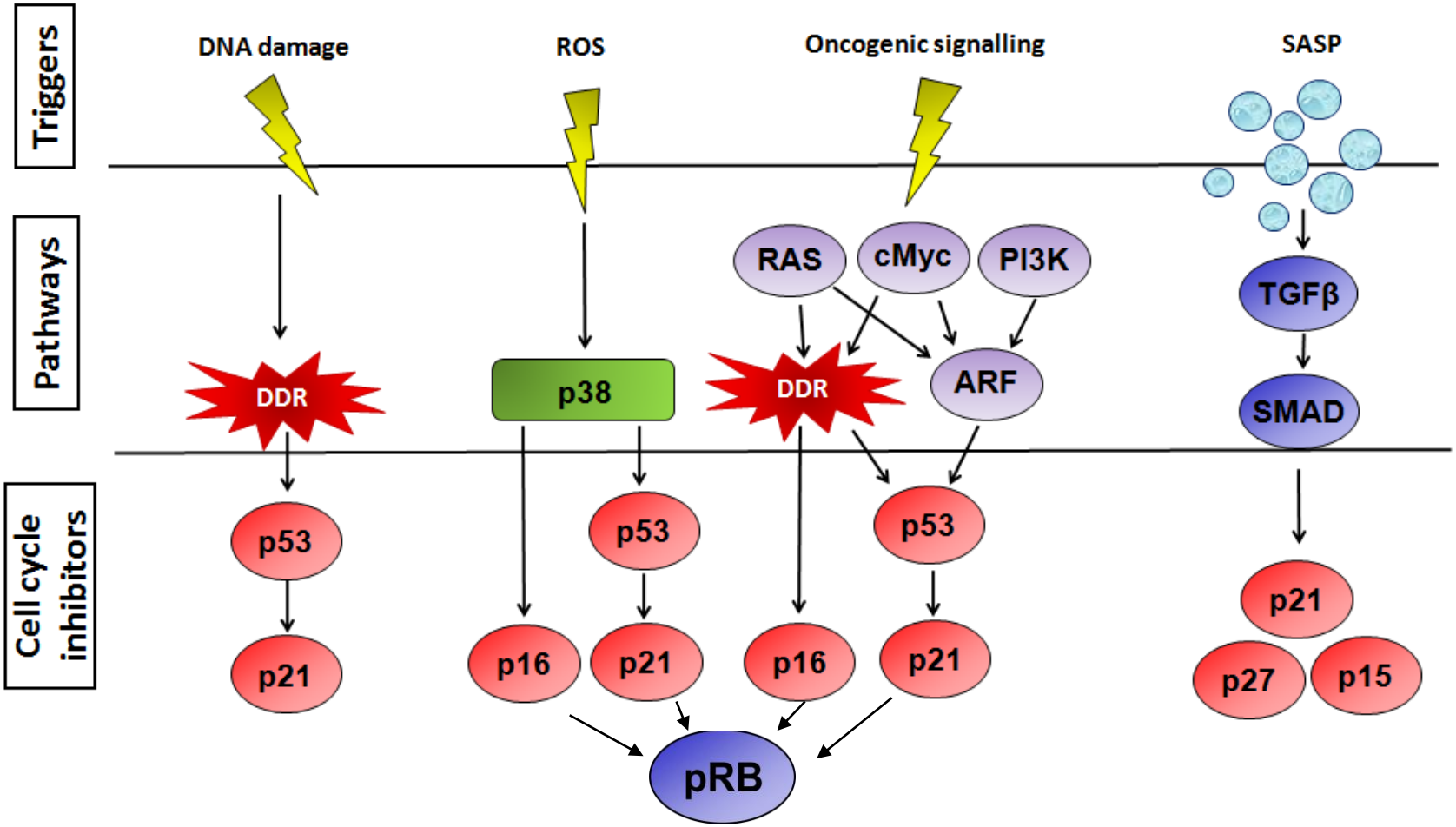


Figure 1.8 ***Senescence signalling (adapted from Campisi, 2007).*** *Triggers, molecular pathways and effectors of cellular senescence. SASP, senescence associated secretory proteins; p38, p38 mitogen activated kinase (MAPK); TGFb, transforming growth factor b; SMAD, TGFb effector.*

1.9.1 The retinoblastoma protein pathway

The retinoblastoma protein (protein name abbreviated pRb) is an archetypal tumour suppressor protein that was first identified in retinoblastoma, a malignant cancer of the retina. This protein is responsible for repressing gene transcription which is essential for transition from G1 to S phase, hence causing G1 growth arrest. This means that pRb has a pivotal role in tumour suppression and negative regulation of the cell cycle. Indeed, mutations in the pRb gene lead to development of retinoblastoma tumours and functional inactivation of pRb is prevalent among many cancers such as Burkitt's lymphoma and cervical cancer (Giacinti and Giordano, 2006).

In G0, the unphosphorylated from of pRb is bound to E2F. E2F proteins are a family of transcription factors which are encoded by the E2F1 gene. These proteins regulate promoters which are required for gene expression. When Rb is bound to any of the E2F family members it blocks their transactivation domains thus causing growth arrest. When pRb is phosphorylated by cyclin dependant kinases (CDKs), it dissociates from E2F. This allows the expression of proteins which are required for transition to S phase (Muller et al., 2007).

The main upstream mediators of pRb are two proteins both encoded by the INK4a-ARF locus (CDKN2A/B) on human chromosome 9p21. These are $p16^{INK4A}$ and $p19^{ARF}$. These proteins trigger the anti-proliferative effects of pRb. Both of these proteins act as inhibitors of the cyclin dependant kinases CDK4 and CDK6, respectively, which phosphorylate and dissociate Rb from E2F thus progressing transcription (Sherr, 2012). Like pRb, the INK4a-ARF locus is commonly deleted or mutated in human cancers and this mimics the phenotype of pRb and p53 co-inactivation. Importantly, the INK4a-ARF locus represents a point of cross talk between the two senescence pathways, as p19 can also negatively regulate p53. p21, which is primarily present in the p53 pathway, can likewise negatively regulate pRb in a manner similar to p16 and p19 (Munoz-Espin and Serrano, 2014).

1.9.1 The p53 pathway

1.9.2.1 p53: Roles and regulation

p53 is an archetypal tumour suppressor protein which has been described as 'the guardian of the genome'. The p53 gene (TP53) is the most commonly mutated gene in human cancers and is altered in over 50% of cases according to the International Agency for Cancer Research TP53 Mutation Database (Voglestein et al., 2010). p53 was discovered in 1979 by six groups working independently (DeLeo et al. 1979, Kress et al. 1979, Lane & Crawford 1979, Linzer & Levine 1979, Melero et al. 1979, Smith et al. 1979). It was considered an oncoprotein until a pivotal discovery in 1989 which uncovered its true function as tumour suppressor (Baker et al., 1989). Since then, it has been at the forefront of human cancer research and so far over 25,000 mutations in the TP53 gene have been identified (Vogelstein and Prives, 2009).

Evidence which confirmed the status of p53 as a tumour suppressor included human studies which concluded that patients with p53 mutations were pre disposed to tumour development (Malkin et al. 1990, Srivastava et al. 1990). Additionally, $p53^{-/-}$ mice were also found to be tumour-prone (Donehower et al. 1992, Lowe et al. 1993). Investigations conducted in cells have been key to understanding the mechanisms by which p53 acts as a tumour suppressor as well the factors which regulate these actions. The first of these which attempted to discern the actions of p53 employed over expression of wild type (wt) p53 *versus* mutant p53and demonstrated that only wt p53 could retard the growth of cultured cells. Subsequently, it became apparent that p53 over expression resulted not only in growth arrest (an inhibition of proliferation) but also in an enhancement of apoptosis, thus regulating the two facets of cell physiology (Yonish-Rouach et al. 1991, Shaw et al. 1992). This bi-functional nature of p53 action has lead to the establishment of p53 as a ubiquitous mediator of cell fate.

Under basal conditions, p53 is expressed in a low level 'housekeeping' state. In this latent state, p53 is subject to constant and rapid turnover by the ubiquitin proteasomal pathway. This is mediated by Mouse double minute 2 homolog protein 2 (Mdm2 - gene name MDM2). Mdm2 negatively regulates p53 via two distinct molecular mechanisms. The first of these is a direct deactivation of p53 activity by binding to the N-terminal motif, which blocks interactions with target proteins of p53. The second is by actively enhancing p53 degradation. Thus, Mdm2 acts as an E3 ubiquitin ligase, attaching ubiquitin molecules to p53 and inducing

its nuclear export and subsequent degradation and turnover by the 26S proteasome (Oren, 2003). Interestingly, Mdm2 is also a transcriptional target of p53, thus negative feedback loops exist which regulate p53 (Momand et al. 1992, Picksley & Lane 1993). In another example of cross talk between the two senescence pathways, $p19^{ARF}$ can form stable complexes with Mdm2, sequestering it in the nucleus to indirectly inhibit MDM2 mediated p53 turnover (Sherr and Weber, 2000).

p53 is a master transcription factor which regulates a diverse range of biological functions including DNA metabolism, apoptosis, cell cycle regulation, senescence, energy metabolism, angiogenesis, immune response, cell differentiation, motility and migration and cell–cell communication (Menedez et al., 2009). p53 can trigger cell fate outcomes based on the nature and intensity of the up-stream stimulus or the extent of cell damage. It remains unknown which molecular mechanisms are in place to determine which downstream pathway becomes activated by p53.

1.9.2.2 p53 and Senescence: p21

To induce growth arrest, one of the most important target genes of p53 is *P21,* which is also known as $p21^{WAF1/Cip1}$. p21 is a CDK inhibitor involved in G1/S regulation that is induced by p53 in response to genotoxicity. p53 mediates the 'DNA damage' checkpoint via transactivation of p21, which in turn promotes cell cycle arrest (Abbas and Dutta, 2009). p21 is a particularly important effector of p53 because in addition to its direct role of inhibiting DNA synthesis by binding to and inhibiting proliferative CDKs, it is a master effector of multiple anti-oncogenic pathways which halt proliferation independent of p53 (Abbas and Dutta, 2009). p21 has two non-overlapping functions which results in its inhibition of DNA synthesis. At the amino terminus, there is an inhibitory domain which inhibits CDK2 and CDK1. Interestingly, CDK2 is responsible for the phosphorylation of pRb and p21 is able to block this interaction, thus p21 can induce growth arrest in the pRb pathway, independent of the p53 pathway (Broude et al., 2007). The inhibition profile determines the stage of cell cycle at which arrest which occurs. At the carboxyl terminus, p21 binds to and inhibits proliferating cell nuclear antigen (PCNA) thereby preventing PCNA binding to DNA polymerase and mediating replication and repair processes (Cayrol et al., 1998). Importantly, p21 can cause growth arrest in p53 null cells, providing further evidence that it can cause growth arrest independently of p53 activation (Cayrol et al., 1998). Microarray studies have

shown that p21 expression correlates with suppression of the cell cycle and induction of senescence associated genes (Chang et al., 2000). Interestingly, some of the genes activated are not linked to inhibition of CDKs or pRb by p21, indicative of its additional roles. In support of this, p21 represses cMyc dependant transcription by directly binding to and inhibiting its dimerisation with MAX (Kitaura et al., 2000). Furthermore, p21 associates with E2F and directly inhibits its transcriptional activity, in another example of cross talk between the p53 and pRb pathways (Delavine et al., 1999). Paradoxically, p21 has been reported to both promote and inhibit apoptosis via p53 dependent and independent mechanisms under differing cellular stresses (Gartel, 2000). Moreover, evidence has emerged which supports a role for p21 in DNA repair, suggesting that p21 has varying roles under different circumstances (Cazzalini et al., 2010).

Interestingly, p21 induced senescence has been linked to ROS and mitochondrial dysfunction. In MRC5 human fibroblasts, telomere dependent or independent DDR resulted in the generation of a positive feedback loop in which long term activation of p21 fuelled mitochondrial dysfunction and potentiated ROS generation. This positive feedback loop was essential for establishing and maintaining a senescent phenotype (Passos et al., 2010).

1.9.2.3 p53 and cell death

p53 has several functions, one of which is to induce cell death or apoptosis. This is well characterised and will not be discussed at length as the focus of this thesis is senescence. The downstream molecules determinant which determine the induction of apoptosis or senescence in response to p53 is still a topic of debate. p53 and apoptosis is reviewed extensively in the following citations (Gottleib and Oren, 1998; Fridman and Lowe, 2003). Briefly, to trigger a cell death response, p53 activates targets such as pro-apoptotic proteins PUMA (Nakano & Vousden 2001, Yu et al. 2001), NOXA (Oda et al. 2000), Bax and Fas, and represses anti-apoptotic gene Bcl-2 (Menedez et al., 2009). p53 can also up regulate expression of caspase 6 (MacLachlan et al., 2002) and induce transcription of components of the extrinisic apoptosis cascade including Fas (Muller et al., 1998), FasL (Maecker et al., 2000) and death receptor 5 (DR5) (Wu et al., 1997). Moreover, p53 has non-transcriptional tumour suppressor functions, evidenced by the fact that over expression of p53 lacking a DNA binding domain can still induce apoptosis in human cells (Haupt et al., 1995; Kakudo et al., 2005; Green and Kroemer, 2009). For apoptosis to occur, p53 must accumulate in the cell. To achieve this, the constant

turnover of p53 by the ubiquitin proteasomal pathway must be halted. There are two main routes to intracellular p53 accumulation. The first is through the induction of p19, which can inhibit Mdm2. The second is by the post translational modification of p53 which renders it resistant to ubiquitination or degradation (Evan and Littlewood, 1998).

1.9.2.4 p53 and ROS

Interestingly, p53 also mediates the transcription of anti-oxidant genes which are pivotal in the cellular response to oxidative stress. In response to low levels of oxidative stress, p53 exhibits anti-oxidant properties which allow the cell to overcome low level ROS-induced damage. Paradoxically, under conditions of high oxidative stress, the function of p53 switches to pro-oxidant, potentiating stress and ultimately leading to cell death (Liu and Xu, 2000). Thus, whether p53 induces a programme of senescence or apoptosis in response to stress is likely a result of the severity of oxidative stress encountered by the cell. This has indeed been shown in both normal and cancer cells, where increase in intracellular ROS and p53 protein expression correlated with the induction of senescence or apoptosis dependant upon the severity of the ROS insult. This led the authors to suggest that a ROS threshold exists which triggers apoptosis as opposed to senescence when it is breached (Macip et al., 2003).

1.9.2.5 p53 in cancer

As previously mentioned, p53 is inactivated in the majority of human cancers. Under normal circumstances and in p53 positive cancers (those which retain p53 function), p53 has combined actions of inducing cell cycle arrest to allow for repair processes, and induction of cell death in the event of unmitigated stresses (Menedez et al., 2009). p53 is altered in human cancers in three different ways: complete loss of wild type p53, mutant p53 with an altered gene modulation profile which can take on a pro-survival role and lastly a dominant-negative p53 which lacks functionality but dampens the suppressive function of wild type p53. When mutations are present, these often occur in the DNA binding region of p53 and, although some are more common than others, almost every codon in this region has been found to be mutated (Leroy et al., 2013). Mutations are often point mutations which in turn lead to a single amino acid change that affects DNA binding to one or more targets. Alternatively, p53 is wild type but is impaired through a reduction in abundance or its enhanced degradation, for

example *via* over expression of Mdm2, which leads to alterations in stress response and cell fate decisions to external or internal stimuli (Vousden and Prives, 2009). Aberrant p53 status in human cancers is linked to poor prognosis and this has fuelled the development of various intervention strategies (Brosh and Rotter, 2009). These aim to restore wild type p53 function to cancers and include activators of down-stream p53 targets, inhibitors of negative regulators of p53, small molecule activators of p53 and adenogene transfer of p53 itself into cells (Muller and Vousden, 2014).

1.9.2.6 p63 and p73

p63 and p73, together with p53, constitute a family of transcription factors. They have high homology and both over-lapping and non over-lapping functions. Each member of the family has three conserved domains: a transactivation domain (TA), a DNA binding domain (DBD) and an oligomerization domain (OD). Importantly, as is the case for p53, p63 and p73 can induce cell cycle arrest and apoptosis (Graziano and Lorenzi, 2011). Moreover, p63 and p73 share particular homology with p53 in the DBD, and all of the essential DNA contact residues have been conserved across p53. p63 and p73(Murray-Zmidjewski et al., 2006).

Tumour protein p63 (TP63) was cloned in 1998, 20 years after the discovery of p53 (Yang et al., 1998). Despite this, phylogenic analysis suggests that p63 was the original family member from which p53 and p73 were formed (Skipper, 2007). Experiments using knockout mice have shown that p63 is essential for epidermal morphogenesis and limb development. Similar experiments in p73 knockout mice have shown that, contrary to p63, p73 is essential for neurological development and p73 deficiency leads to chronic inflammation and susceptibility to infection (Khoury and Bourdon 2011).

There are multiple isoforms of both p63 and p73. Paradoxically, isoforms which have a transactivating domain (TA) exert functions similar to p53 whereas isoforms lacking the N-terminal transactivation domain (ΔNp63/p73) induce a functional block against p53 by acting as a dominant negative (Benard et al., 2003). Interestingly, both p63 and p73 genes can be transcribed from 2 different promoters: one upstream of exon 1 (the distal promoter) and another located within intron 3 (the internal promoter). The distal promoter leads to the expression of TAp63 and TAp73, respectively, while the internal promoter leads to the expression of isoforms deleted of the N-terminal domain, ΔNp63 and ΔNp73, respectively.

Unlike p53, p63 and p73 are rarely mutated or deleted in human cancers. In spite of this, studies have shown that p63 and p73 are implicated in response to cancer therapy. Indeed, aberrant splicing that the ΔN forms of p63 and p73 have been reported in cancers and this has been linked to poor clinical outcomes (Inoue and Fry, 2014). Furthermore, a definitive role for p73 as a tumour suppressor was confirmed by genetic experiments using mice lacking TAp73 but retaining Δp73. These mice were more prone to sporadic tumour development as well as tumour development in response to DMBA (7,12-dimethylbenz[a]anthracene) (Tomasini et al., 2008). Further work by the same group confirmed that TAp73$^{-/-}$ mice were sensitised to chemical carcinogens and demonstrated that TAp73 has a role in maintaining genomic stability (Tomasini et al., 2009). In support of these findings, Δp73 knockout mice showed impaired tumour formation due to an inhibition of signal transduction from sites of DNA damage to the DDR pathway (Wilhelm et al., 2010). These findings, taken together, suggest that the balance between TA and Δ forms of p63 and p73 might affect p53 regulation.

The close functional interplay between p63, p73 and p53 as well as the over-lapping functions they exert make p63 and p73 attractive targets for treatment of human cancer, particularly where p53 is mutated or deleted. Understanding the regulation and role of p63 and p73 in proliferation, senescence and growth arrest could ultimately lead to novel therapeutic approaches for cancer treatment.

1.10 Sphingolipids and senescence

Cellular senescence is thought to have evolved to limit cancer growth. Sphingolipids, as mediators of cell fate, have a key role in mediating induction of senescence and the shift between survival, senescence or apoptosis.

The majority of literature surrounding sphingolipids and senescence focuses on ceramide. This is because ceramide has a long established role in regulating both replicative senescence and premature senescence. This was first shown in WI-38 lung fibroblasts where it was reported that endogenous ceramide levels increased 4 fold as cells approached natural senescence. Importantly, these elevations were not net sphingolipid elevations but were unique to ceramide, suggesting a shift in the sphingolipid rheostat (Venable et al., 1995). In the same study, exogenous ceramide addition recapitulated the effects of natural senescence and this was accompanied by the de-phosphorylation of Rb and inhibition of DNA synthesis.

This mimicked the basic biochemical and molecular changes of replicative senescence. In this setting, the Rb pathway was altered as opposed to the p53 pathway. This supports previous observation - that the Rb pathway is the primary pathway implicated in replicative senescence and the p53 pathway is more associated with premature senescence or stress induced senescence (Venable et al., 1995). Since this hallmark study, investigations into the mechanism behind the ceramide increase and the pathways involved have shown that the sphingomyelinase pathway (predominantly via neutral sphingomyelinase) is responsible for the shift in sphingolipid metabolism as cells reach replicative senescence (Venable et al., 2006). Interestingly, ceramides also mediate telomere shortening in cells via a multitude of mechanisms (Saddhoughi et al., 2008).

SKs have received less attention with respect to senescence. The interconnected nature of sphingolipid signalling and the close relationship between SK and ceramide make it highly likely that SKs also have some role in cellular senescence. This was indeed shown to be the case when a link between SK1 and p53 was established in an *in vivo* mouse model. p53 null mice had elevated SK1 levels which were concurrent with a tumorigenic sphingolipid dysregulation characterised by a decreased ceramide to S1P ratio. Interestingly, genetic deletion of SK1 in p53 null mice attenuated tumorigenesis, completely abrogated thymic lymphomas and increased life span by ~30%. In the same study, it was reported that SK1 was proteolysed in response to UV irradiation (genotoxic stress) and this was rescued in p53 KO cells (Heffernan-Stroud et al., 2012). These findings suggest that the sphingolipid signalling pathway and the p53 pathway work as collaborators to control cell fate and modulation of these pathways together could prove beneficial in the treatment of cancer.

SK2 has also been linked to senescence via its role as a producer of nuclear S1P. Thus, SK2-derived S1P bound to the histone deacetylases HDAC1 and HDAC2 and inhibited their enzymatic activity. Indeed, SK2 was found in complexes with HDAC1/2, which were enriched at the promoter region of *P21* thus enhancing p21 transcription (Hait et al., 2009). An additional role of SK2 derived S1P in senescence was recently demonstrated in human and mouse fibroblasts. S1P was shown to stabilise the catalytic sub unit of hTERT by binding to it at residue D684. S1P binding to hTERT enhanced it's stability by inhibiting the interaction between hTERT and makorin ring finger protein 1 (MKRN1), an E3 ubiquitin ligase that tags hTERT for degradation by the ubiquitin proteasomal system. S1P was suggested to mimic phosphorylation of hTERT, which normally functions to stabilise it.

Inhibiton of this interaction of S1P and hTERT reduced the stability of hTERT and promoted senescence. Thus, SK2 inhibitors may have potential to induce cancer cell senescence by destabilising hTERT (Panneer Selvam et al., 2016).

1.11 Senescence as a cancer therapy

One of the major hallmarks of cancer cells is that they proliferate independently of apoptotic signals. Senescence, which is a permanent cell cycle arrest, has recently been hailed as a potential means of blocking proliferation of cancer cells, thereby halting tumour progression or enhancing conventional chemotherapy (Campisi and Di Fagagna, 2007).

The promise of harnessing senescence as a means of cancer treatment is evident by the fact that one of the cells natural responses to oncogenic transformation is to induce a state of senescence termed oncogene induced senescence (OIS). OIS is triggered by oncogene activation e.g. of genes such as Ras^{G12V}. Several independent studies on cancer lesions have identified populations of cells which have undergone OIS. Importantly, as these tumours progressed, the amount of senescent cells decreased, becoming completely absent in the most advanced tumours (Chen et al., 2005; Braig et al., 2005). This suggests that cancer deactivates senescence in order to advance. Interestingly, cancellation of senescence genes in nevi, which are benign pre-melanomas, is enough to transform these into melanomas (Gray-Schopfer et al., 2006). This need for cancer cells to bypass senescence in order to develop further highlights a crucial role for senescence in cancer progression.

Several mediators of senescence have been identified as potential targets for anti-oncogenic therapy. These are tumour suppressor p53, cell cycle regulators such as p16, p19 and p21 and oncogenic cMyc (Campisi and di Fagagna, 2007).

p53 reactivation or up regulation has been a long intended outcome of cancer intervention. The therapeutic promise of this is demonstrated in the fact that transgenic mice with extra copies of p53 or other senescence markers such as p16 exhibit extended cancer protection than wild type counterparts (Serrano and Blasco, 2007). Furthermore, several therapeutic compounds have been developed which aim to exploit p53 induction for cancer treatment, such as nutlin, which is an inhibitor of Mdm2, the p53 E3 ubiquitin ligase (Acosta and Gil, 2012). Drugs are also currently being tested which can restore wild type p53 function in p53

mutant cancers such as PRIMA-1 (Wilman, 2006). Indeed, in mouse models, restoration of p53 in tumours with mutant p53 status using a Cre-loxP expression system resulted in regression of lymphomas via induction of apoptosis. In sarcomas however, cells senesced as opposed to undergoing apoptosis, which suggests that the role of p53 in cancer is cell type dependant (Ventura et al., 2007). shRNAs have also been utilised to assess the therapeutic efficacy of p53 reactivation in cancer. A tet-inducible shRNA which regulates endogenous p53 levels was used in a mosaic mouse model of hepatocellular carcinoma (HCC) to reactivate p53 following chronic down regulation and assess tumour regression. In this system, even a brief p53 reactivation was able to cause tumour regression. This was accompanied in the large part by senescence rather than apoptosis, which was negligible (Xue et al., 2007).

Similarly, drugs which aim to exploit tumour addiction to cMyc have been developed in order to down regulate cMyc function. For example, recently synthesised small inhibitor compounds JQ1 and I-BET151 have shown promise in acute myeloid leukaemia, multiple myeloma and mixed-lineage leukaemia. In multiple myeloma, JQ1 treatment resulted in cMyc down regulation followed by induction of senescence and cell cycle arrest (Delmore et al., 2011). Importantly, in murine models of cancer exhibiting cMyc addiction, switching off cMyc using a tet-dependant cMyc expression system resulted in senescence of lymphomas, osteosarcomas or hepatocellular carcinomas (HCCs) (Wu et al., 2007). Another avenue of cMyc exploitation aims to use a dominant negative cMyc termed OmoMyc, which heterodimerises with wild type cMyc and sequesters it. In advanced adenocarcinoma, OmoMyc decreased proliferation which was a result of inducing senescence (Soucek et al., 2011).

Senescence was directly demonstrated to contribute to the efficacy of chemotherapeutic treatment in a murine model of lymphoma. In this study, tumours were unable to undergo apoptosis in response to chemotherapeutic agents due to oncogenic mutations or transformation. However, they entered a programme of senescence instead. This was associated with an accumulation of senescence markers. As these tumours progressed mutations in p53 or $p16^{INK4a}$ resulted in tumour burden becoming terminal. Importantly, mice bearing tumours which were capable of inducing a programme of cytostatic senescence had better prognosis than counterparts bearing tumours with mutations in senescence genes (Schmitt et al., 2002). Thus, there is rationale for utilising senescence as a tumour-

suppressive mechanism. Importantly, senescence stops cancer cells from proliferating, which, as well as preventing tumour growth, would halt accumulation of additional somatic mutations (Campisi and di Fagagna, 2007).

1.12 Sphingosine kinase and prostate cancer

Prostate cancer is the most common cancer affecting men in the U.K. and constitutes 13% of all new cancer cases diagnosed each year. It is also one of the top three cancers affecting men worldwide (lung, prostate and colorectal). Incidence rates for prostate cancer are projected to rise by 12% in the UK between 2014 and 2035 (Cancer research U.K. web page). Advent of early detection techniques such as the prostate specific antigen (PSA) blood test has resulted in a reduction in mortality rates in recent years. Despite this, prostate cancer mortality rates are still high and no curative therapies are currently available for metastatic cancers.

Early stage prostate cancers are normally dependent on androgen receptor (AR) stimulation for growth (Denmeade et al., 1996). This androgen dependence is exploited to treat locally advanced or metastatic prostate cancer, using methods which block androgen receptor signalling. These include pharmacological approaches using AR antagonists or surgical/chemical castration. These methods often lead to tumour regression but subsequent recurrence, usually occurring around 18 months post treatment. This acquisition of androgen independence and castration resistance results in incurable prostate cancer with a median survival of around 18 months (Lee et al., 2008). The mechanisms underlying progression towards androgen independence are still not fully understood.

Several studies indicate a role of SK1 in maintaining prostate cancer cell survival. In cell models of prostate cancer (LNCaP and PC3 cells), over-expression of SK1 enhances prostate cancer cell proliferation (Brizuela et al., 2010) whereas siRNA knockdown of SK1 inhibits prostate cancer cell proliferation (Pchejetski et al., 2005). Interestingly, pharmacological inhibitors of SK1 induced apoptosis via shifting the sphingolipid rheostat. Importantly, these effects were reversed by over-expression of SK1. When SK1 was over expressed, SK inhibitors failed to sufficiently shift the sphingolipid rheostat towards ceramide and induce apoptosis (Pchejetski et al., 2008; Pchejetski et al., 2010). Moreover, in PC3 cells, short term androgen removal resulted in SK1 inhibition which in turn resulted in reduced cell growth (Dayon et al., 2009). Furthermore, SK1 may be involved in resistance to oxidative stress in

prostate cancer cells. Acute hypoxia increases SK1 and SK2 expression whereas chronic hypoxia increases only SK1 expression (Ahmad et al., 2006) and exposure of cells to hypoxic conditions activates SK1 which ultimately leads to an increase in hypoxia inducible factor 1α (HIF1α) expression (Ader et al., 2008).

Importantly, as prostate cancer progresses, SK1 expression is altered. In stage four androgen sensitive LNCaP AI cells, SK1 expression was significantly higher than in less advanced (stage 3) LNCaP cells. This was true for both isoforms of SK1 – SK1a and SK1b (Loveridge et al., 2010). This indicates that SK1 fuels cancer progression in these cells or that these cells require high SK1 levels for survival, supporting the 'non-oncogene addiction' theory of SK1 in cancer. Interestingly, this study demonstrated that resistance to apoptosis by chemotherapeutic agents was primarily conferred by an increase in SK1b, which is a more stable variant of SK1 with 86aa N terminal extension. In androgen sensitive LNCaP cells, down regulation of SK1a and SK1b resulted in an apoptotic response to SKi, whereas in LNCaP AI cells, SK1b evaded the proteasome and rendered these cells resistant to apoptosis. However, siRNA treatment in combination with SKi, which removed both SK1a and SK1b, resulted in apoptosis of these cells (Watson et al., 2012; Loveridge et al., 2010).

In vivo studies have also demonstrated a role of SK1 in maintaining prostate cancer survival. In nude mice injected with prostate cancer cells, injection of SK1 over expressing cells results in formation of larger tumours than control cells which express endogenous SK1. Targetting SK1 in this system using SK inhibitors induced tumour regression via a shift in the Ceramide/S1P ratio. These studies also indicated the SK1/S1P up-regulation conferred resistance to chemotherapy (Pchejetski et al., 2008).

In clinical samples, SK1 was shown to be pivotal in prostate cancer progression, adaptation to hypoxia and in tumour angiogenesis. In tumour samples from cancer patients, a 2 fold increase in the enzymatic activity of SK1 was observed *versus* control specimens. In this study, high SK1 activity correlated with higher tumour volume and higher surgical failure rate (Malavaud et al., 2014).

Several studies have also concluded a role for SK2 in prostate cancer. SK2 inhibition using ABC294640 was shown to reduce prostate cancer cell viability, survival and proliferation (Gestaut et al., 2014). Furthermore, inhibition of SK2 by ABC294640 was reported to induce

the down regulation of several critical oncogenes in prostate cancer such as cMyc and AR (Schrecengost et al., 2015). A limitation of these studies however is that they solely attribute the effects of ABC294640 to SK2 inhibition and do not consider additional effects of this inhibitor which have been reported such as Des1 inhibition and SK1 degradation (Cingolani et al., 2011; Venant et al., 2015; McNaughton et al., 2016).

Thus, there is rationale for utilising drugs which alter sphingolipid signalling and target SKs in prostate cancer therapy, especially in advanced stage, castrate resistant prostate cancer, where survival and proliferation is associated with increased SK1 expression.

1.13 Project aim

Pre-clinical studies strongly implicate a role of SK1 in maintaining prostate cancer cell survival and indicate that advanced stage prostate cancer cells acquire resistance at the same time as expressing higher levels of SK1. Other evidence suggests a role for SK2 in maintaining prostate cancer cell survival. Therefore, SKs represent a promising target for the development of novel therapeutics in this disease. This, taken with the fact that induction of senescence represents a novel and promising avenue of cancer therapy, provides clear rationale for investigating SKs and senescence in prostate cancer cells. Thus, the role of SKs in regulating senescence in prostate cancer cells was examined with the aim of identifying novel signalling networks and chemotherapeutic targets.

CHAPTER 2:

MATERIALS AND METHODS

2. CHAPTER 2 - Materials and Methods

2.1 Materials

2.1.1 General Reagents

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

GE Healthcare (UK)

Hybond™ ECL™ Nitrocellulose membrane

Christiansen-Linhart, Munich

Kodak LX 24 developer, Kodak industrex fixer, CEA RP New X-ray film

Thermo Scientific

Pierce BCA Protein Assay kit

Qiagen (Crawley, UK)

QIAGEN Plasmid plus Kits, Scrambled SiRNA (ALLSTARS Negative control)

Dharmacon (Cromlington, UK)

DharmaFECT™2 reagent; ON-TARGETplus SMARTpool® SK1 siRNA; ON-TARGETplus SMARTpool® SK2 siRNA; ON-TARGETplus SMARTpool® DES1 siRNA Solaris qPCR Gene Expression Assay kits

2.1.2 Cell Culture

RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), OptiMEM®, European foetal calf serum (EFCS), Penicillin-streptomycin (10,000 units/ml penicillin and 10,000µg/ml streptomycin), L-glutamine, 2mM non-essential amino acids (NEAA), trypsin/EDTA solution and Lipofectamine™ 2000 were purchased from Invitrogen (Paisley, UK). Charcoal filtered foetal bovine serum was purchased from Sera Laboratories International Ltd (Seralab Ltd). Sodium pyruvate was from Sigma-Aldrich (UK). 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). HEK293T cells were gifted from Professor Anthony Futerman (Weizmann Institute, Israel).

2.1.3 Antibodies

Abcam (UK)

Rabbit monoclonal antibody to DEGS1(EPR9680), Custom made monoclonal anti-SK1 antibody (48:2)

New England Biolabs Ltd. (Hitchin, UK)

monoclonal anti-p21 antibody (#2947)

Santa Cruz Biotech Inc (Poole, UK)

monoclonal anti-c-Myc (9E10)

Sigma-Aldrich (Poole, UK)

Monoclonal anti-p53 (DO-7) antibody (#P8999), polyclonal anti-actin antibody (#A2066), reporter horseradish peroxidase-conjugated anti-mouse IgG (#A9169), reporter horseradish peroxidase-conjugated anti-rabbit IgG (#A0545). tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG; fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG

2.1.4 Agonists and inhibitors

Avanti Polar Lipids (Alabaster, USA)

C2 Ceramide

Calbiochem (Nottingham, UK)

2-(p-Hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi)

Enzo Life Sciences (Exeter, UK)

Fumonisin B1

MedChem Express (USA)

(3-(4-chlorophenyl)-adamantane-1-carboxylicacid(pyridin-4-ylmethyl)amide) (ABC294640), Fenretinide

Merck Biosciences (Nottingham, UK)

Cycloheximide, CA074Me

Promega (Southhampton, UK)

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

Sigma-Aldrich (Poole, UK)

N-acetyl L-cysteine, MG132, Nutlin

(*R*)-FTY720 methyl ether (ROMe) (Lim *et al*., 2011) and RB-005 (Baek *et al*., 2013) were gifted from Professor Robert Bittman (Queens College of the City University of New York, New York, USA).

Thermo Scientific

Pierce BCA Protein Assay kit

2.1.5 Molecular Biology

Addgene (Cambridge, USA)

WT MDM2 plasmid construct (pcDNA-MDM2)

Bioline (London, UK)

HyperLadder™ I

Invitrogen (Paisley, UK)

UltraPure Agarose, Lipofectamine™2000, TOP10 chemically competent cells, PureLink® HiPure Plasmid Miniprep Kit

New England Biolabs Inc.

SafeView Nucleic Acid Stain

2.1.6 Radioisotopes

PerkinElmer (UK)

[Methyl-3H] thymidine (37MBq/ml)

[32P]-γ-ATP

2.2 Methods

2.2.1 Cell Culture

Maintenance of HEK293T and LNCaP-AI Cells

Human embryonic kidney (HEK293T) or LNCaP-AI cell lines were maintained in DMEM medium or RPMI-1640 medium and supplemented with 10% (v/v) EFCS or 10% (v/v) dilapidated serum respectively, 50 U/ml penicillin/50 μg/ml streptomycin and 1% (v/v) L-glutamine. HEK293T medium was also supplemented with 1 % (v/v) sodium pyruvate. 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 DMEM or RPMI 1640 medium, respectively. 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).

2.2.2 Treatment protocol

For experimentation, cells were plated on 12 well plates, 6 well plates or 25 cm^2 flasks, as required, and grown to approximately 70% confluence. Cells were treated with inhibitors or vehicle for 8-48 h, as indicated. When cells were treated for 48 hours, the inhibitors or vehicles were replaced after 24 hours.

N-acetyl L-cysteine was reconstituted in sterile distilled H_2O before being added to the cell culture medium. Cyclohexemide (CHX) was reconstituted in methanol (MeOH) at a final concentration of MeOH <0.4%. All other inhibitors were reconstituted in DMSO. The final concentration of DMSO added to cells was <0.5%.

2.2.3 Transient cell transfections

HEK293T or LNCaP AI cells were transiently transfected with plasmid constructs as described in the figure legends or vector plasmid (pcDNA3.1) as control. On the day of transfection the media in each well of a 12 well plate was replaced with transfection medium

consisting of serum free medium or Opti-MEM® supplemented with 1% (v/v) of serum for HEK293T cells or LNCaP AI cells, respectively. For each well of cells to be transfected, 1µg of the appropriate plasmid DNA and 1.5µl of Lipofectamine™2000 were diluted separately in 200µl of serum free media and incubated at room temperature for 20 minutes before 200µl of the DNA complexes was added to each well. Cells were incubated at 37°C in 5% CO_2 for 24 hours. Cells were then treated as described in the figure legends and harvested by aspirating medium and then boiled in boiling sampling buffer [125mM Tris-Base (pH6.7), 0.5mM Na4P2O7, 1.25mM EDTA, 1.25% (v/v) glycerol, 0.5% (w/v) SDS, 25mM 1.4-Dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue]. Cells were then scraped off the bottom of wells using a 1ml pipette tip and homogenised by repeated passage through a 0.24mm guage needle.

2.2.3 SiRNA transfection

LNCaP AI cells were transiently transfected with siRNA constructs as described in the figure legends or scrambled siRNA as control at a final concentration of 200nM. Cells were grown to approximately 60% confluence and for each well a mixture of 4µM siRNA in 50µL of DNAse/RNAse free water and 1.6µL of DharmaFECT® 2 transfection reagent in 150µL of serum free, antibiotic free media was prepared. These preparations were incubated for 5 minutes at room temperature to mix and then combined and incubated for 20 minutes at room temperature to allow formation of siRNA and DharmaFECT® 2 transfection reagent complexes. Next, 800 µl of antibiotic-free RPMI 1640 medium supplemented with 10% (v/v) delipidated and 1% L-glutamine were added to the transfection mix, which was then gently mixed. This was then used to replace cell culture medium and cells were incubated at 37°C in 5% CO_2 for 48 hours. Cells were then treated as described in the figure legends or harvested by aspirating medium and then boiled in boiling sampling buffer [125mM Tris-Base (pH6.7), 0.5mM Na4P2O7, 1.25mM EDTA, 1.25% (v/v) glycerol, 0.5% (w/v) SDS, 25mM 1.4-Dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue]. Cells were then scraped off the bottom of wells using a 1ml pipette tip and homogenised by repeated passage through a 0.24mm guage needle.

Samples were then either used immediately or stored at -20°C prior to analysis by western blotting.

2.2.4 Cell lysate preparation for western blotting analysis

Preparation of LNCaP-AI cell lysate from 25cm^3 flasks

LNCaP-AI cells were treated as indicated and scraped into their own medium. Cells were then pelleted by centrifugation for 3 minutes at 180 *g* before being re-suspended in ice cold phosphate buffered saline (PBS) and re-centrifuged for a further 3 minutes at 180 *g* to wash. The PBS was then aspirated and the resultant pellet re-suspended in 250µl of ice cold lysis buffer consisting of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 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). Lysates (kept on ice at all times) were then homogenised by repeated passage through a 0.24mm guage needle , transferred to 1.5ml eppendorf tubes and mixed for 30 minutes at 4°C. Tubes were then stored at -20°C if not immediately used for protein concentration determination.

The bicinchoninic acid (BCA) assay (Smith et al., 1985) was used to determine protein concentration of cell lysate harvested from 25cm^2 flasks. The BCA assay is a spectrophotometric method for determination of protein concentration which compares the absorbance of cell lysate at 562nm (562_{OD}) to absorbancies of known protein concentrations used to generate a standard curve. Measurements for each sample were performed in duplicate and the mean used in final protein concentration determination.

2.2.6 SDS-PAGE and Western Blotting

SDS-PAGE

Polyacrylamide gel for electrophoresis consisting of a resolving and stacking layer was prepared as 10% (v/v) acrylamide:bis-acrylamide, 0.375 M Tris- Base (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.025% (w/v) tetramethylethylenediamine (TEMED) for the resolving layer and 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 persulphate and 0.1% (v/v) of TEMED for the stacking layer.

The Bio-Rad Mini-Protean II electrophoresis kit was used to electrophorese each sample which was loaded by a Hamilton syringe into the gel. A set of molecular weight standards containing proteins of known molecular weight was also added to one well in order to

identify the relative mobility of the protein of interest. Samples were electrophoresed at 120V for 2 hours in electrophoresis running buffer consisting of 25 mM Tris-Base, 0.21 M glycine and 0.1% (w/v) SDS.

Resolved proteins were then transferred from the gel to a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot kit at 100V for 1 hour in transfer buffer consisting of 25 mM Tris-Base, 0.21 M glycine and 20% (v/v) methanol.

Western blotting

Nitrocellulose membranes were incubated in a blocking solution 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] for 1 hour. Membranes were then incubated primary antibody diluted in 1% (w/v) BSA in TBST at 4°C overnight with gentle agitation. Membranes were washed 3 times for 7 minutes in TBST and incubated in 1% (w/v) non-fat dry milk in TBST containing 1:80,000 horseradish peroxidase-conjugated secondary antibody (either mouse or rabbit depending on the origin of primary antibody). Cells were washed a further 3 times for 7 minutes.

Developing solution was prepared by mixing equal volumes of 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) to make enhanced chemiluminescence (ECL) reagent. Membranes were incubated in ECL for 2 minutes at room temperature before being blotted of excess and inserted between two transparent plastic sheets in a metal cassette and exposed to an X-ray film, which was then developed by passing it through an X-Omat machine for an appropriate time to get a chemiluminescent signal from the immunoreactive proteins. Molecular weight was determined by comparison to a pre stained marker containing standards of known molecular weights as follows:

Standard	**Source**	**Molecular weight (kDa)**
α2-macroglobulin	equine serum	180
β-galactosidase	*E. coli*	116
Lactoferrin	human milk	90
Pyruvate kinase	rabbit muscle	58
Fumarase	porcine heart	48.5
Lactic dehydrogenase	rabbit muscle	36.5
Triosephosphate isomerase	rabbit muscle	26.6

Stripping and re-probing

Membranes were able to be stripped of antibody and re-probed a finite number of times by incubating them at 70°C for 45 minutes in stripping buffer consisting of 62.5 mM Tris-HCl (pH 6.7), 2% (w/v) SDS and 100 mM β-mercaptoethanol. Blots were washed three times for ten minutes and then re-probed using an antibody to detect a protein known to be un-affected by treatments to ensure comparable protein levels between samples (normalised).

Quantification

Band density was determined using Image J software and expressed as integrated density units (IDU).

Molecular weights of antibodies used in western blotting experiments

Protein	**Molecular weight (kDa)**
cMyc	35
Des1	116
p21	21
p53	53
SK1	62

2.2.7 Plasmid transformation and purification

Escherichia coli transformation

Gifted plasmid DNA was recovered from filter paper by 5 minute rehydration in 50µl 10 mM Tris, pH 7.6. The mix was briefly centrifuged and the supernatant added to TOP10 chemically competent *E.coli* cells. The mixture was then incubated on ice for 30 minutes before being heat shocked for exactly 30 seconds at 42°C and then placed on ice for 2 minutes. 250µl of pre-warmed SOC media [containing 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl2, 10mM MgSO4, 20mM glucose] was added to the cells before 1 hour incubation at 200rpm and 37°C in a shaking incubator. Either 20µl or 200µl of the mixture was then spread onto an Lysogeny broth (LB) agar plates [containing 10% (w/v) tryptone, 5% (w/v) yeast extract and 10% NaCl] (with ampicillin 100µg/ml) and incubated overnight at 37°C. The next day, an individual colony was selected and used to inoculate 5ml of LB broth (with ampicillin 100µg/ml) and the culture grown

overnight at 200rpm and 37°C in a shaking incubator. 700µl of the overnight culture was then added to 300µl of 50% (v/v) sterile glycerol and stored at -80°C for future use. The remainder was used for plasmid purification.

Plasmid purification

1.5ml of overnight culture was added to a sterile centrifuge tube before being pelleted by for 3 minutes at 120 *g*. The supernatant was aspirated and a further 1.5ml of overnight culture was added and again pelleted by centrifugation before the supernatant was aspirated. The remaining pellet was purified using the PureLink® HiPure Plasmid Miniprep Kit as per the manufacturer's instructions. The DNA yield was determined by measuring absorbance at 260nm using the Nanodrop2000 UV-Vis Spectrophotometer and Nanodrop2000/2000c Software.

2.2.8 [3H] Thymidine uptake proliferation assay

HEK293T or LNCaP AI cells were seeded in 1mL of complete media on to 24 well plates. When 60-70% confluent, cells were treated and incubated as described in the figure legend. 5 hours before the end of the incubation period, [3H] thymidine (37KBq) was added to each well. Cells were then harvested by aspirating media and washing three times for ten minutes on ice with 1mL of 10% (w/v) ice cold trichloroacetic acid (TCA) solution. Nuclear material was then dissolved in 0.25ml of 0.1% SDS/0.3M NaOH. Samples were then transferred to scintillation vials and 2mL of scintillation cocktail was added before DNA synthesis was quantified using liquid scintillation counting.

2.2.9 MTT assay

LNCaP AI cells were seeded onto 96 well plates at a density of 10,000 cells per well in 50µL of complete media. Treatments were added to each well as indicated in 50µL serum free media and incubated for 24 hours. Media was then aspirated and replaced with 50µL MTT reagent (5mg/ml in sterile PBS). Plates were wrapped in foil and incubated at 37ºC for 2 hours. MTT reagent was then discarded and replaced with 100µL of MTT stop reagent (containing 4mM hydrochloric acid and 0.1% (w/v) NP-40 in propan-2-ol). Plates were then re-wrapped in foil and formazan crystals were solubilised by gentle agitation for 30 minutes before absorbance was read at 620nm.

2.2.10 Immunofluorescence microscopy

Cells were plated on autoclaved glass coverslips (13mm diameter) in 12-well plates, and treated as described in figure legends. 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 2 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 in a light proof box at 4°C with antibodies as indicated, spotted on to parafilm (at 1:100 dilution in blocking solution). Coverslips were then dipped into 3 beakers of PBS and washed twice with PBS by aspirating and re-filling of a 12 well plate. Cover slips were then 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 dilutions in blocking solution, spotted on to parafilm in a light proof box. 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 and left to set overnight. Fluorescence was visualised using a Nikon E600 epifluorescence microscope.

2.2.11 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 forms a luciferase substrate following proteasomal cleavage, which in turn generates a luminogenic signal. The intensity of the luminogenic signal is therefore proportional to the proteasomal activity of cells against the exogenous substrate.

Cells were seeded in 96 well-plates at a density of 20,000 cells per well and allowed to adhere overnight at 37°C in 5% CO2. Cells were then treated as indicated in the Results sections. Proteasome activity was then assessed using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit as per the manufacturer's instructions. Briefly, the luminogenic substrate was equilibrated to room temperature before being dissolved in a cell permeabilization buffer (also equilibrated to room temperature) and mixed with the luciferase enzyme. Media was aspirated from cells and replaced with the previously made proteasome assay mixture. Cells were wrapped in foil, shaken at 600rpm for 2 minutes and then incubated for 10 minutes at room temperature, luminescence (350-650 nm) was then

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.

2.2.12 SK activity assay

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 re-suspended 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 MgCl2 and 1 μCi of [32P]-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 [32P]-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 [32P]-γ-ATP, which after each centrifugation was removed using a glass pasteur. The organic phase was then transferred to scintillation vials and the [32P]-S1P content was quantified by counting the dpm (disintegrations per minute) using a scintillation counter.

2.2.13 Cell cycle analysis

Cells were plated on 12 well plates then treated in triplicate as indicated. Cells were then trypsinised before being collected in FACS tubes in 750uL of PBS. Cells were then centrifuged (5 minutes, 1500 rpm) and re suspended in 150uL of PBS and then fixed by addition of 350uL of ethanol and 20 minute incubation at 4°C. 1mL of PBS was added to cells and then the mixture was centrifuged (10 minutes, 3000 rpm) and the supernatant discarded. Finally, cells were re suspended in 250uL of PBS and 5uL of RNAse A (50μg/mL) and 13.5uL of propidium iodide (PI) were added. Cells were then analysed immediately using

immediately analysed using a BD FACSCanto system and BD FACSDiva software (both BD Biosciences).

2.2.14 Statistical analysis

Results are representative of experiments which have been repeated at least three times. Data was analysed using GraphPad Prism software and is expressed as mean with standard error of the mean (SEM) or mean with standard deviation (SD) as indicated. Differences between groups were analysed using the student's t test or one way ANOVA as indicated and a p value of <0.05 was deemed significant. The following criteria were used when assigning statistical significance to a data set, as per the GraphPad Prism manual:

*=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

CHAPTER 3:

CHARACTERISATION OF SPHINGOSINE KINASE INHIBITORS IN LNCAP AI CELLS

3. CHAPTER 3 - Characterisation of SK inhibitors in LNCaP AI cells.

3.1 Introduction

There are currently several reports in the literature which provide strong evidence that SK1 has a role in regulating prostate cancer metabolism. These studies range through from studies conducted in cells to clinical studies involving prostate cancer patients. There are a multitude of cellular studies which prove that SK1 regulates prostate cancer cell proliferation and survival. SK1 knockdown using non specific inhibitors or SK1 siRNA knockdown reduces prostate cancer viability (Pchejetski et al., 2005; Akao et al., 2006; Brizuela et al., 2010). These findings were affirmed when it was shown for the first time that this also occurred in response to SK inhibitor SKi rather than general chemotherapeutics (French et al., 2003; Loveridge et al., 2010). In agreement with cell based studies, clinical studies demonstrate that SK1 protein expression is increased in prostate cancer and demonstrate that this correlates with disease progression (Malavaud et al., 2012). Importantly, this assigns clinical relevance to findings in cells.

The androgen independent LNCaP AI cell line was used in this study as an *in vitro* model of castrate resistant prostate cancer. LNCaP cells were established from a lymph node metastasis of a human prostate carcinoma. Importantly, LNCaP cells maintain malignant properties and are tumourigenic when injected into nude mice (Horoszewicz et al., 1980). Androgen independent LNCaP AI cells were established by culturing LNCaP cells in conditions of androgen deprivation for lengthy periods (Halkidou et al., 2003). LNCaP AI cells are a good model cell for advanced stage prostate cancer as these cells mimic what happens in progression towards androgen independence in patients treated with androgen ablation therapy who develop castrate resistant tumours. These cells grow independently of AR receptor stimulation but nonetheless have androgen receptors present and can still respond to stimulation of AR and AR associated genes (Halkidou et al., 2003).

It has previously been demonstrated that SKi (defined above) induces the proteasomal degradation of SK1a and SK1b (which has an 86 amino-acid N-terminal extension compared to SK1a) in androgen-sensitive LNCaP prostate cancer cells and this results in a reduction in S1P levels and an increase in sphingosine and C22:0 and C24:0 ceramide levels and the induction of apoptosis (Loveridge et al., 2010). SKi also induces proteasomal degradation of SK1a in androgen-independent LNCaP AI cells, but fails to reduce SK1b levels (Loveridge et

al., 2010) and does not increase C22:0 and C24:0 ceramide levels. Androgen-independent LNCaP AI cells are in fact resistant to apoptosis induced by SKi. Nevertheless, SKi is still able to inhibit DNA synthesis indicative of promoting growth arrest of these cells. The inability of SKi to reduce SK1b expression levels appears due to a compensatory increase in SK1b mRNA levels. Thus, combined treatment with SK1 siRNA (to prevent mRNA translation of SK1a and significantly, SK1b) and SKi was required to induce apoptosis of androgen-independent LNCaP AI cells (Loveridge et al., 2010). The current study focuses on SK1a and western blots herein are for SK1a.

Recent studies have demonstrated that like SK1, SK2 might also have an important role in cancer. For instance, the aryladamantane compound, ABC294640 (3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide) which is reported to be a selective inhibitor of SK2 activity is in phase 1 clinical trials for solid tumours and phase 2 clinical trials for diffuse B-cell lymphoma. ABC294640 is a competitive (with respect to sphingosine) inhibitor of SK2 activity with a Ki of 9.8 μM, and reduces S1P formation in cancer cells (French et al. 2010). ABC294640 suppresses the proliferation of tumour cell lines, and inhibits tumour cell migration associated with the loss of microfilaments. Oral administration of ABC294640 to mice with mammary adenocarcinoma xenografts results in dose-dependent anti-tumour activity associated with reduced tumour S1P levels and apoptosis. ABC294640 also reduces clonogenic survival and viability of ovarian cancer lines and increases caspase cleavage and apoptosis of Kaposi's sarcoma-associated herpes virus positive patient-derived primary effusion lymphoma cells (Qin et al., 2014). ABC294640 can also promote autophagic death of A-498 kidney carcinoma cells, PC-3 prostate and MDA-MB-231 breast adenocarcinoma cells (Gao et al., 2012; Maines et al., 2008; Antoon et al., 2011). Indeed, ABC294640 delays tumour growth in severe combined immunodeficient mice with A-498 xenografts via an autophagic mechanism (Gao et al., 2012). ABC294640 also decreases tumour incidence in an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model of colitis driven colon cancer suggesting that SK2 has a role in inflammation-induced cancer progression (Chumanevich et al., 2010).

Recently, the SK2 selective inhibitor, ABC294640 was demonstrated to induce proteasomal degradation of cMyc and myeloid cell leukemia 1 (Mcl-1) in multiple myeloma cells (Venkata et al., 2013). Interestingly, Watson and colleagues (Watson et al., 2013) have previously demonstrated that the SK1/2 inhibitor, SKi also induced the proteasomal degradation of cMyc in androgen-sensitive LNCaP prostate cancer cells. In contrast, the SK2

selective inhibitor ((R)-FTY720 methylether (ROMe (Lim et al., 2011b)) failed to modulate cMyc expression in these cells (Watson et al., 2013). Therefore, the mechanism underlying the ability of ABC294640 to induce proteasomal degradation of cMyc is unclear. Consequently, it was of interest to examine whether the effect of ABC294640 on cMyc might be mediated through an indirect effect on SK1 expression and its ubiquitin-proteasomal degradation in androgen-independent LNCaP AI cells.

Previous studies have shown that inhibitors which exert catalytic activity towards SK1 induce the proteasomal degradation of SK1 in human pulmonary artery smooth muscle cells (hPASMCs) as well as several solid cancer cell lines including LNCaP AI cells (Loveridge et al., 2010; Tonelli et al., 2010). Therefore, the first part of this study focuses on characterising this effect with respect to the panel of inhibitors which will be used throughout the study, including SK2 inhibitors which have previously not been characterised in this manner. To establish confidence in the results produced in LNCaP AI cells and to gain insight into the reproducibility of findings in an alternative cell line, there are some results which are also presented in human embryonic kidney cells (HEK293T cells). In this chapter, these cells were solely used to validate findings in LNCaP AI cells.

A panel of available SK inhibitors was selected as study tools to investigate the role of SK isoforms, SK1 and SK2, in regulating senescence in LNCaP AI cells. These were first characterised in LNCaP AI cells for effects on SK1 protein expression, cMyc and cell viability. Each of these inhibitors has been described extensively so will only be briefly described herein (see Introduction 3.8). Of these inhibitors, some are selective for SK1 (PF-543 (Schnute et al., 2012) and RB-005 (Baek et al., 2015)), some are selective for SK2 (ABC294640 (French et al., 2010), F-02 (Byun et al., 2013) and ROMe) and one is selective for SK1 and SK2 (SKi (French et al., 2013)).

3.2 Results

3.2.1 ABC294640 induces the proteasomal degradation of SK1: reversal by MG132

For reasons aforementioned, the ability of ABC294640 (1-25μM) to degrade SK1 protein in LNCaP AI cells was tested in the presence and absence of proteasomal inhibitor MG132 (10μM).

Indeed, ABC294640 (10-25μM) reduced the expression of SK1 (Mr=42kDa) in androgen-independent LNCaP AI cells and this was prevented by pre-treating cells with a specific, potent and cell permeable proteasomal inhibitor, MG132 (Figure 3.1). Therefore, this is the first demonstration that ABC294640 (a SK2 selective inhibitor with no activity against SK1 at concentrations as high as 100μM (French et al., 2010)), is able to remove SK1 from cells. As ABC294640 does not bind to SK1, an additional mechanism is indicated which is able to remove SK1 from these cells independent of inhibitor binding. This effect was similar to that seen with the dual SK1/SK2 inhibitor, SKi, which can activate the proteasome and promote accelerated ubiquitin-proteasomal degradation of SK1 in androgen-sensitive and androgen independent prostate cancer cells (Loveridge et al., 2010). To confirm the ability of ABC294640 to activate the proteasome, the ability of this inhibitor to influence the the protein level of cMyc was also measured, which is known to be degraded via the ubiquitin-proteasome pathway. Indeed, treatment with ABC294640 (10-25μM) promoted the degradation of cMyc (Mr=57kDa) in LNCaP AI cells. This suggests that, like SKi, ABC294640 is an activator of the ubiquitin proteasomal pathway.

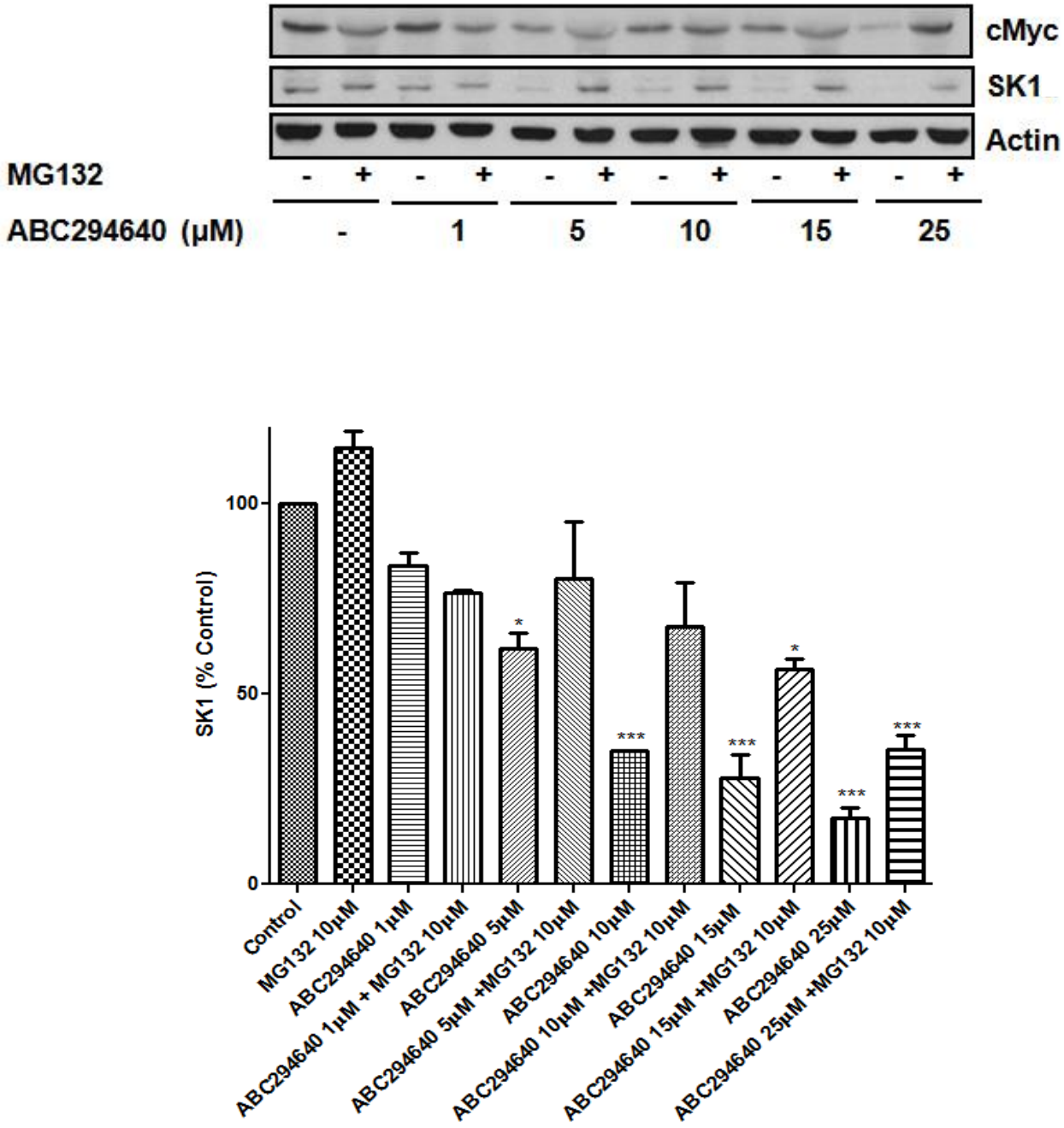


Figure 3.1 ***Effect of ABC294640 treatment on SK1 and cMyc protein levels in LNCaP AI cells: reversal by MG132.*** *Western blot showing the effect of ABC294640 (1–25 μM, 24 hours) on the expression of SK1 and cMyc in the presence and absence of MG132 (10μM, 30 minutes pre-treatment) (representative blots from 3 experiments). Also shown is a bar graph of the quantification of ABC294640 (1–25 μM) on the proteasomal degradation of SK1 with protein levels normalised to actin and expressed as a percentage of control. Results are expressed as means +/− SEM for n = 3 experiments. *p < 0.05, ***p < 0.001 versus control.*

3.2.2 Effect of SK inhibitors on the proteasomal degradation of SK1

As previously mentioned, a common feature of SK1 inhibitors is that they induce the ubiquitin-proteasomal degradation of SK1 in solid cancer cell lines (Loveridge et al., 2010; Tonelli et al., 2010; Lim et al., 2011a) and proliferating vascular smooth muscle cells (Loveridge et al., 2010; Baek et al., 2013; Byun et al., 2013). The ubiquitin-proteasomal degradation of SK1 is linked with binding and/or inhibition of SK1 catalytic activity as it correlates with inhibitory potency. However, other inhibitors of SK1 and SK2 such as SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole)) induce the proteasomal degradation of SK1 via a mechanism that is only partially dependent on direct binding to SK1 (Loveridge et al., 2010).

To this end, a previously described panel of inhibitors including SK1 selective inhibitors (PF543 and RB005), SK2 selective inhibitors (ABC294640, F-02 and ROMe) and an SK1 and SK2 selective inhibitor (SKi) were used in combination with the proteasomal inhibitor MG132. The effect of these inhibitors on the proteasomal degradation of SK1 was compared in order to establish whether other SK2 inhibitors had a similar effecr. In this regard, the SK1 selective PF-543 (Schnute et al. 2012), and RB-005 (Baek et al., 2013) induced the proteasomal degradation of SK1 in LNCaP AI cells (Figure 3.2) However, treatment of LNCaP AI cells with the SK2 selective inhibitor ROMe or F-02 (Byun et al., 2013) failed to reduce SK1 expression (Fig 3.2). Notably, of the three SK2 inhibitors tested, only ABC294640 was able to degrade SK1 (see discussion 3.3.1). The treatment of LNCaP AI cells with SKi (10 μM) was confirmed to induce a decrease in the expression of SK1 and this could be reversed by pre treatment of the cells with MG132.

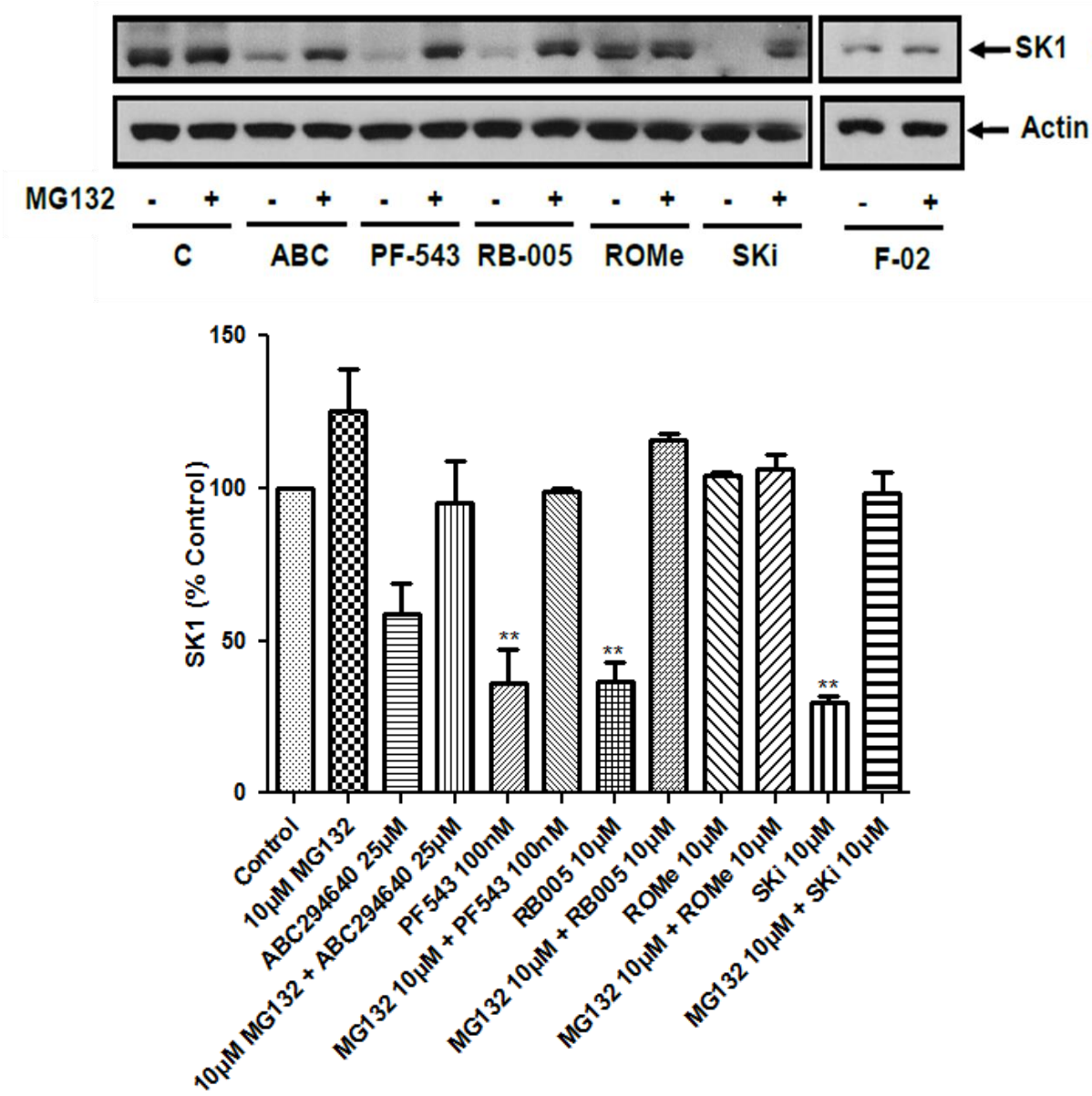


Figure 3.2 ***Effect of proteasomal inhibition on degradation of SK1 by SK inhibitors.*** *Cells were pre-treated with MG132 (MG132 10μM, 30 minutes) before addition of inhibitors (24 hours) or vehicle (DMSO 0.1% v/v) as indicated. Also shown is the quantification of the effect of SK inhibitors on SK1 levels expresesd as mean +/- SEM. n=3 with protein levels normalised to actin*

3.2.3 Effect of SK inhibitors on SK1 expression in HEK293T cells.

Because differences exist between cell types in terms of SK1 and cMyc regulation and expression and in order to establish that the observed effect of the SK1 and SK2 inhibitors employed here on SK1 expression were not unique to LNCaP AI cells, a widely recognised and commercially available cell line (HEK293T) was used. In agreement with findings in LNCaP AI cells, ABC294640 (25μM) and SKi (10 μM) reduced SK1 protein levels (Figure 3.2). Similar to the observations made in LNCaP AI cells (Figure 3.2), SK1 inhibitors (PF543 and RB-005) also reduced SK1 protein whereas the SK2 inhibitors (ROMe and F02) did not. Blockade of the SK inhibitor-induced SK1 degradation by the proteasomal inhibitor MG132 was attempted but could not be tested in HEK293T cells because it was toxic in these cells (at <1μM) and resulted in cell death at 24hr. This is perhaps because of the high metabolic activity of these cells, which means inhibition of normal proteasomal function would quickly result in a toxic build up of mis-folded and aggregated proteins. Therefore, MG132 could not be used to confirm the involvement of the proteasome in the removal of SK1 in HEK293T cells.

Significantly, both ABC294640 and SKi decreased cMyc protein levels, which is consistent with the data in LNCaP AI cells. However, these effects were not recapitulated by RB005, PF543 (SK1 inhibitors, which reduce SK1 levels) or ROMe or F02 (SK2 inhibitors, with no effect on SK1 levels) which shows that induction cMyc degradation is not a common property of all SK inhibitors This suggests that the expression of SK1 and cMyc are regulated differently and not linked (degradation of SK1 does not necessarily affect cMyc expression) and that the inhibitors ABC294640 and SKi may affect SK1 and cMyc expression via an increase in overall proteasomal flux. In contrast, SK inhibitors affect SK1 expression via an action involving direct binding to the enzyme and increasing its susceptibility to basal proteasome activity rather than up-regulating proteasomal flux *per se*.

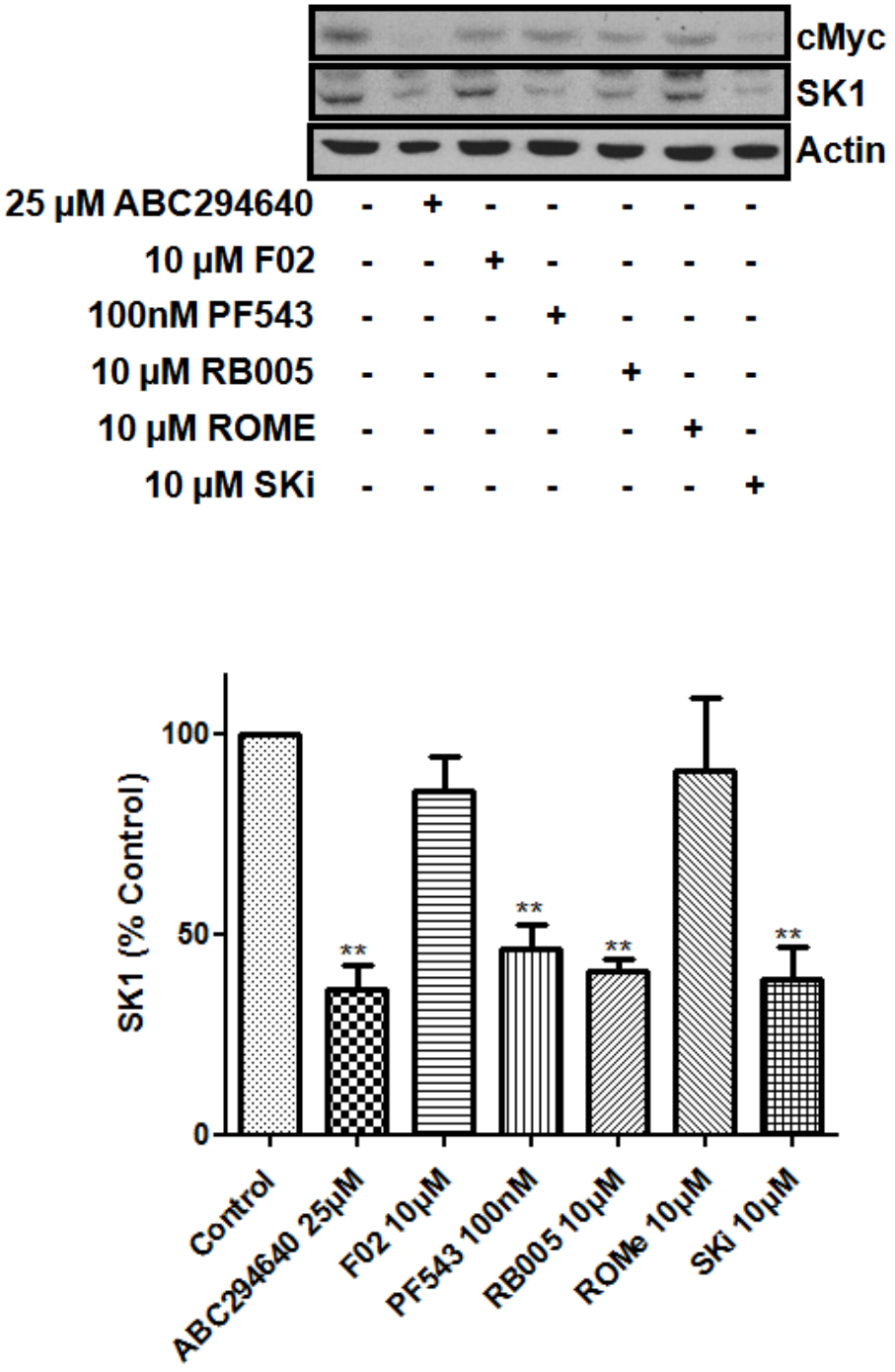


Figure 3.3 ***Effect of SK inhibitors SK1 and cMyc protein levels in HEK293T cells.*** *Western blot showing the effect of SK inhibitors (24 hrs) on the expression of SK1 and cMyc in HEK293Tcells (representative blot from 3 experiments). Also shown is a bar graph of the quantification of the effect of SK inhibitors on the proteasomal degradation of SK1 with protein levels normalised to actin and expressed as a percentage of control. Results are expressed as means +/− SEM for n = 3 experiments. *p < 0.01 versus control;*

3.2.4 ABC294640 activates the ubiquitin proteasomal system in LNCaP AI cells and HEK293T cells.

It has previously been shown that SK1/SK2 selective inhibitor SKi induces the proteasomal degradation of SK1 via a mechanism that is only partially dependent on direct binding to SK1 (Loveridge et al., 2010). It has also previously been demonstrated that SKi activates the ubiquitin proteasomal system in LNCaP AI cells (Loveridge et al., 2010).

It was therefore of interest to determine whether ABC294640 similarly activated the ubiquitin proteasomal system. To this end, a cell based luminescent proteasome assay was used which measures 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. SKi (10μM) was used as a reference compound in these experiments.

As expected, SKi (10μM) and ABC294640 (24 μM) accelerated proteasomal activity in both LNCaP AI cells (Figure 3.4A) and HEK293T cells (Figure 3.4B). Interestingly, data suggested that HEK293T cells are more sensitive to the effects of SK inhibitors on the proteasome. Alternatively, HEK293T cells have a higher basal proteasomal activity hence are more sensitive to proteasomal manipulation. This theory is supported by the fact that HEK293T cells have higher basal values for proteasomal activity than LNCaP AI cells (data not shown).

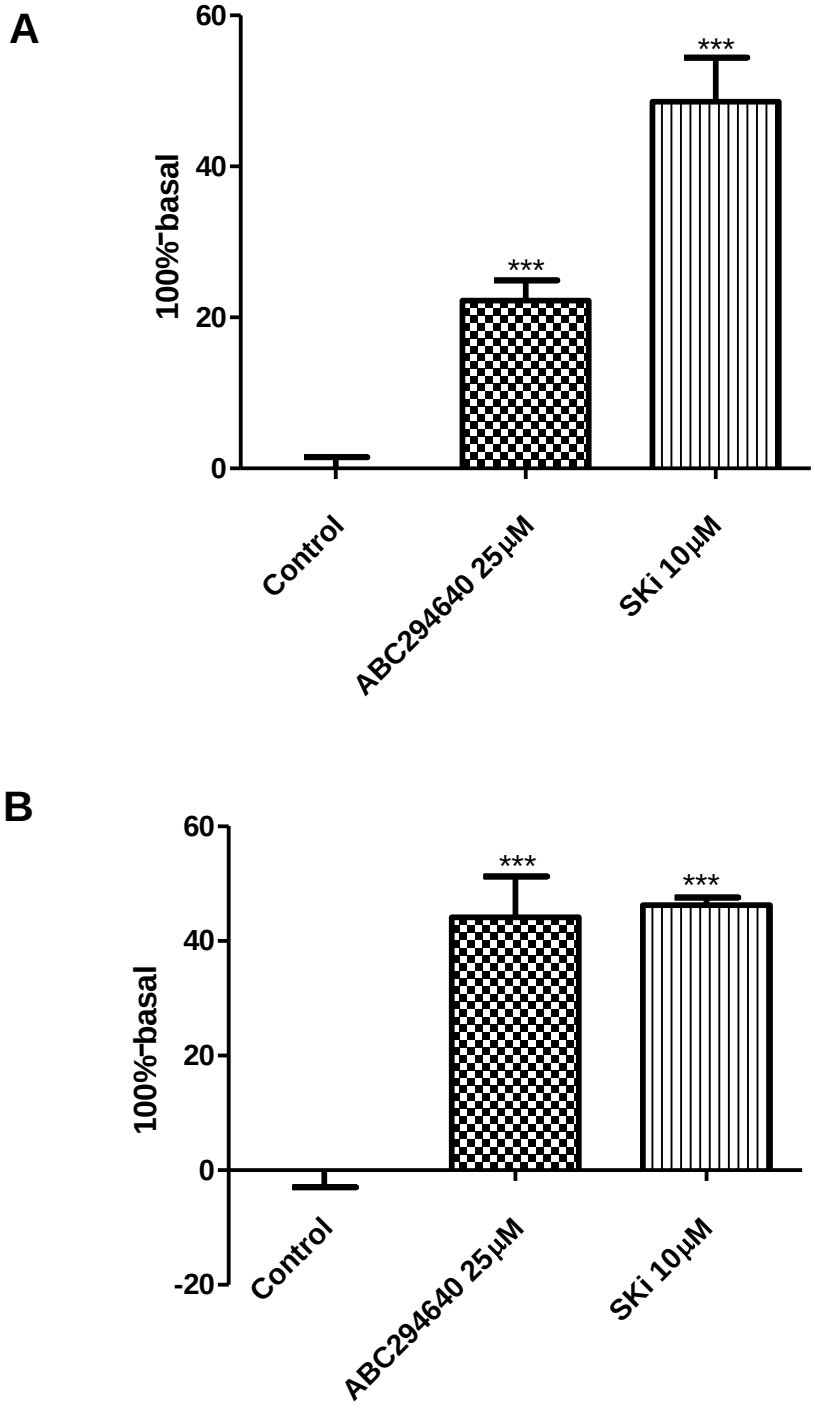


Figure 3.4 ***Effect of ABC294640 and SKi on proteasomal activity in LNCaP AI and HEK293T cells.*** *Histograms showing the effect of ABC294640 (25 μM, 24 hours) and SKi (10 μM, 24 hours) on proteasomal activity in (A) LNCaP AI and (B) HEK293T cells. Results are expressed as means +/− S.E.M for n = 3 experiments. ***p < 0.001 versus control.*

3.2.5 The effect of SK inhibitors on cell viability in LNCaP AI cells.

To give an indication of potency and in order to test the ability of SK inhibitors to affect cancer cell viability, a range of concentrations of each inhibitor was tested and cell viability assessed by MTT assay (which measures total mitochondrial activity and indirectly reflects viable cells). All of the SK inhibitors tested (ABC294640, PF543, RB-005, F-02, ROMe and SKi) affected LNCaP AI cell viability in a concentration dependant manner. Importantly, the IC_{50} values for reduction in cell viability (Figure 3.5) correlate well with published IC_{50} data for inhibition (see introduction 3.8) of the relevant sphingosine kinase isoform(s) for each compound, with the exception of PF543 (see Introduction 3.8). PF543 affected cell viability only at a concentration more than ten times its documented IC_{50} value for SK1 inhibition (Schnute et al., 2012). For this reason, only a partial curve is shown for PF543. A comparison of their relative potencies was made (see discussion 3.3.3).

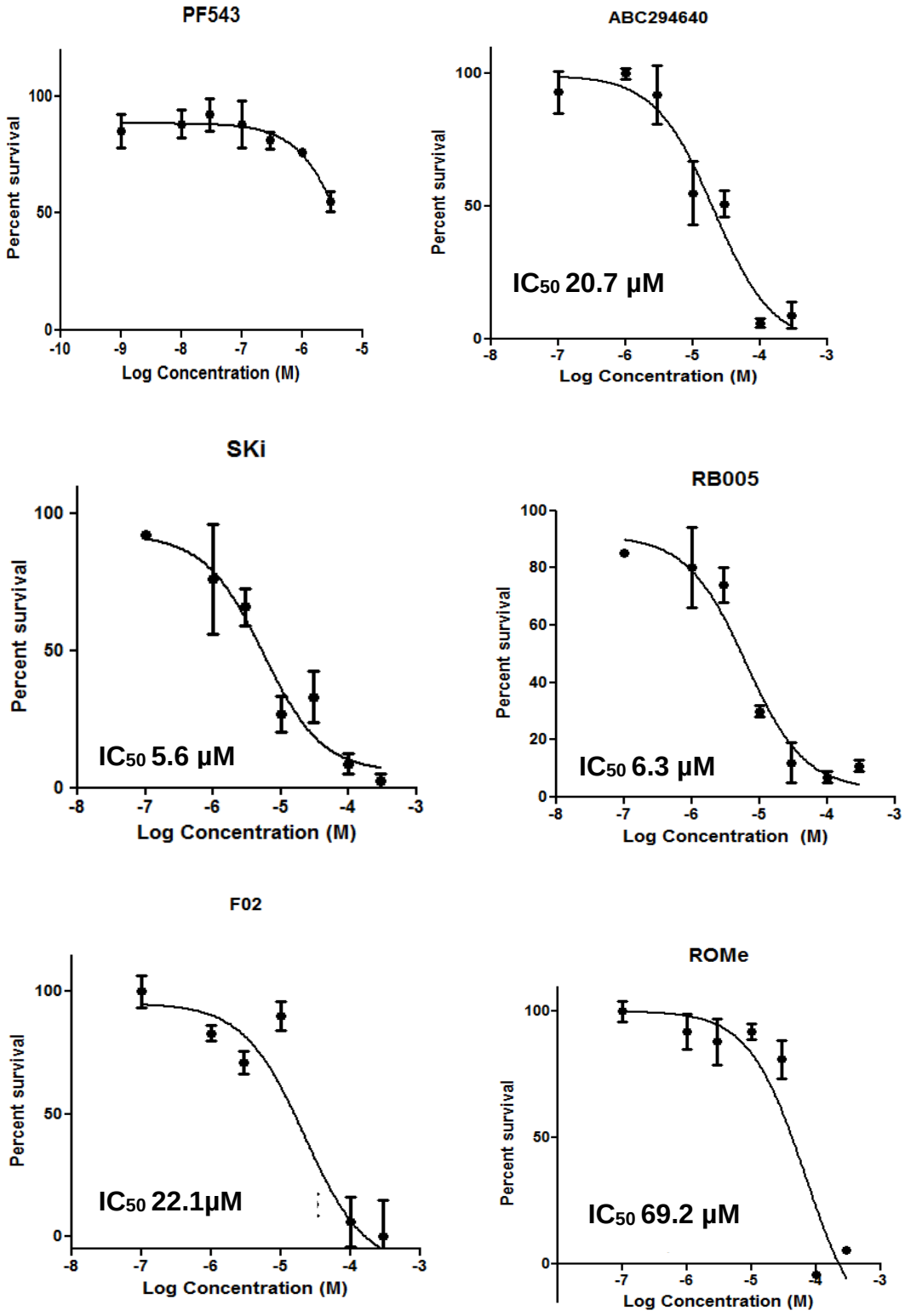


Figure 3.5 ***Effect of SK inhibitors on cell viability in LNCaP AI cells.*** *LNCaP AI cells were treated with inhibitors a range of concentrations of SK inhibitor as indicated (24 hours) and analysed by non linear regression using GraphPad Prism. Data are expressed as mean ± S.E.M. n=3. Results are representative of data from at least three experiments.*

3.2.6 The effect of SK inhibitors on cell morphology in LNCaP AI cells.

It was an incidental finding of the study that the SK inhibitors tested had distinct effects on cell morphology. In agreement with cell viability experiments (see 3.2.5), PF-543 treatment induced no notable morphological changes in LNCaP AI cells. This was in contrast to SK1 inhibitor RB-005 and SK2 inhibitor ROMe treatment which resulted in LNCaP AI cells taking on a rounded morphology with apparent blebbing which is perhaps consistent with apoptosis (Saraste and Pulkki, 2000; Ziegler and Groscurth, 2004). Interestingly, both ABC294640 and SKi treatment resulted in similar morphological changes with cells taking on a flattened, star shaped morphology in response to these compounds. There are several reports in the literature of this being consistent with senescence (Chen et al 2000; Georgakopoulou et al., 2013; Sikora et al., 2016). In addition to this, treatment with ABC294640 and SKi resulted in acidification of the cell culture media, as evidenced by the medium's pH indicator, phenol red (data not shown). These findings further support the notion that ABC294640 and SKi induce different phenotypes from inhibitors which are selective for only a single SK isoform in LNCaP AI cells.

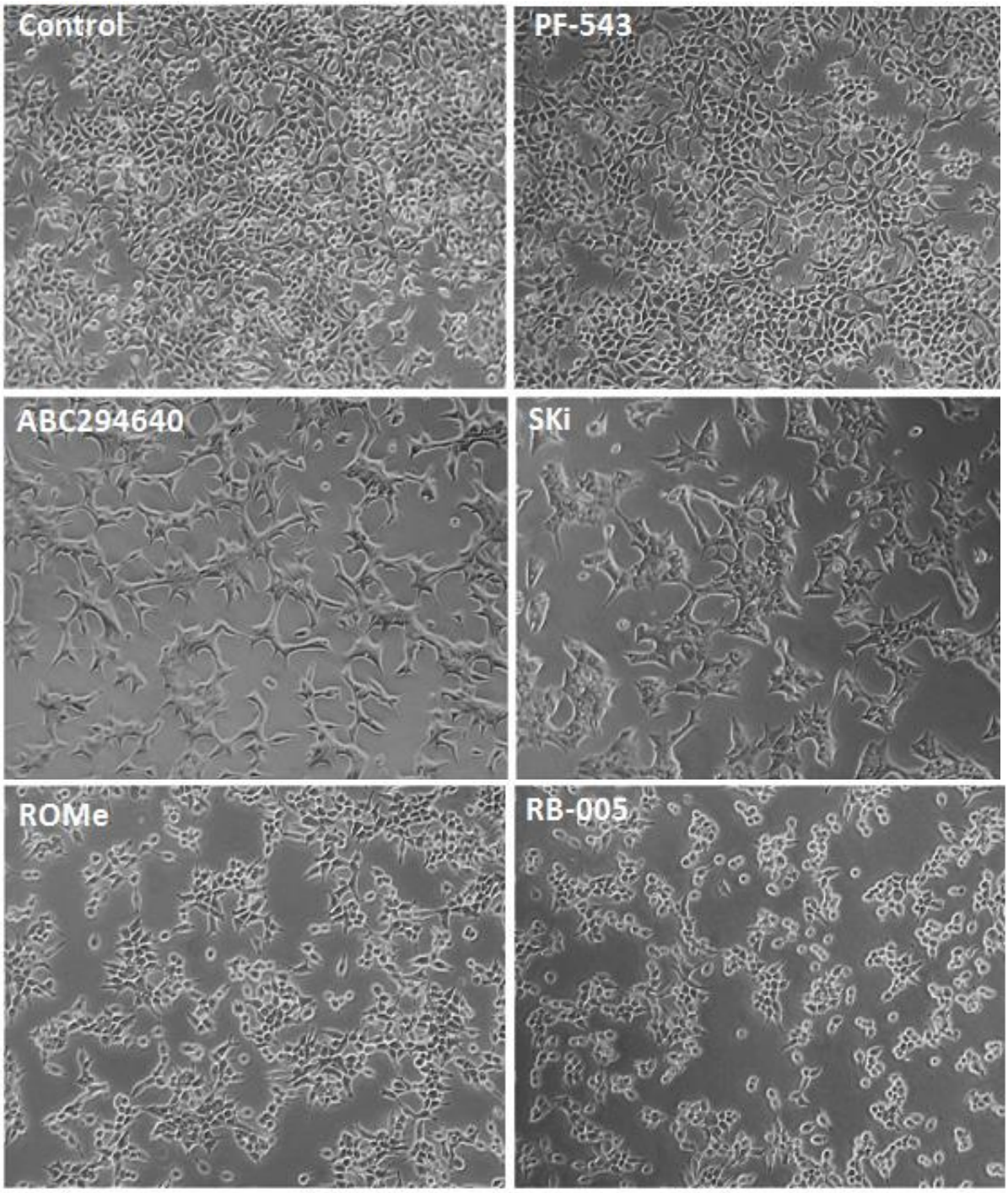


Figure 3.6 ***Effect of SK inhibitors on LNCaP AI cell morphology***. *LNCaP AI cells were pre-treated with SK inhibitors as indicated (PF-543, 100 nM; ABC294640 25 μM; SKi 10μM; ROMe 10μM RB-005, 10μM, 24 hours) or vehicle (DMSO 0.1% v/v) as indicated. Results are representative of cell morphology from at least three separate experiments.*

3.2.7 The effect of lysosomal inhibition on the degradation of SK1.

The degradation of proteins can occur through either the ubiquitin-proteasomal pathway or the lysosomal pathway. In a study using podocytes by Ren and colleagues, SKi down regulated SK1 protein without affecting SK1 mRNA expression. SK1 degradation in podocytes was reversed by the broad specificity lysosomal inhibitor chloroquine as well as the specific Cathepsin B inhibitor CA047Me (10μM). These findings indicated that the primary mechanism of SK1 degradation in podocytes was lysosomal (Ren et al., 2010).

Thus, to examine whether there was a role for lysosomes in mediating the degradation of SK1 in response to SK inhibitors in LNCaP AI cells, SK inhibitors were tested in combination with CA047Me. CA047Me was chosen in this study over chloroquine as it is a more selective inhibitor of the lysosome and it is known that chloroquine exhibits additional off target effects which could skew interpretation of results. For example, chloroquine is also an inhibitor of autophagy (Shintani and Klionsky, 2004) and it is known that several SK inhibitors also affect autophagy such as ROMe (Evangelesti et al., 2014), SKi (Tonelli et al., 2013) and ABC294640 (Beljanski et al., 2010).

It is shown here that the pre treatment of LNCaP AI cells with CA074Me had no effect on the ability of ABC294640, PF-543, RB-005 or SKi to induce a decrease in SK1 expression (Figure 3.7), thereby excluding lysosomal involvement; therefore it is likely that differences exist between LNCaP AI cells and podocytes in the mechanisms of SK inhibitor-induced SK1 protein degradation.

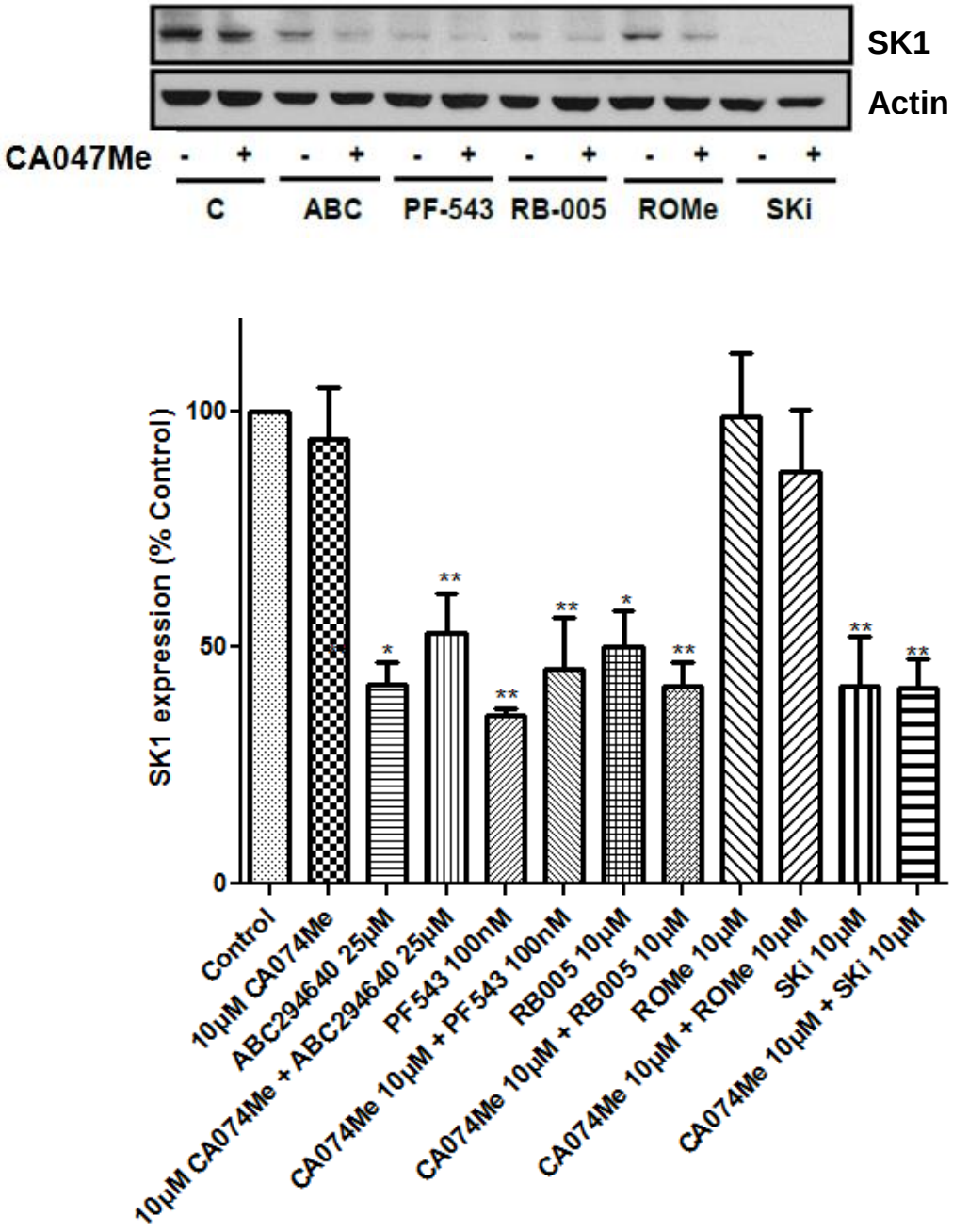


Figure 3.7 ***Effect of lysosomal inhibition on degradation of SK1 by SK inhibitors.*** *Cells were pre-treated with CA047Me (10μM, 30 minutes) before addition of inhibitors or vehicle (DMSO 0.1% v/v) (24 hours). Also shown is a bar graph of the quantification of the effect of SK inhibitors (24 hours) on the proteasomal degradation of SK1 expressed as mean +/- SD n=3 with protein levels normalised to actin.*

3.2.8 The effect of ceramide synthase inhibition on the degradation of SK1 by SK inhibitors.

According to the 'sphingolipid rheostat', inhibition of SK results in ceramide accumulation. Indeed, when SK1 or SK2 is knocked down, or upon treatment with SK inhibitors, ceramide accumulation occurs (Pchejetski et al. 2007; Tonelli et al., 2013). Furthermore, ceramide accumulation has been linked to activation of the proteasome (Kroesen et al., 2003). It was therefore of interest to see whether the proteasomal degradation of SK1 by SK inhibitors could be blocked under conditions where ceramide accumulation via the salvage pathway (i.e. reacylation of sphingosine) was prevented. To test this, fumonisin was used. Fumonisin B1 (FB1) is a ceramide synthase inhibitor which prevents the back conversion of sphingosine to ceramide (Merrill et al., 1993; Merrill et al., 2001), thus preventing a shift in the sphingolipid rheostat from S1P back, via sphingosine, to ceramide.

Pre-treatment of LNCaP AI cells with FB1 failed to reverse the degradation of SK1 by various SK inhibitors (Figure 3.8), suggesting that ceramide accumulation following a shift in the sphingolipid rheostat is not responsible for the induction of the proteasomal degradation of SK1 by these compounds.

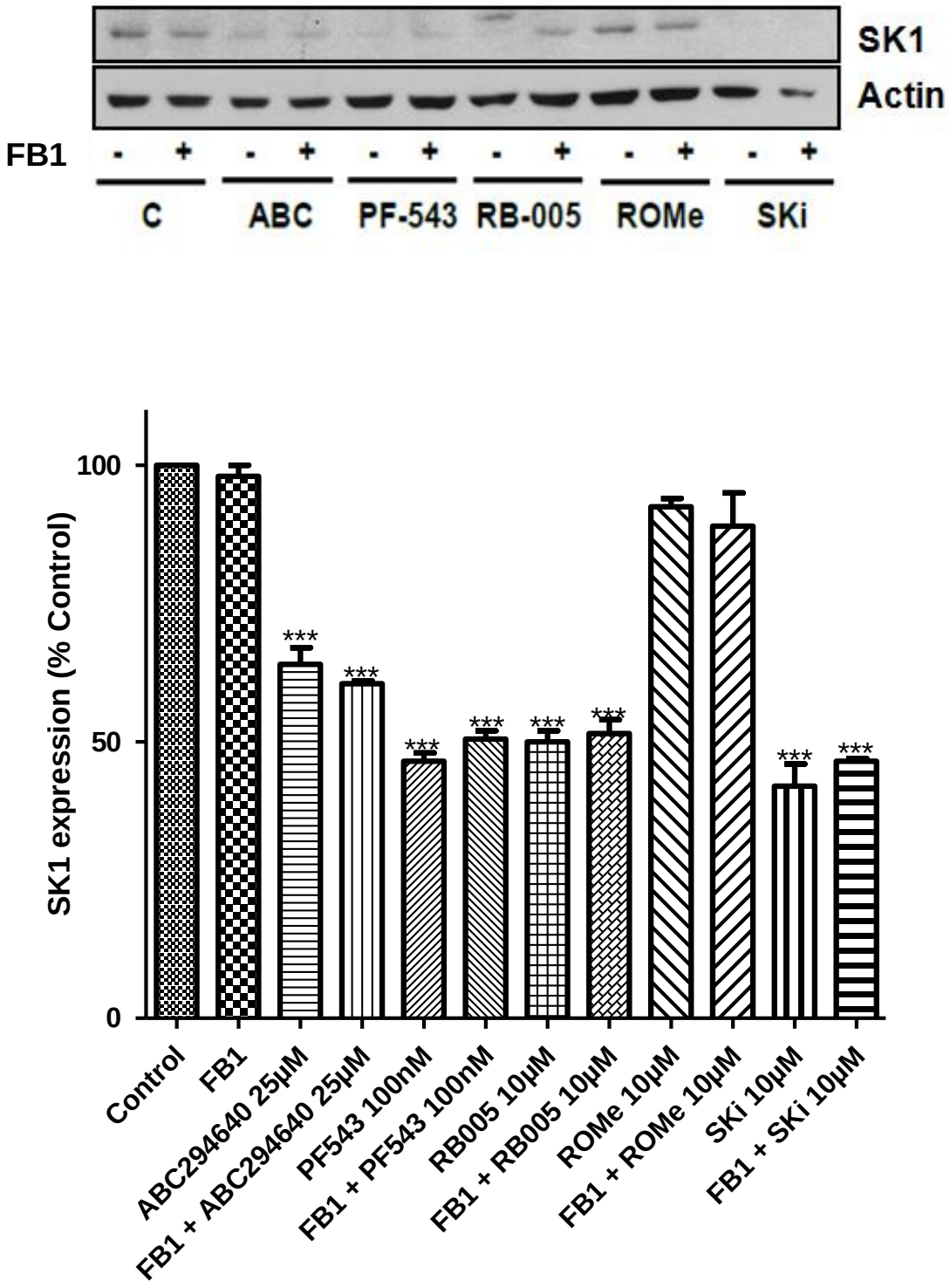


Figure 3.8 ***Effect of ceramide synthase inhibition on degradation of SK1 by SK inhibitors.*** *Cells were pre-treated with fumonisin B1 (100nM, 1 hour) before addition of SK inhibitors (24 hours) or vehicle (DMSO 0.1% v/v) as indicated. Quantification is also shown expressed as mean +/- SEM. Western blot results are representative of at least three separate experiments. *** = p<0.001 vs control normalised to actin reprobe.*

3.2.9 The effect of inhibiting *de novo* ceramide synthesis on the degradation of SK1 by SK inhibitors.

Having shown that accumulation of ceramide generated via the salvage pathway (i.e. reacylation of sphingosine by ceramide synthase) is not responsible for the degradation of SK1 by SK inhibitors, it was of interest to test whether *de novo* ceramide synthesis could play a role.

To investigate whether ceramide formed via the *de novo* pathway affects the SK inhibitor-induced degradation of SK1, cells were pre-treated with myriocin (100nM for 2 hours – Mullen et al., 2011), which inhibits serine palmitoyltransferase which catalyses the rate limiting step of *de novo* ceramide synthesis. Interestingly, myriocin failed to affect the reduction in SK1 in LNCaP AI cells treated with SK inhibitors (Figure 3.9), suggesting that ceramide formed from the *de novo* pathway is not involved in SK inhibitor induced activation of the proteasome.

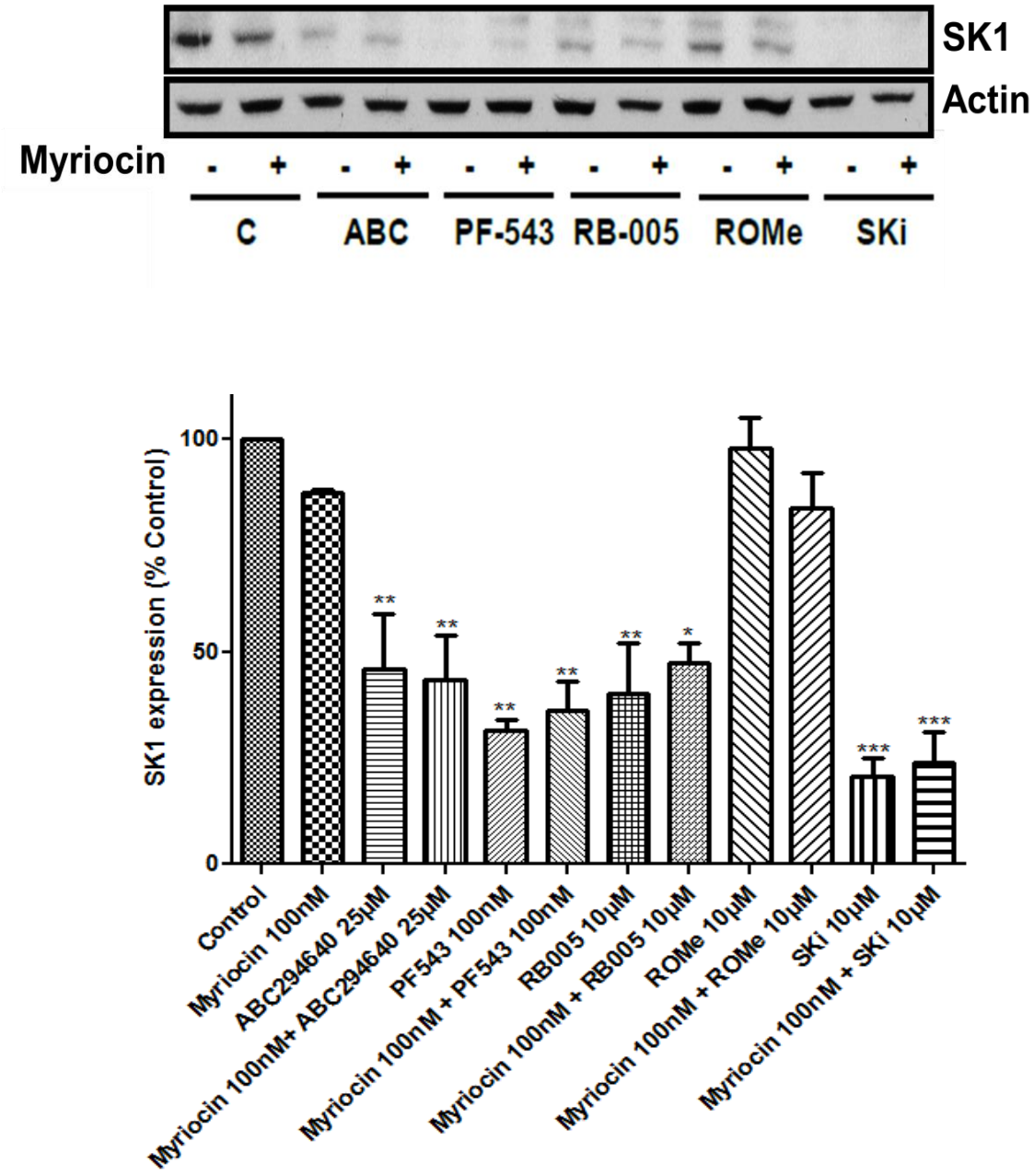


Figure 3.9 ***Effect of de novo sphingolipid synthesis inhibition on degradation of SK1 by SK inhibitors.*** *Cells were pre-treated with myriocin (100nM, 2 hours) before addition of inhibitors (24 hours) or vehicle (DMSO 0.1% v/v) as indicated. Also shown is quantification of the effect of myriocin on the degradation of SK1 by SK inhibitors normalised to actin reprobe, expressed as mean +/- SEM. Western blot results are representative of at least three separate experiments. *=p<0.05, **=p<0.01, ***=p<0.001.*

3.3 Discussion

3.3.1 ABC294640 induces the proteasomal degradation of SK1 and cMyc.

It was recently demonstrated that ABC294640 induced the proteasomal degradation of cMyc in multiple myeloma cells (Venkata et al., 2014). This is reminiscent of the properties of the SKi which, in LNCaP and LNCaP AI prostate cancer cells, induces the proteasomal degradation of SK1, cyclin D1 (Loveridge et al., 2010) and cMyc (Watson et al., 2013). Furthermore, cMyc expression is decreased in Apc/SK1 knockout mice. This suggests that SK1 regulates cMyc expression (Kohno *et al.*, 2006). Taken together these findings raised the possibility that ABC294640 might also degrade SK1, despite the finding that this compound fails to directly inhibit SK1 (Gao et al., 2012).

Indeed, the treatment of LNCaP AI cells and HEK293T cells with SK2 selective inhibitor ABC294640 resulted in the proteasomal degradation and removal of SK1 and cMyc from both HEK293T (Figure 3.2) and LNCaP AI cells (Figure 3.1). This is the first demonstration of an indirect effect of ABC294640 on SK1 expression (McNaughton et al., 2016). These effects were prevented by pre-treating cells with the proteasomal inhibitor, MG132 (Figure 3.6). The degradation of SK1 in response to SK inhibitors appears to require their binding to SK1 as this response correlates well with the IC_{50} values for each inhibitor. However, this is not the case for ABC294640 (which does not inhibit SK1 in an activity assay (Gao et al., 2012)) and therefore involves a different mechanism, most likely via causing an increase in proteasomal flux or affecting another target which enhances ubiquitination of proteins or modifies them in another way which targets them to the proteasome. Ultimately, ABC294640 cannot be considered an SK2 selective inhibitor in cells due to its removal of SK1 protein. Thus, the current study highlights that care must be taken when attributing the effects of ABC294640 solely to SK2 inhibition. This is widespread in the literature (French et al., 2010; Beljanski et al., 2011; Yang et al, 2015) This finding carries further significance as there are clinical trials being conducted which are based on the inhibition of SK2 by ABC294640 (Britten et al., 2017).

The treatment of LNCaP AI and HEK293T cells with ABC294640 or SKi increases the flux of cellular proteins through the ubiquitin proteasomal pathway (Figure 3.3). This is supported by both measurements of proteasomal activity and by the finding that the levels of cMyc, which is subject to constant rapid proteasomal degradation is also markedly reduced in response to ABC294640 and SKi, but not other SK inhibitors (Figure 3.1; Figure 3.2).

Furthermore, the effects of the inhibitors on SK1 and on cMyc are reversed by proteasome inhibitor MG132 (Figure 3.1; Figure 3.6). This is consistent with previous reports which demonstrate that SKi activates the proteasome in MCF-7 cells (Loveridge et al., 2010) and LNCaP AI cells (Tonelli et al., 2012).

The finding that the proteasome is activated by ABC294640 is further supported by the measurement of proteasome activity in response to this compound (Figure 3.4). However, the proteasomal degradation of SK1 by ABC294640 was not fully reversed by MG132 at higher concentrations of the SK inhibitor. This is recapitulated for cMyc (Figure 3.1). This may suggest that an additional mechanism of SK1 protein regulation, perhaps involving reducing SK1 mRNA. PCR would need to be used to investigate this. Interestingly, others have shown that ABC294640 reduces cMyc expression in B-ALL cells through a mechanism involving modulation of S1P/HDAC regulation, although this is thought to be via its inhibition of SK2 (Wallington-Beddoe et al 2014).

The degradation of cMyc by ABC294640 and SKi is significant and could represent an additional mechanism of action of these compounds with therapeutic promise. cMyc is an oncoprotein that is commonly dysregulated in prostate cancer. Furthermore, cMyc is one of the primary oncogenes responsible for promoting progression to an aggressive and chemotherapeutic resistant form of human prostate cancer (Taylor et al., 2010). High cMyc expression also correlates with poor prognosis in advanced forms of the disease (Taylor et al., 2010; Schrecengost et al., 2013). Therefore, targeting cMyc in prostate cancer could potentially inhibit tumour growth and could represent a novel therapeutic option. Indeed, in multiple myeloma, ABC294640 was shown to induce the proteasomal degradation of cMyc and myeloid cell leukemia-1 (Mcl-1) which resulted in caspase-3 mediated apoptosis (Venkata et al., 2014). Moreover, ABC294640 down regulated cMyc expression in acute lymphoblastic leukaemia which resulted in a reduction in cell viability (Wallington-Bedoe et al., 2014). Furthermore, in LNCaP and C4-2 prostate cancer cells, ABC294640 down regulated both cMyc expression and mRNA and this inhibited prostate cancer cell viability and proliferative signalling (Schresengost et al., 2015). However, it is unclear from these published studies whether the reduction in cell viability and growth induced by ABC294640 is linked to SK1 degradation, SK2 inhibition, cMyc degradation and/or other actions. The current study demonstrates that proteasomal degradation had a role in cMyc depletion in response to ABC294640 in prostate cancer cells and was reversed using proteasome inhibitor MG132. This is consistent with the findings of Schrecengost and colleagues (Schrecengost et

al., 2015). It is highly likely that ABC294640 is causing an overall increase in proteasomal flux (Figure 3.3) which causes increased degradation of all proteins subject to turnover by this route. Alternative mechanisms include one where SK2-derived S1P inhibits histone de-acetylase (HDAC) in the nucleus (Hait et al., 2009), driving accumulation of histone H3 lysine 9 acetylation (H3K9ac) at the MYC gene promoter. In this model, decreasing nuclear S1P using ABC294640 would result in decreased cMyc transcription (Wallington-Beddoe et al., 2014). However, findings of the current study suggests that SK2 is unlikely to be involved in the regulation of cMyc expression as SK2 inhibitors ROMe and F-02 do not reduce cMyc expression in HEK293T cells (Figure 3.3).

Interestingly, ABC294640 was also shown to down regulate AR expression in LNCaP and C4-2 prostate cancer cells and this was also reversed using MG132 (Venant et al., 2015; Schrecengost et al., 2015). This is reminiscent of the effects of SKi in LNCaP AI cells, which down regulated AR expression in an oxidative stress dependant manner (i.e. reversed by the antioxidant N-Acetyl Cysteine (NAC)) in LNCaP AI cells (Tonelli et al., 2013). These findings, taken together, suggest that ABC294640 modulates multiple signalling pathways through mechanisms involving oxidative stress and activation of the proteasome. Importantly, like the degradation of cMyc induced by ABC294640, degradation of AR represents an additional potential therapeutic facet of ABC294640 treatment. Indeed, most of the current therapies for prostate cancer (including androgen deprivation therapy and AR antagonists) aim to modify this protein as aberrant AR signalling is essential for prostate cancer progression and correlates with disease severity (Patel et al., 2014). Overall, these findings highlight ABC294640 as a multiple modifier of a plethora of proteins of relevance to prostate cancer progression, including cMyc, AR and SK1.

3.3.2 ABC294640 is a proteasomal activator

To be targeted to the proteasome, proteins must first undergo lysine-48-linked polyubiquitination. Furthermore, it is known that the proteasome is a barrel shaped structure with enclosed proteolytic sites. Therefore, the proteasome is naturally repressed and for substrates to gain access, activators or facilitators are required (Stadtmueller and Hill, 2011). SK1 is ubiquitinated and degraded by the proteasome under basal conditions (Kihara et al., 2006; Loveridge et al., 2010) and this increases in response to treatment with SKi (Loveridge et al., 2010) or ABC294640, which appears to enhance proteasomal flux (Figure 3.3). ABC294640 and SKi could do this either by up regulating proteasomal machinery or by activating an up-stream component of the degradation process by, for example, activating an E1 ubiquitin activator (UAE) or an E2 ubiquitin conjugating enzyme or an E3 ubiquitin ligase. This would in turn enhance ubiquitination of proteins and target them to the proteasome. The E3 ligase (or E3 ligases) responsible for the ubiquitination of SK1 are currently unknown, although it is reported that E3 ligase TRAF 2 binds to SK1 (Alvarez et al., 2010). It would be of interest to determine if the E3 ligase that ubiquitinates SK1, cMyc and/or AR is the same ligase, because if this is not the case, the target is likely to be a common upstream target. Interestingly, both cMyc and AR are reported to be ubiquitinated by E3 ligases CHIP and Skp2 (von der Lehr et al., 2003; Li et al., 2014; Paul et al., 2014). In light of this, it would be of interest to determine if either of these are responsible for SK1 ubiquitination. The mechanism of proteasomal activation by ABC294640 or SKi has yet to be elucidated and additional experiments targeting up stream components of the ubiquitin proteasomal pathway are required to resolve this. For example, the use of a ubiquitination resistant SK1 could be informative to determine whether enhanced ubiquitination or enhanced activation of proteasomal machinery is implicated in the degradation of SK1 by ABC294640 and SKi.

The proteasome is involved in regulating many essential cellular functions such as the cell cycle, cell differentiation, signal transduction, antigen processing, stress signalling, inflammatory responses, and apoptosis (Huang and Chen, 2011). Consequently, the therapeutic capacity of both proteasome inhibitors and activators has been investigated in cancer and other diseases (Crawford et al., 2011). However, the therapeutic potential of proteasomal activators is not well studied. Interestingly, oleuropein, which is a natural product derived from the olive tree that has been shown to activate the proteasome has strong anti-cancer properties in cells and murine cancer models. It inhibited the proliferation and

migration of advanced-grade tumour cell lines *in vitro* by stunting invasiveness, motility and replication and regressed tumours *in vivo* in 9-12 days when administered to mice (Hamdi and Castellon, 2005). Furthermore, betulinic acid (BA), which is a lupene-type pentacyclic triterpene derived from the birch tree, is capable of activating the proteasome at low micromolar concentrations. BA has been studied as an anti-tumour agent in a multitude of cancer cell lines and interestingly was selectively cytotoxic towards some cell cancer lines and not others (Kessler et al., 2007). This suggests that the potential of proteasome activators as anti-cancer compounds is cancer type and potentially disease stage specific. Indeed, it has been shown that some activators of the proteasome promote colon cancer by providing cancer cells with protection from apoptosis and enhanced clearance of irregular proteins (Bauer et al., 2011). Paradoxically, it has also been shown that proteasome activators have therapeutic potential through their ability to enhance removal of aberrant proteins from the cell (Huang et al., 2009; Leestemaker et al., 2017). Proteasome inhibitors have also been developed as anti-cancer agents (Crawford et al., 2011). For example, bortezomib is proteasome inhibitor which is used clinically for the treatment of multiple myeloma (Field-Smith, 2006). Overall, work still needs to be done to stratify the role of proteasome modulators in cancer cell metabolism. Importantly, findings of the current study suggest an additional property of ABC294640 and SKi with potential therapeutic application which has not previously been investigated.

3.3.3 Effect of SK inhibitors on cell viability of LNCaP AI cells

MTT experiments demonstrated that all of the SK inhibitors tested (except PF543) affect cell viability of LNCaP AI cells at concentrations which have been shown to inhibit SK (Figure 3.5). This suggests that both SK1 and SK2 have a role in maintaining survival of LNCaP AI cells. Of the inhibitors tested, the inhibitors which effect SK1 either catalytically or via reducing SK1 expression (ABC294640, RB-005 and SKi) had more profound effects on cell survival than the inhibitors which are fully selective for SK2 (F-02 and ROMe). This suggests that SK1 has a more prominent role in promoting survival of LNCaP AI cells.

In general the cell viability data generated in this study correlates well to current available data on each compound. For example, ABC294640 was shown by French et al. (French et al., 2012) to exert anti-tumour activity in a number of cancer cell lines including prostate cancer cells. Importantly, in the study by French and colleagues, ABC294640 treatment markedly shifted the sphingolipid rheostat in favour of ceramide with C16 ceramide, C22 ceramide, C24 ceramide, and C24:1 ceramide species elevated concomitant with S1P being reduced with an IC_{50} of 26μM. Furthermore, cell viability experiments conducted in DU145 prostate cancer cells demonstrated an IC_{50} of 21.9μM (French et al., 2010). This correlates well with the IC_{50} for reduction in cell viability reported herein for LNCaP AI prostate cancer cells (20.6μM) (Figure 3.5). Interestingly, the range of IC_{50} values exhibited for ABC294640 in the French study across different cell types varied greatly and ranged from 6μM to 48.1μM, suggesting differences in susceptibility to ABC204640 of different cancer cell types (French et al., 2010).

Although F02 has not been previously characterised for its effects on cell viability, the IC_{50} for cell viability reported herein (22.1μM) is almost identical to the IC_{50} for SK2 inhibition (21.8μM) (Byun et al., 2013). This is a good indicator of causality between SK2 inhibition and reduction in cell viability exerted by this compound.

For the final SK2 inhibitor tested, ROMe, the IC_{50} for its effect on cell viability was unexpectedly high (69.2μM). In MCF7 cells, ROMe inhibited DNA synthesis with an IC_{50} of 15μM, which correlates well with the IC_{50} for its inhibition of SK2 (27μM) and Ki for sphingosine (16.5μM) (Lim et al., 2011). The higher IC_{50} observed in the current study may be due to cell type differences. However, it is also important to note that there DNA synthesis assays and cell viability assays are different measures and do not always yield similar results. Indeed, DNA synthesis experiments in LNCaP AI cells demonstrate that ROMe (10μM),

reduces DNA synthesis to <50% (see chapter 4, figure 4.1) which is consistent with the reports of Lim et al. (Lim et al., 2011) Thus, it is likely that SK2 inhibition by ROMe causes cancer cell growth arrest at sub toxic concentrations. With the exception of PF543, SKi is the most potent SK1 inhibitor of the panel with an *in vitro* IC50 for SK1 inhibition of 0.5μM. SKi was also the most potent compound tested against LNCaP AI cancer cell viability with an IC_{50} of 5.6μM. This could indicate that catalytically inhibiting a combination of SK1 and SK2 is the most effective means of killing cancer cells. Previous studies have also shown that SKi is a mixed inhibitor of SK1 which inhibits both ATP and Sph binding (Lim et al., 2012). Lim et al. importantly showed that SKi reduced S1P levels in LNCaP AI cells but interestingly did not increase ceramide levels (Lim et al., 2012). This may be due to the inhibition of dihydroceramide desaturase by SKi (Cingolani et al., 2015) which would prevent an increase in ceramides. The reduction of S1P by SKi was also shown by French and colleagues in multiple other cancer cell types (French et al., 2010). In their study, IC_{50}s for cell viability for SKi are reported to range from 0.9μM in MCF7 cells to 4.6μM in T24a cells. This correlates well with the findings of the current study in LNCaP AI cells (5.6μM) (Figure 3.5). Unexpectedly, almost 10 years after the publication of the original study on SKi, Gao and colleagues reported SK2 inhibition by SKi. In the latter study, SKi was reported to favour SK2 inhibition over SK1 inhibition, with IC_{50}s of 20μM and 35μM, respectively (Gao et al., 2012). This IC_{50} for SK1 is considerably different from the original IC_{50} reported by French and colleagues although it is possible that batch variation or different assay parameters could account for this (French et al., 2003). Consistent across both studies however is the fact that SKi is able to reduce cancer cell viability in line with its Ki and IC_{50} for SK inhibiton, indicating target specificity (Gao et al., 2012).

RB005, which is an SK1 selective inhibitor, also potently reduced cancer cell viability with an IC_{50} of 6.3μM (Figure 3.5). This is a good indicator that SK1 is a more promising target for cancer treatment in these cells than SK2. As with the other compounds the IC_{50} for reduction of cell viability for RB005 is similar to its reported IC_{50} for SK1 inhibition which is 3.6μM (Baek et al., 2013). Importantly, this is a good indicator that the reduction in cell viability by RB005 is a result of SK1 inhibition by this compound.

Unlike the other SK inhibitors tested, the nanomolar potent SK1 selective inhibitor PF543 (Ki 3.6nM) had no effect on LNCaP AI cell viability until low micro molar concentrations. This is consistent with the findings of Schnute and colleagues who reported that PF543 treatment had no effect on the viability or proliferation of 1483 head and neck carcinoma cells (Schnute

et al., 2012). Although PF543 was able to disrupt the cellular S1P/Sph ratio in these cells, supporting a shift in the sphingolipid rheostat, there was no significant increase in the levels of C18:0, C24:0 or C24:1 ceramides. Increases in ceramide were not observed until concentrations of >3μM. Interestingly, this correlates with the concentration of PF543 at which a decline in viability is observed in the current study (Figure 3.5). It is therefore possible that to affect cell viability, the accumulation of ceramide as well as a decrease in cellular S1P is required. It is also possible that PF543 may affect another target in the sphingolipid signalling pathway when used at micromolar concentrations. Indeed, PF543 is a sphingosine competitive inhibitor of SK1 (Ki 2-3.6 nM; Schnute et al., 2012) that inhibits SK2 with a Ki of 356 nM. Therefore, it is possible that it has effects on other targets of Sph. Interestingly, there is only a 2 fold increase in Sph observed in the study versus a 10 fold decrease in S1P (Schnute et al., 2012). This suggests that either the S1P could be subject to enhanced conversion to degradation products hexadecenal and phosphoethanolamine (both of which have signalling functions of their own) or is rapidly being converted to another sphingolipid mediator, via Sph or ceramide, for example dhCer or dhSph. Both of these lipids also have signalling functions of their own which could ultimately confer resistance to PF543 treatment on cell viability or proliferation. Further experiments are required to elucidate this including quantification of the entire sphingolipid metabolome in response to PF543 treatment in of LNCaP AI cells.

3.3.4 SK1 selective inhibitors induce the degradation of SK1

Although many of the novel small molecule inhibitors of SKs have been described and sometimes characterised pharmacologically, most of these inhibitors have not been characterised uniformly in a single cell type. Furthermore, for the inhibitors which have been characterised, only one or two cell types have been included in the studies and often differences between cell types are reported. These differences and the fact that there is limited published information available on the novel isoform specific SK inhibitors in a cellular context highlighted a need to characterise these in the cell system being studied. This need is made further evident in the knowledge that SKi which had classically been accepted as an SK1 selective SK inhibitor (French et al., 2003) was shown to also inhibit SK2 (Gao et al., 2012). Moreover, this has been affirmed by the finding that ABC294640 - a well characterised inhibitor which has numerous published accounts attributing its effects to inhibition of SK2 - degrades SK1 protein in cells (Figure 3.1).

The current study is consistent with previous studies which show that catalytic inhibitors of SK1 induce its proteasomal degradation (Loveridge et al., 2010; Lim et al., 2011; Tonelli et al., 2012; Baek et al., 2013). However, this is the first study to consider the potential of SK2 selective inhibitors (such as ROMe, F-02) to degrade SK1 protein in cancer cells. Furthermore, several distinct metabolic components were targeted in combination with SK inhibitors with the aim of further discerning the mechanism of the SK1 degradation by SK inhibitors.

As expected, (except for ABC294640 which has been previously discussed at length in section 3.3.1), only inhibitors which exert catalytic inhibition of SK1 induced its proteasomal degradation (Figure 3.3.2). Importantly, SK2 selective inhibitors ROMe and F02 did not induce the degradation of SK1 (Figure 3.6). For F02, this is consistent with published information regarding the lack of effect of F02 on SK1 protein levels in PASMCs (Byun et al., 2013). These findings were consistent in both LNCaP AI cells and HEK293T cells (Figure 3.2; Figure 3.6). It appears that the degradation of SK1 by SK1 inhibitors involves their target engagement as the concentrations that induce SK1 degradation correlate with inhibitory potency, i.e. binding of the inhibitor to SK1 is required to induce its proteasomal degradation. This could potentially involve a structural change being induced in response to inhibitor binding which renders SK1 more susceptible to degradation by the proteasome or more susceptible to ubiquitination. Although the degradation of SK1 in response to these

inhibitors is rescued by MG132 pre-treatment (Figure 3.6), it is unlikely that these inhibitors activate the proteasome in a similar manner to ABC294640 or SKi. This is evident in the fact that only ABC24640 and SKi also induce the degradation of cMyc, which is indicative of activation of the proteasome as it is also normally subject to turnover via this route (Figure 3.1; Figure 3.3).

It was reported by Ren and colleagues that the degradation of SK1 in podocytes was via the lysosomal route (Ren et al., 2010). Moreover, in MCF-7 cells, it was shown by the Obeid group that SK1 is a substrate for lysosomal protease Cathepsin B (Tata et al., 2005). In light of these observations, it was of interest to determine the role of the lysosome in regulating the degradation of SK1 in LNCaP AI cells. Pre-treatment of LNCaP AI cells using a specific inhibitor of lysosomal protease cathepsin B (CA047Me) did not reverse the effects of SK inhibitors on SK1 protein expression (Figure 3.7), suggesting the proteasome rather than the lysosome is the primary protein degradation pathway implicated in SK1 regulation in LNCaP AI cells. The differences in cell type between the current study and the Ren and Tata studies could account for the different observations (Tata et al., 2005; Ren et al., 2010). Indeed, it has been shown that cell type differences exist in the relative contribution of lysosomal and proteasomal pathways to protein clearance (Clague and Urbè, 2010).

Next, the role of ceramide accumulation following SK inhibition was investigated as a potential trigger for the proteasomal degradation of SK1. Indeed, ceramide accumulation has previously been linked to activation of the proteasome. In B-cells, B cell receptor (Bcr) activation induced up-regulation of C16 ceramide in the short term (6h) and very long chain ceramides over longer times (20-24h). This resulted in proteasome activation and subsequent activation of effector caspases (Kroesen et al., 2003). Interestingly, these effects were blocked by myriocin, an inhibitor of SPT, which catalyzes the first and rate-limiting step in the *de novo* formation of ceramide. In the current study, neither myriocin nor fumonisin B1, which inhibits ceramide synthases (thereby blocking the back conversion of Sph to ceramide) was able to reverse the effects of SK inhibitors on SK1 degradation (Figure 3.8; Figure 3.9). This suggests that ceramide accumulation upon SK inhibition is not involved in activating the proteasome. Cell permeable ceramides could be added to the cells and the proteasome assayed to provide further information about this.

Undoubtedly, the removal of SK1 protein by SK1 inhibitors as well as SKi or ABC294620 constitutes a mode of action of these compounds. Importantly, this could markedly enhance

inhibitory potency and in turn improve therapeutic efficacy (Pyne et al., 2011). Indeed, *in vitro* SK1 activity assays conducted by Ren and colleagues concluded only a very weak direct inhibition by SKi of SK1 in HEK293T lysates (Ren et al., 2011). They concluded that inhibition of SK1 by SKi is limited *in vitro* and that the degradation of SK1 by this compound was a stronger contributor to the inhibitory effect observed upon treatment in various cell types (Ren et al., 2011). Further work is required to establish the contribution of the degradation of SK1 to the therapeutic efficacy of SK inhibitors. For example, ubiquitination deficient or proteasome resistant SK1 mutants could be used to assess the differences in effects of SK inhibitor treatments on cell viability. Indeed, it is reported that resistance of LNCaP AI cells to SKi is due to their higher expressing of proteasome resistant SK1b. Since SK1b is more stable than SK1a, it is able to evade the proteasome in response to SKi treatment, rendering LNCaP AI cells resistant to apoptosis (Tonelli et al., 2010).

3.4 Summary

A major finding of the current study is that the SK2 selective inhibitor ABC294640 induces the proteasomal degradation of SK1. This finding highlights a novel mechanism of action for ABC294640, which is currently in phase 2 clinical trials for haematological cancers and phase 1 clinical trials for solid tumours. Furthermore, the finding that ABC294640 is a proteasomal activator highlights another facet of ABC294640 action which has not previously been reported. Not only does this begin to account for some of the unexplained anti-cancer effects of this compound compared to other drugs which target SK2 specifically but these observations also highlight a short coming of previous studies which have attributed the actions of this compound solely to SK2 inhibition.

The present study also adds to current knowledge surrounding the effect of SK inhibitors on SK1 protein degradation. The ability of SK2 selective inhibitors to degrade SK1 protein has previously not been considered. Furthermore, additional knowledge has been provided surrounding the cellular mechanism of SK1 degradation. It would be of great interest to similarly determine the effects of the SK inhibitors on SK2 protein. However, at the time of the current study, the commercially available SK2 antibodies are not sensitive enough to detect endogenous SK2 protein (data not shown).

Current data also begins to explore the down regulation of cMyc by SK inhibitors and introduces the idea of certain SK inhibitors activating the proteasome. This particular effect

may have potential to be therapeutically exploited due to the removal of several oncogenic proteins including AR, cMyc and SK1.

CHAPTER 4:

THE ROLE OF SPHINGOSINE KINASES IN SENESCENCE IN LNCAP AI CELLS

4. CHAPTER 4 - The role of SK1 and SK2 in regulating senescence in LNCaP AI cells.

4.1 Introduction

It has previously been shown that LNCaP cells undergo apoptosis in response to SKi (as evidenced by PARP cleavage), but that LNCaP AI cells are resistant to this compound (no PARP cleavage) (Loveridge et al, 2010). In addition, treatment with SKi resulted in an increase in p53 expression in LNCaP AI cells (Tonelli et al., 2013), although the mechanism underlying this effect was not investigated. p53 is a master regulator of senescence, autophagy and apoptosis, and can be used as a biomarker of cellular senescence (reviewed in Aubrey et al., 2018). Interestingly, there are several reports in the literature which assign a role to SK in regulating senescence and which link SKs and p53 (see introduction 3.10). These relate to both SK1 and SK2. For example, a functional interaction has been recently described between SK1 and p53 (Heffernan-Stroud et al., 2012). In particular, the authors of this study demonstrated that p53 negatively regulates SK1 expression. More recently, it was demonstrated that SK1 is proteolysed in response to doxorubicin in breast cancer cells downstream of caspase 2 activation by p53 (Carroll et al., 2018). SK2 was also implicated in the induction of p21 by doxorubicin although this was found to be independent of p53 (Sankala et al., 2007).

It is possible that SK inhibitors which induce the removal of SK1 may induce senescent growth arrest of LNCaP AI cells as opposed to apoptosis. The current study confirms that SKi, which inhibits both SK1 and SK2 (Gao et al 2102), induces the proteasomal degradation of SK1 as first observed by Loveridge et al. (Loveridge et al 2010). Moreover, the current study has established for the first time that the inhibitor of SK2 catalytic activity, ABC294640, also induces the proteasomal degradation of SK1, therefore affecting both SK1 and SK2 in LNCaP AI cells whereas other inhibitors of SK2 are without effect on SK1. Therefore, these and other SK inhibitors, together with other pharmacological and molecular tools, were used to investigate the role of each SK isoform in regulating senescence in LNCaP AI cells. p53 was used as a cellular marker of senescence together with p21, which is a cyclin dependent kinase (CDK) inhibitor protein that promotes growth arrest in response to many external stimuli. It is also a tumour suppressor protein and a marker which is up-regulated only in senescence (Abbas and Dutta, 2009).

4.2 Results

4.2.1 SK inhibitors inhibit DNA synthesis in LNCaP AI cells.

To investigate the effect of SK inhibitors on DNA synthesis, [^{3}H] thymidine incorporation into newly synthesised DNA was measured in LNCaP Ai cells. Cells were treated with a fixed concentration of various SK1 inhibitors (RB-005) and SK2 inhibitors (ABC294640, F-02, ROMe) or SK1/SK2 inhibitors (SKi) (all 10μM, based on previously published data (see introduction)). The exception to this was PF543 (100nM, in line with supramaximal SK1 catalytic inhibition (Schnute et al., 2012)). Pre-treatment of LNCaP AI cells with ABC294640 (French et al., 2010), RB-005 (Baek et al., 2013), F-02 (Byun et al., 2013), K145 (Liu et al., 2013), ROMe (Lim et al., 2011) and SKi (French et al., 2003) significantly reduced [^{3}H] thymidine incorporation into DNA, indicating that these compounds can inhibit proliferation of these cells (Figure 4.1). Although it appears that inhibiting both isoforms of SK is able to reduce DNA synthesis, SK2 inhibitors F-02 and ROME have a lesser effect than SK1 inhibitor RB-005, which is similar to inhibitors SKi and ABC294640 which affect both SK1 and SK2 (see chapter 3). In contrast, PF543 did not reduce DNA synthesis in LNCaP AI cells. This result is consistent with that of Schnute et al. (Schnute et al., 2012), who did not report any inhibition of proliferation in 1483 head and neck carcinoma cells (See discussion 3.3.1). The latter is also consistent with MTT experiments in which PF543 failed to affect LNCaP AI cell viability at concentrations below 3μM (Figure 3.5).

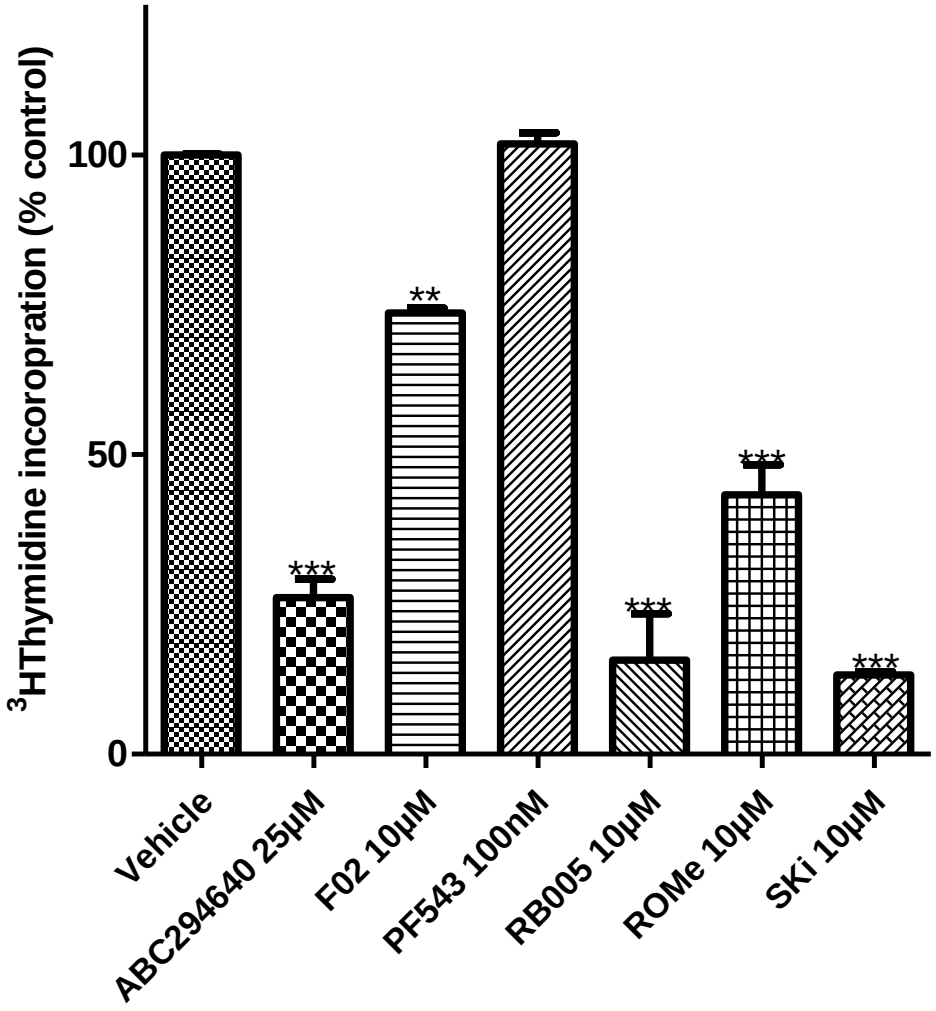


Figure 4.1 ***Effect of SK inhibitor treatment on DNA synthesis in LNCaP AI cells.*** *Histogram showing the effect of SK inhibitor treatment (24 hours) on DNA synthesis in LNCaP AI cells. LNCaP AI cells were treated with ABC294640 (25 μM), F02 (10 μM), PF543 (100 nM), RB005 (10 μM), ROMe (10 μM) or Ski (10 μM) for 24 hours prior to measuring the [^{3}H] Thymidine incorporation. Results are expressed as means +/− S.E.M for n = 3 experiment and were analysed by one way ANOVA. **p < 0.01, ***p < 0.001 versus vehicle-treated control.*

4.2.2 Some SK1 or SK2 selective inhibitors do not up regulate p53 expression in LNCaP AI cells.

Due to reduced DNA synthesis in SK inhibitor treated cells (Figure 4.1), the potential involvement of senescence/growth arrest was investigated. Indeed, SKi has previously been reported to increase p53 expression, which can be associated with growth arrest, in LNCaP AI cells (Tonelli et al., 2013). However, it was not established whether this was due to its effect on SK1 and/or SK2 or another mechanism. Therefore, the previously characterised panel of inhibitors were tested for their ability to induce an increase in p53 expression in LNCaP AI cells. 48 hour treatment time was selected in these experiments to achieve maximal p53 induction and in line with previous studies which showed that 48 hour treatment was optimal for p53 induction by SKi (Tonelli et al., 2012).

As expected, treatment of LNCaP AI cells with SKi induced the expression of p53, which confirms the observation of Tonelli et al (2012). Interestingly, of the inhibitors tested, only SKi induced an increase in p53 protein expression (Figure 4.2). This suggests that sole inhibition of SK1 or SK2 is not responsible for the p53 increase by SKi, as it is not recapitulated by SK1 selective PF543 or RB-005 or SK2 selective ROMe. This raises the possibility that inhibition of both SK isoforms together is required for induction of p53.

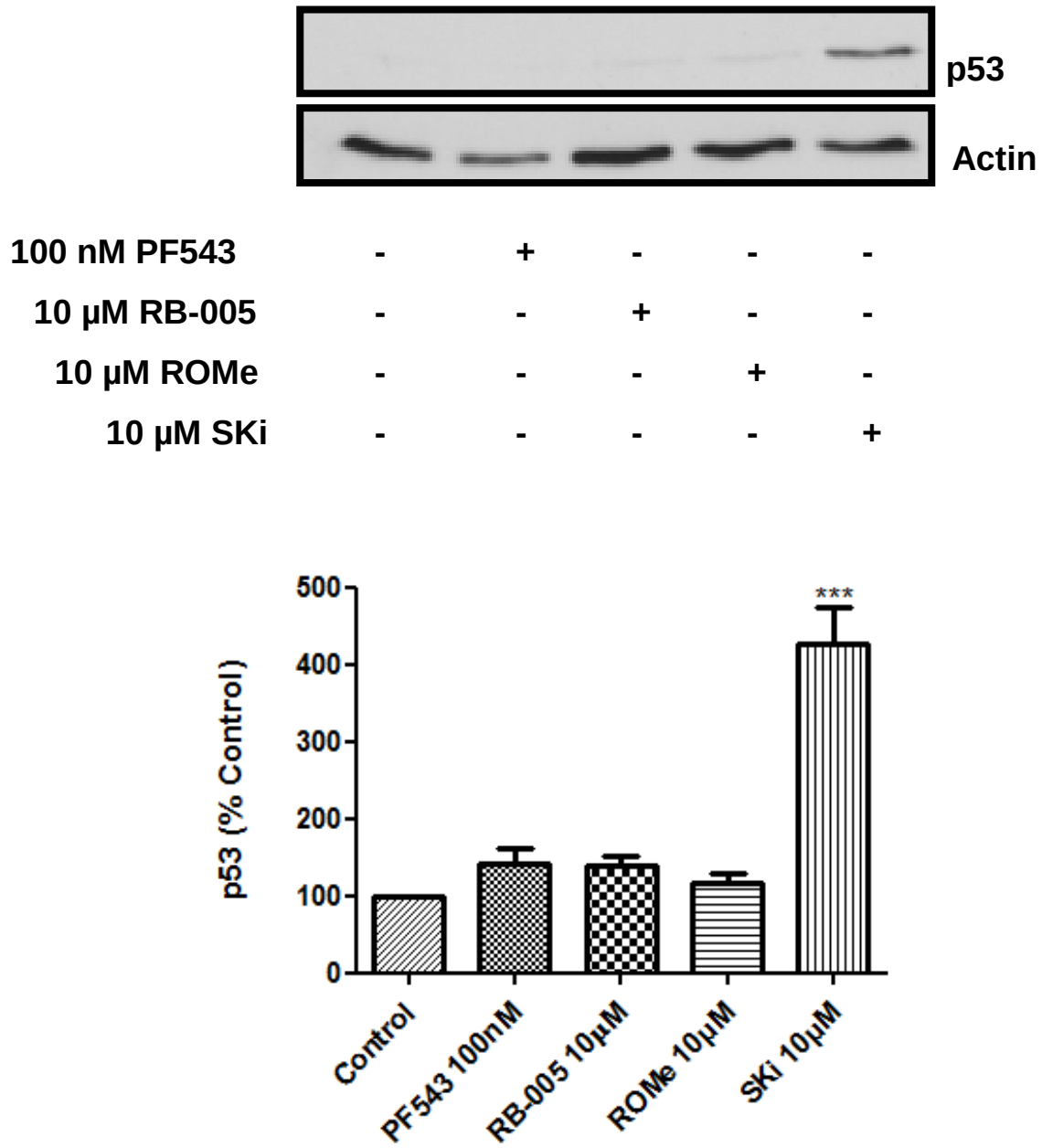


Figure 4.2 ***Effect of SK inhibitors on p53 protein levels in LNCaP AI cells.*** *Western blot showing the effect of SK inhibitors on the expression of p53 in LNCaP AI cells. Cells were treated with PF543 (100 nM), RB-005 (10 µM), ROMe (10 µM) or SKi (10 µM) for 48 hours and relative p53 protein expression measured by Western blotting. Also shown is a bar graph of the quantification of the effect of SK inhibitors on p53 protein levels, normalised to actin. Results are expressed as means +/− S.E.M for n = 3 experiments and analysed by one-way ANOVA. ***p < 0.001 versus DMSO control.*

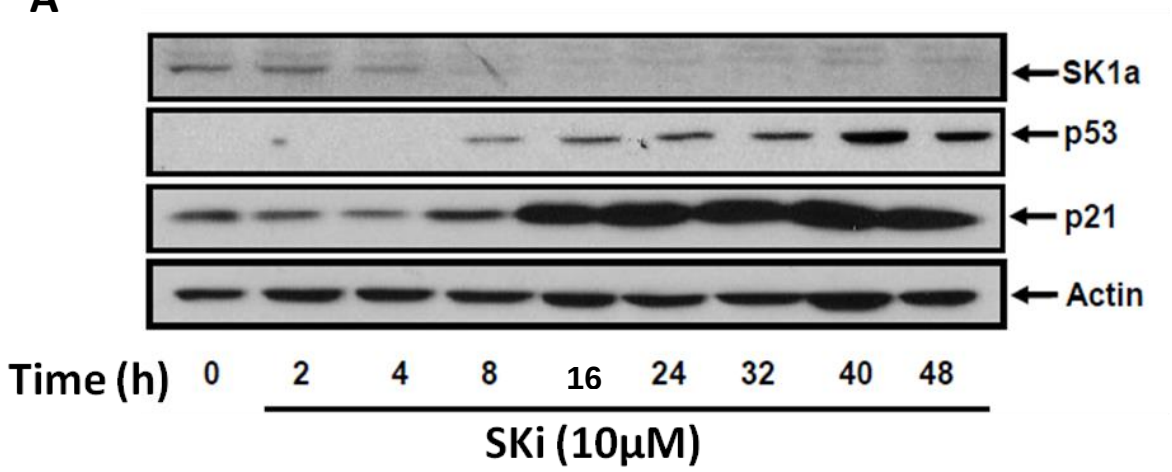
4.2.3 ABC294640 and SKi induce senescence in LNCaP AI cells.

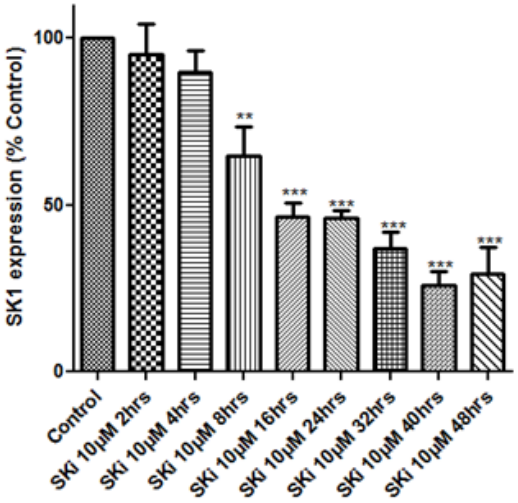
Because of the multi faceted nature of p53 actions (as a regulator of apoptosis, senescence and autophagy), additional markers are required to establish the specific phenotype and biochemical processes set in train by the treatment with SKi. As previously mentioned, p21 is a cyclin dependent kinase (CDK) inhibitor which promotes growth arrest in response to many external stimuli. It is also a tumour suppressor protein and a marker which is upregulated only in senescence (Abbas and Dutta, 2009). Because of the previously observed similarities between ABC294640 and SKi (see chapter 3), ABC294640 was similarly tested for its ability to induce senescence

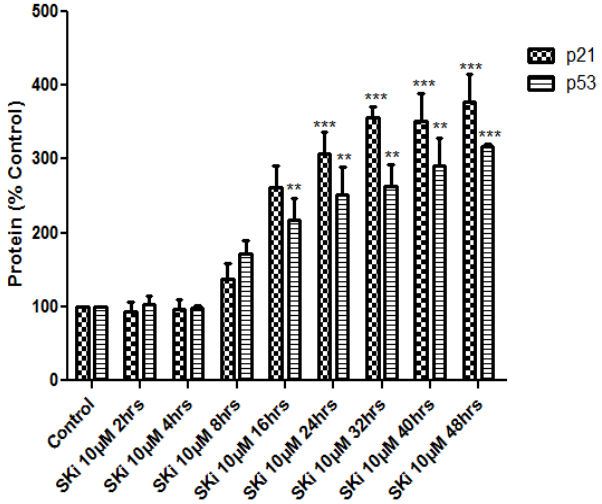
Figure 4.3 illustrates the time course of the (A) SKi and (B) ABC294640 induced increase in p53 and p21 expression, which is accompanied by SK1 removal. This data shows for the first time that ABC294640 also increases p53 protein. The induction of p21 together with p53 suggests that these two SK inhibitors are inducing senescence of LNCaP AI cells. Down regulation of SK1 is apparent at 4 hours and is significant at 8 hours for both compounds. This suggests that this precedes p21 induction. The onset of p21 the increase is significant at at 8 hours for SKi and at 16 hours for ABC294640. In addition, the extent of p21 expression is greater for SKi-treated cells than for those treated with ABC294640. p53 follows a similar pattern to p21 and is significantly increased at 16 hours for SKi treated cells but only significant at 32 hours in ABC294640 treated cells.

Overall, these findings indicate that inhibition of SK2 activity and down-regulation of SK1 expression (which therefore indirectly reduces SK1 activity) by ABC294640 or dual inhibition of SK1 (and its proteasomal removal) and SK2 by SKi are associated with the induction of p53 and p21 expression and senescence. This could be contingent upon down regulation of SK1 protein as it precedes the onset of p53 and p21 induction.

A


SK1a
p53
p21
Actin
Time (h) 0 2 4 8 16 24 32 40 48
SKi (10μM)


SK1 expression (% Control)
100
50
0
Control
SKi 10μM 2hrs
SKi 10μM 4hrs
SKi 10μM 8hrs
SKi 10μM 16hrs
SKi 10μM 24hrs
SKi 10μM 32hrs
SKi 10μM 40hrs
SKi 10μM 48hrs


Protein (% Control)
500
400
300
200
100
0
p21
p53
Control
SKi 10μM 2hrs
SKi 10μM 4hrs
SKi 10μM 8hrs
SKi 10μM 16hrs
SKi 10μM 24hrs
SKi 10μM 32hrs
SKi 10μM 40hrs
SKi 10μM 48hrs

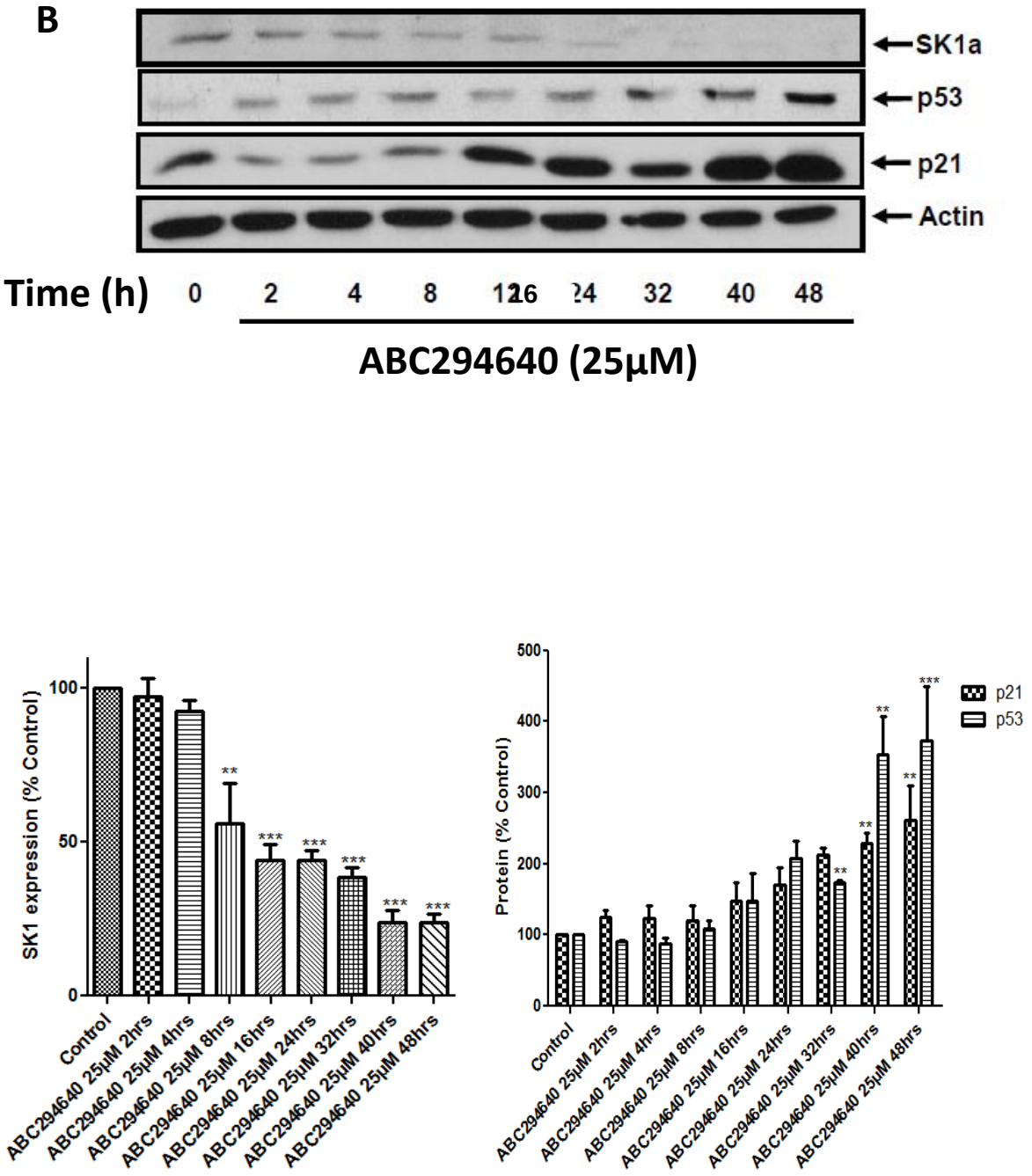


Figure 4.3 ***Effect of ABC294640 and SKi on p53, p21 and SK1 protein levels in LNCaP AI cells.*** *Cells were treated with (A) SKi (10µM) or (B) ABC294640 (25 µM) at time points indicated or with the vehicle alone (DMSO 0.1% v/v). Protein levels determined using SDS PAGE and Western blotting. Actin was used to verify protein loading. Quantification is*

*shown below. Results are expressed as means relative to actin +/− S.E.M for n = 3 experiments, and analysed by one-way ANOVA. **p<0.01, ***p < 0.001 versus control.*

4.2.4 ABC294640 and SKi inhibit DNA synthesis in LNCaP AI cells.

Due to the measured increase in p21 and p53 by ABC294640 and SKi, further evidence that these compounds can induce growth arrest was sought. One approach was to further characterise their effect on the inhibition of cell proliferation. The concentration dependence for inhibition of [^{3}H]-thymidine uptake into newly synthesised DNA was established for both (A) ABC294640 and (B) SKi. As expected, the IC_{50} for SKi was lower than for ABC294640, 1.29µM and 19.3µM, respectively (Figure 4.4). This is consistent with findings from western blotting experiments in which it appears that ABC294640 can induce a milder phenotype (marker induction/degradation) than that induced by SKi, at concentrations which correlate to the IC_{50} for SK inhibition (French et al., 2003) and/or degradation for each compound (see chapter 3).

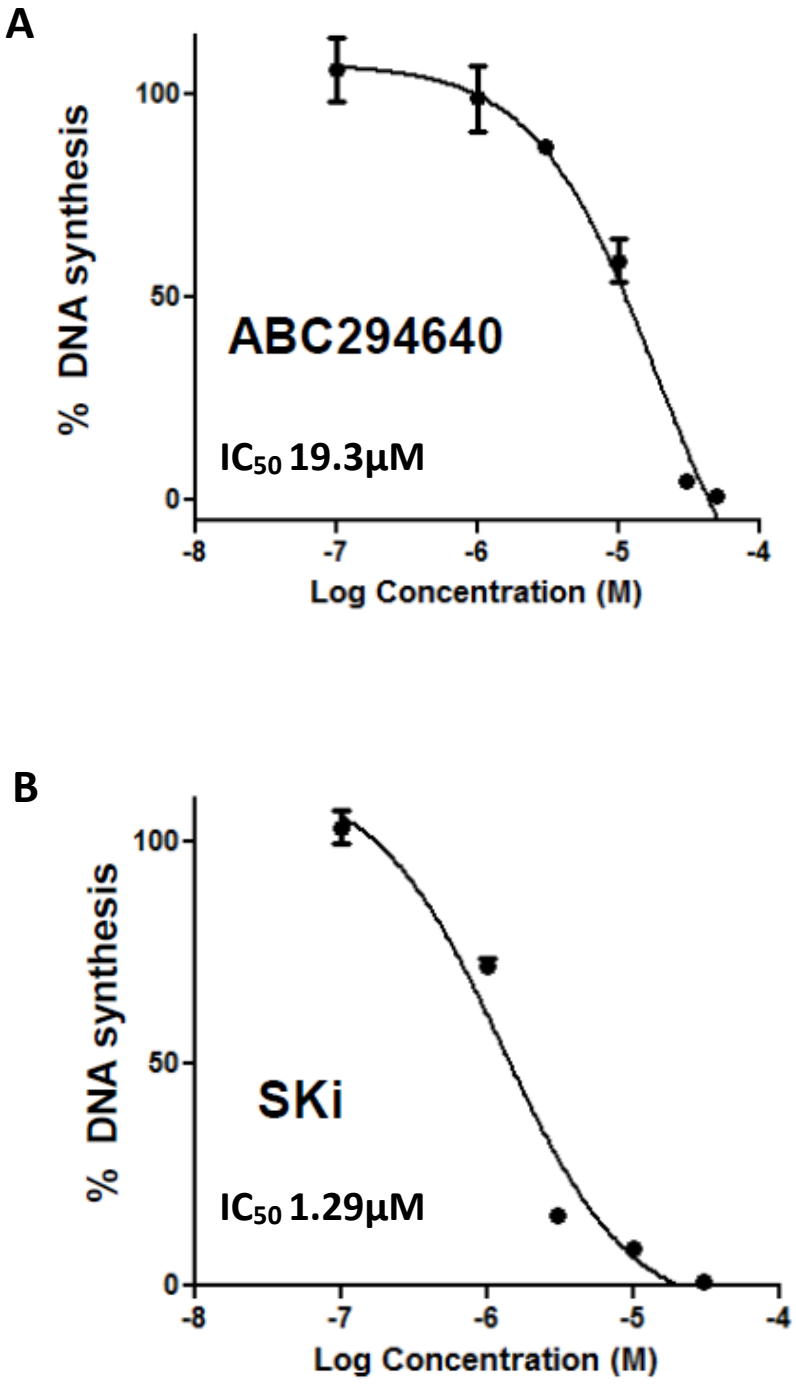


Figure 4.4 ***Effect of ABC294640 and SKi treatment on DNA synthesis in LNCaP AI cells.*** *The effect of (A) ABC294640 (24 hours) or (B) SKi (24 hours) on DNA synthesis in LNCaP AI cells with IC_{50} values indicated. Results are expressed as means +/− S.E.M for n = 3 experiments.*

4.2.5 Cell cycle analysis of ABC294640 and SKi treatment in LNCaP AI cells.

To establish the cell cycle stage at which ABC294640 and SKi induce growth arrest, cell cycle analysis using flow cytometry was conducted using propidium iodide (PI). PI intercalates into the major binding groove of DNA and emits a highly fluorescent signal. The amount of fluorescent signal generated by any given cell is directly proportional to the amount of DNA in the cell at a given time, which is in turn indicative of the phase of the cell cycle that the cell is currently in (G0/G1, S or G2/M) (Krishan, 1975).

As expected, given the effect of the compounds on inhibition of DNA synthesis and the increase in p53 and p21, both ABC294640 (25μM) and SKi (10μM) caused a shift in cell cycle stage versus vehicle treated cells. Both ABC294640 and SKi induced growth arrest at G1/0 phase with a decrease in the proportion of cells in G2/M and S phase (Figure 4.5).

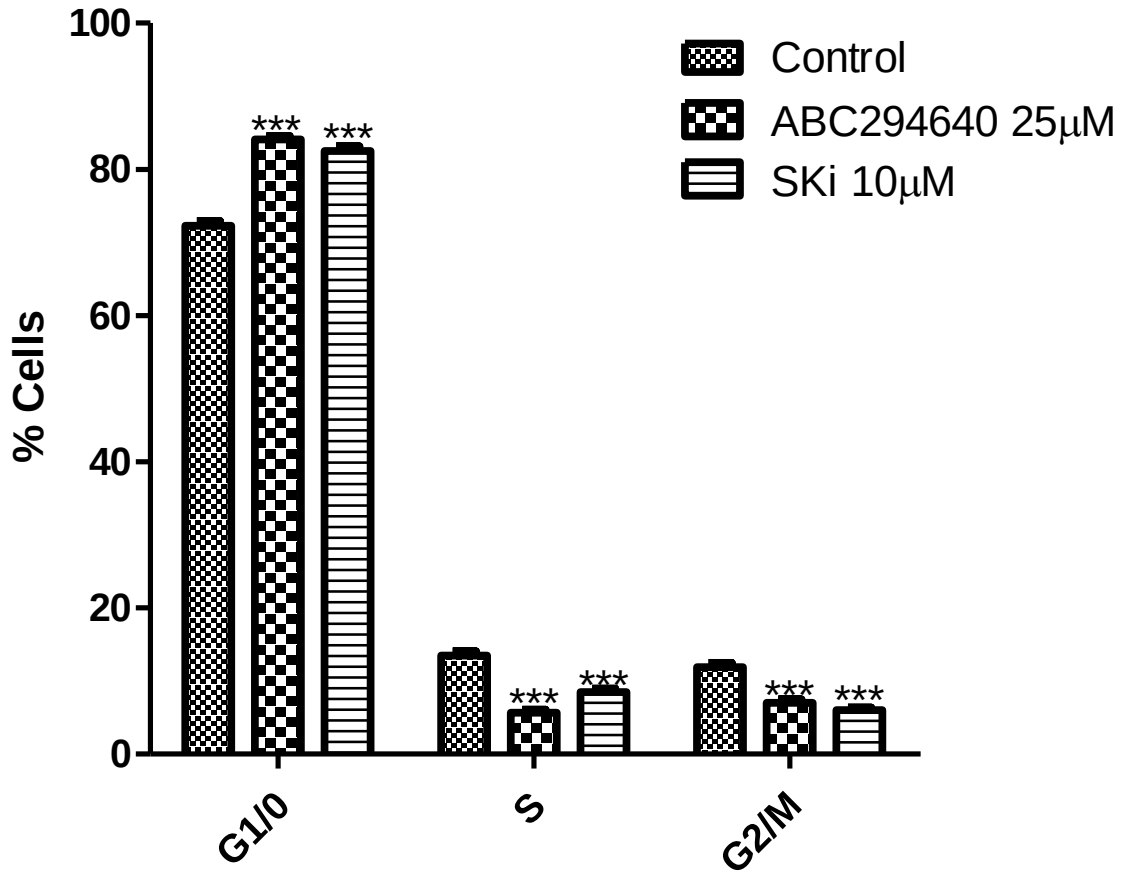


Figure 4.5 ***FACS analysis of LNCaP AI cells treated with ABC294640 or SKi.*** Cells were *treated with ABC294640 (25μM) or SKi (10μM) for 24 hours and cell cycle analysis was carried out using FACS analysis after DNA staining with PI. Data is expressed as means +/- S.E.M. and represents combined data from n=3 experiments using means generated from triplicate samples. Results were subjected to one-way ANOVA analysis. ***p < 0.001 versus control.*

4.2.6 ABC294640 and SKi up regulate p53 and p21 in an oxidative stress dependant manner in LNCaP AI cells.

It has been established that treatment with SKi, which inhibits both SK1 and SK2, resulted in increased p53 expression and that these effects were not recapitulated by sole SK1 or SK2 inhibition (figure 4.2). It has also been established that ABC294640 removes SK1 protein from cells via proteasomal degradation (see chapter 3). Therefore, evidence has been presented to demonstrate that ABC294640 dually affects both SK1 and SK2 at 24 hours treatment and also increases p53 and p21 protein (figure 4.3)

Metabolomic analysis has previously revealed that treatment of LNCaP AI cells with SKi results in an oxidative stress response (Watson et al., 2013). This was characterised by cells diverting glucose 6-phosphate into the pentose phosphate pathway to provide NADPH, which serves as an antioxidant to counter an oxidative stress response (Watson et al., 2013). Furthermore, it has been demonstrated that SKi treatment results in down regulation of AR protein and this is reversed by anti-oxidant scavenger molecule N-acetyl cysteine (NAC) (Tonelli et al., 2013). Thus, it was of interest to determine whether the up-regulation of p53 and p21 by SKi and ABC294640 was similarly able to be reversed using the anti-oxidant scavenger NAC. Indeed, a full reversal of the increase in p53 and p21 induced by ABC294640 and SKi was observed when cells were pre-treated with NAC (30 minute pre-treatment, optimised from previous experiments (data not shown)). Treatment with NAC alone was without effect on p53 or p21 expression. This suggests that ROS generation in response to treatment with these compounds is required for the increase in p53 and p21 expression induced.

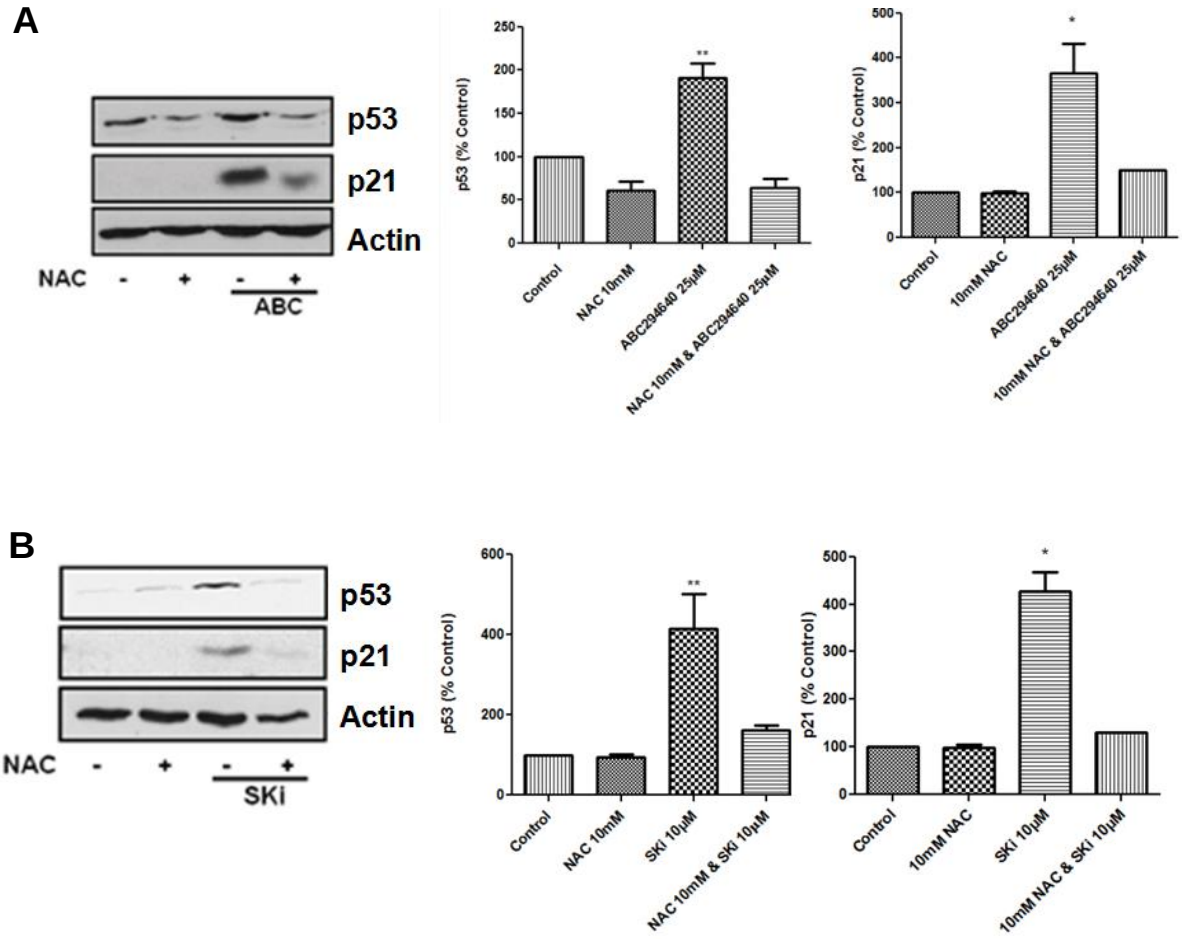


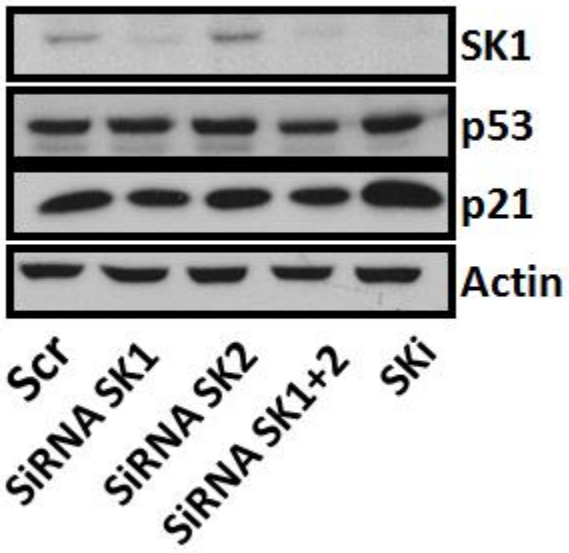
Figure 4.6 ***Effect of ABC294640 and SKi on p53 and p21 protein levels: reversal by NAC.*** *Cells were pre-treated with NAC (10mM, 30 minutes) before addition of (A) ABC294640 or (B) SKi or vehicle (DMSO 0.1% v/v) (48 hours). Also shown are bar graphs of the quantification of the effect of ABC294640 and SKi on p53 and p21 protein, relative to actin. Results are expressed as mean +/- S.E.M for n=3 experiments and analysed by one way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.*

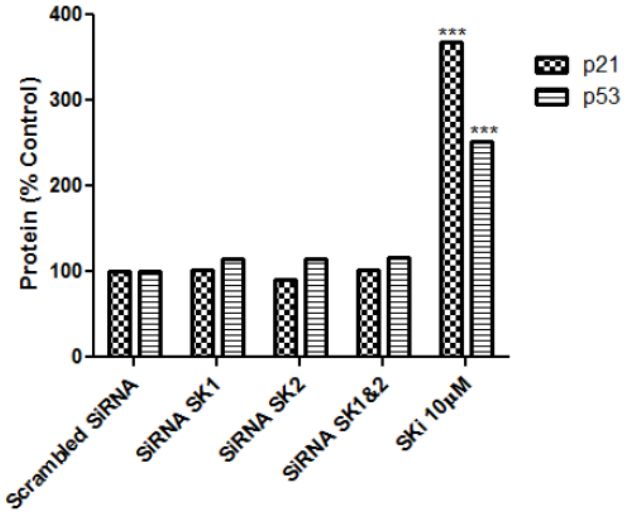
4.2.7 siRNA knockdown of SK1/SK2 does not induce senescence in LNCaP AI cells.

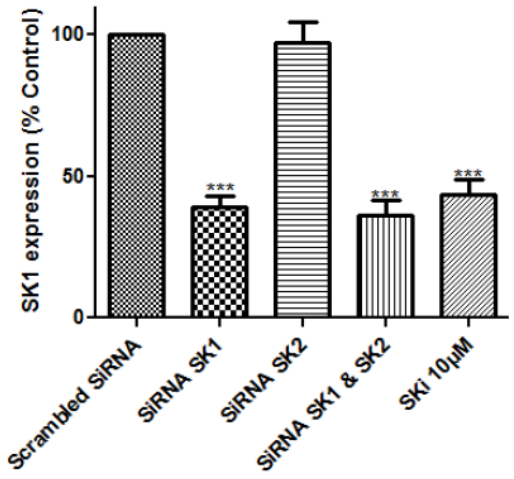
To affirm pharmacological data showing that targeting both SK1 and SK2 together induces a senescent phenotype a molecular approach was taken to investigate the induction of senescence by the removal of either SK1 or SK2 or both SK isoforms simultaneously. LNCaP AI cells were either treated using SK1 siRNA, SK2 siRNA or a combination of both SK1 and SK2 siRNA. Each treatment was assessed for (A) the ability to induce p53 and p21 protein expression and (B) the ability to inhibit DNA synthesis of LNCaP AI cells. These experiments were limited by the inability to quantify SK2 expression by western blotting due to the absence of a SK2 antibody of sufficient selectivity and affinity to detect endogenous levels of SK2.

Interestingly, whilst siRNA knockdown of SK2 was unable to reduce DNA synthesis in LNCaP AI cells, siRNA knockdown of either SK1 or SK1 and SK2 together was effective, as evidenced by measurement of ^{3}H thymidine uptake into newly synthesised DNA (Figure 4.7B). Surprisingly, an individual role for SK1 or SK2 in regulating p53/p21 expression was excluded based on the finding that siRNA knockdown of either of these kinases or both together had no effect on p53 or p21 expression. These findings suggest that the effects of ABC294640 or SKi on p53 and p21 expression and the induction of senescence involve a mechanism that does not require SK1 or SK2 inhibition or their down-regulation.

A


SK1
p53
p21
Actin
Scr
SiRNA SK1
SiRNA SK2
SiRNA SK1+2
SKi


Protein (% Control)
400
300
200
100
0
***
***
p21
p53
Scrambled SiRNA
SiRNA SK1
SiRNA SK2
SiRNA SK1&2
SKi 10μM


SK1 expression (% Control)
100
50
0
***
***
***
Scrambled SiRNA
SiRNA SK1
SiRNA SK2
SiRNA SK1 & SK2
SKi 10μM

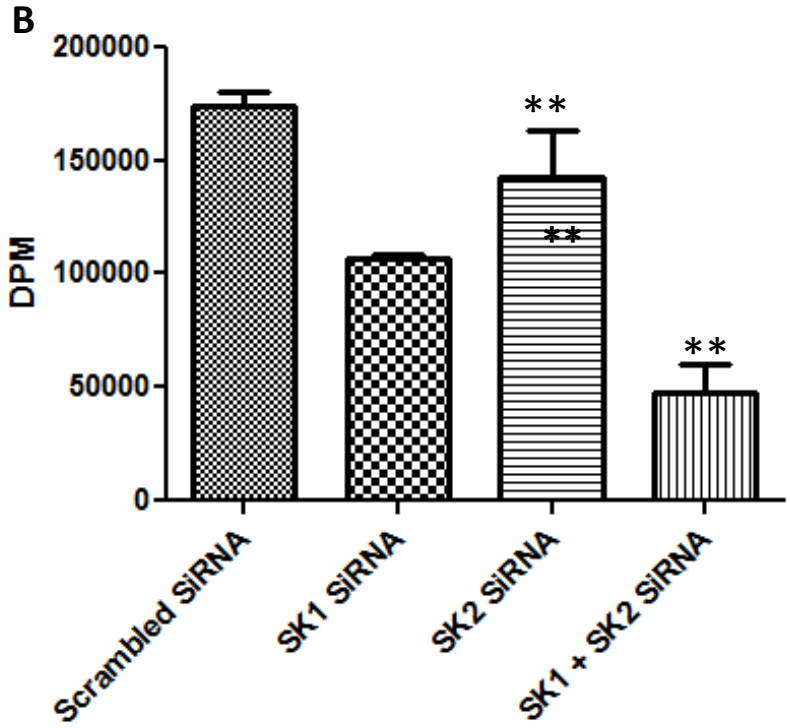


Figure 4.7 ***Effect of siRNA targeting SK1 or SK2 on SK1, p53 and p21 protein levels and on DNA synthesis in LNCaP AI cells.*** *Cells were treated with siRNA specific for SK1, SK2 or a combination of both SK1 and SK2 or with scrambled siRNA (48hrs) as described in the methods section. (A) Cell lysates were analysed using SDS PAGE and Western blotting for*

*SK1, p53 and p21. Actin was used to verify similar protein loading. Also shown is the quantification of the effect of the siRNA treatment indicated on SK1, p53 or p21 protein. (B) Cells were assayed for DNA synthesis measured as [^{3}H]-thymidine uptake into newly synthesised DNA. Results are expressed as means +/− SEM for n = 3 experiments and analysed by one-way ANOVA **p<0.01, ***p < 0.001 versus control.*

4.2.8 Anti-oxidant NAC does not reverse the degradation of SK1 by ABC294640 or SKi in LNCaP AI cells.

In light of the finding that the up-regulation of p53 and p21 by ABC294640 and SKi is reversed using NAC (Figure 4.6), the requirement for ROS production was similarly tested in relation to the degradation of SK1 protein by ABC294640 and SKi.

Treatment of LNCaP AI cells with NAC alone was without effect on SK1 expression. Moreover, in contrast to the results observed for p53 and p21, pre-treatment of LNCaP AI cells with NAC was unable to reverse the SK1 degradation induced by ABC294640 or SKi (Figure 4.8). This data suggests that either ROS generation in response to SKi or ABC294640 is downstream from SK1/SK2 inhibition and SK1 degradation and that p53 (and thereby p21) up-regulation and SK1 degradation exist on separate signalling pathways. In addition, this is consistent with the siRNA data showing that SK1 expression is not directly related to p53 and p21 expression (Figure 4.7)

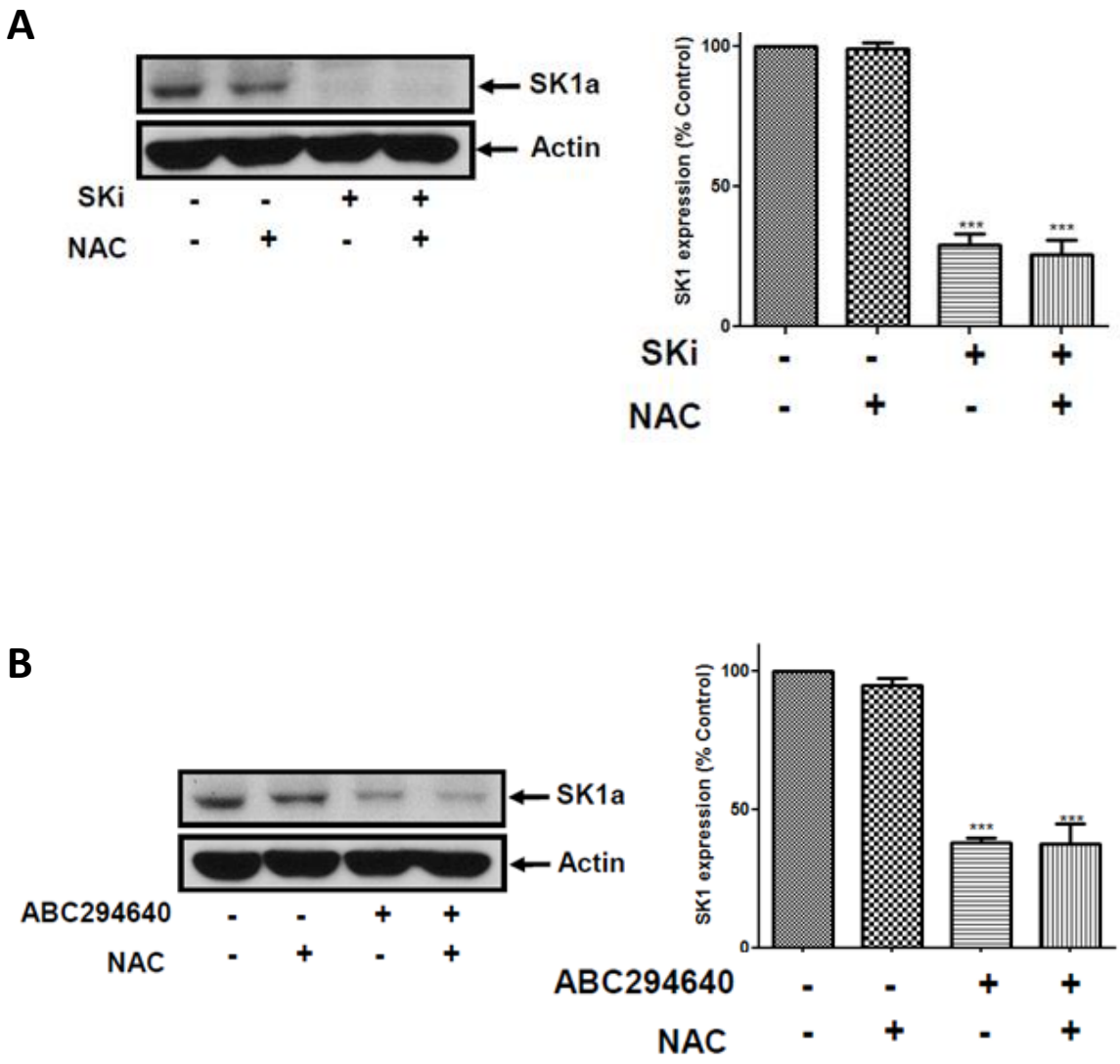


Figure 4.8 ***Effect of NAC on the degradation of SK1 by ABC294640 and SKi.*** *Cells were pre-treated with NAC (NAC 10mM, 30 minutes) before addition of (A) SKi (10μM) or (B) ABC294640 (25μM) or vehicle (DMSO 0.1% v/v)(48 hours). Also shown is a bar graph of the quantification of the effect of NAC on the degradation of SK1 by ABC294640 and SKi, normalised to actin protein levels. Results are representative of at least three separate experiments are expressed as means +/− S.E.M and analysed by one-way ANOVA versus control. ***p < 0.001.*

4.2.9 Alternative antioxidants do not reverse the degradation of SK1 by ABC294640 or SKi in LNCaP AI cells.

To support findings using NAC (Figure 4.8), alternative antioxidant compounds were tested for their ability to reverse SK1 degradation by ABC294640 and SKi.

To this end, two alternative compounds - glutathione (GSH) and 4-hydroxy-Tempo (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) (TEMPO) were selected. GSH is the main endogenous anti-oxidant present in mammalian cells. The thiol group acts as a reducing agent to unstable molecules such as ROS, thereby protecting the cell from oxidative stress (Nikolova-Karakashian et al., 2011). 4-Hydroxy-Tempo detoxifies ROS by catalysing the neutralisation of superoxide, facilitating hydrogen peroxide metabolism (Wilcox and Pearlman, 2008).

As shown in Figure 4.5, 30 minutes pre-treatment of LNCaP AI cells with GSH (10mM) (Sultan et al., 2006) or TEMPO (10-1000μM) was unable to reverse the SK1 degradation induced by ABC294640 or SKi. This is consistent with previous observations made using NAC (Figure 4.9), thereby confirming that SK1 degradation by ABC294640 and SKi is independent of ROS.

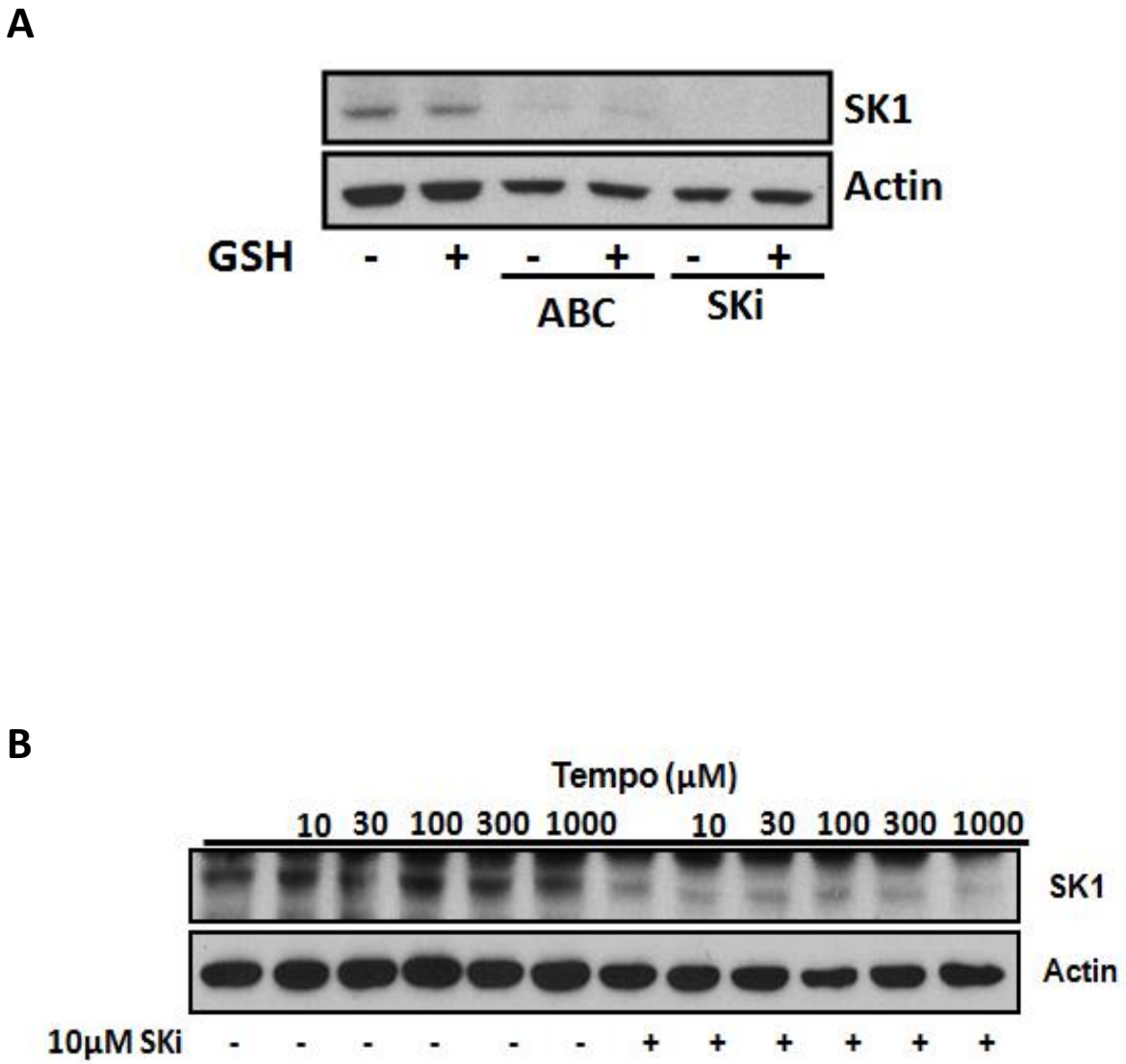


Figure 4.9 ***Effect of GSH and Tempo on the degradation of SK1 by ABC294640 and SKi.*** *Cells were pre-treated with (A) GSH (10mM, 30 mins) or (B) Tempo (30 mins, concentrations as indicated) or vehicle (DMSO 0.1% v/v) before addition of ABC294640 (25μM) or SKi (10μM) as indicated. Samples were then analysed for SK1 protein levels by western blotting and protein levels were verified using actin. Results are representative of at least three separate experiments.*

4.2.10 p53 localises to the nucleus in response to ABC294640 and SKi in LNCaP AI cells.

p53 function is subject to tight regulation by transcription and translation, protein modifications, protein stability and finally by sub-cellular localisation (Brate and Giannakakou, 2003). p53 contains three nuclear localisation signals (NLS) and two nuclear export signals (NES). In the nucleus, p53 is most stable, transcriptionally active, and protected from degradation by the proteasome. This occurs via p53 tetramerisation which results in shielding of its NES (El-Deiry et al., 1992). p53 is most commonly sequestered in the nucleus in response to DNA damage, where it activates transcription of DNA damage response genes. MDM2 is the most important negative regulator of this process, which, as previously mentioned (see introduction), binds to p53 and poly-ubiquitinates it, before its movement to the proteasome for degradation (Boyd et al., 2000).

It has been established by Western blotting that treatment of LNCaP AI cells with ABC294640 or SKi results in p53 accumulation. It was therefore of interest to determine whether the increase in abundance of p53 was accompanied by its cellular re-distribution, particularly in light of the knowledge that ABC294640 and SKi are proteasomal activators. Indeed, the p53 upregulation in response to these compounds is paradoxical, as it is known that p53 is subject to constant turnover by the ubiquitin proteasome.

The subcellular distribution of p53 in vehicle treated cells was cytoplasmic with very little nuclear distribution (Figure 4.10). Interestingly, p53 was shown to localise to the nucleus in response to treatment with either (A) ABC294640 (25μM, 14hrs) or (B) SKi (10μM, 14hrs) (Figure 4.10). This localisation, which enhances p53 stability, could account for the increase in p53 and be a mechanism of p53 evading the proteasome in response to these compounds.

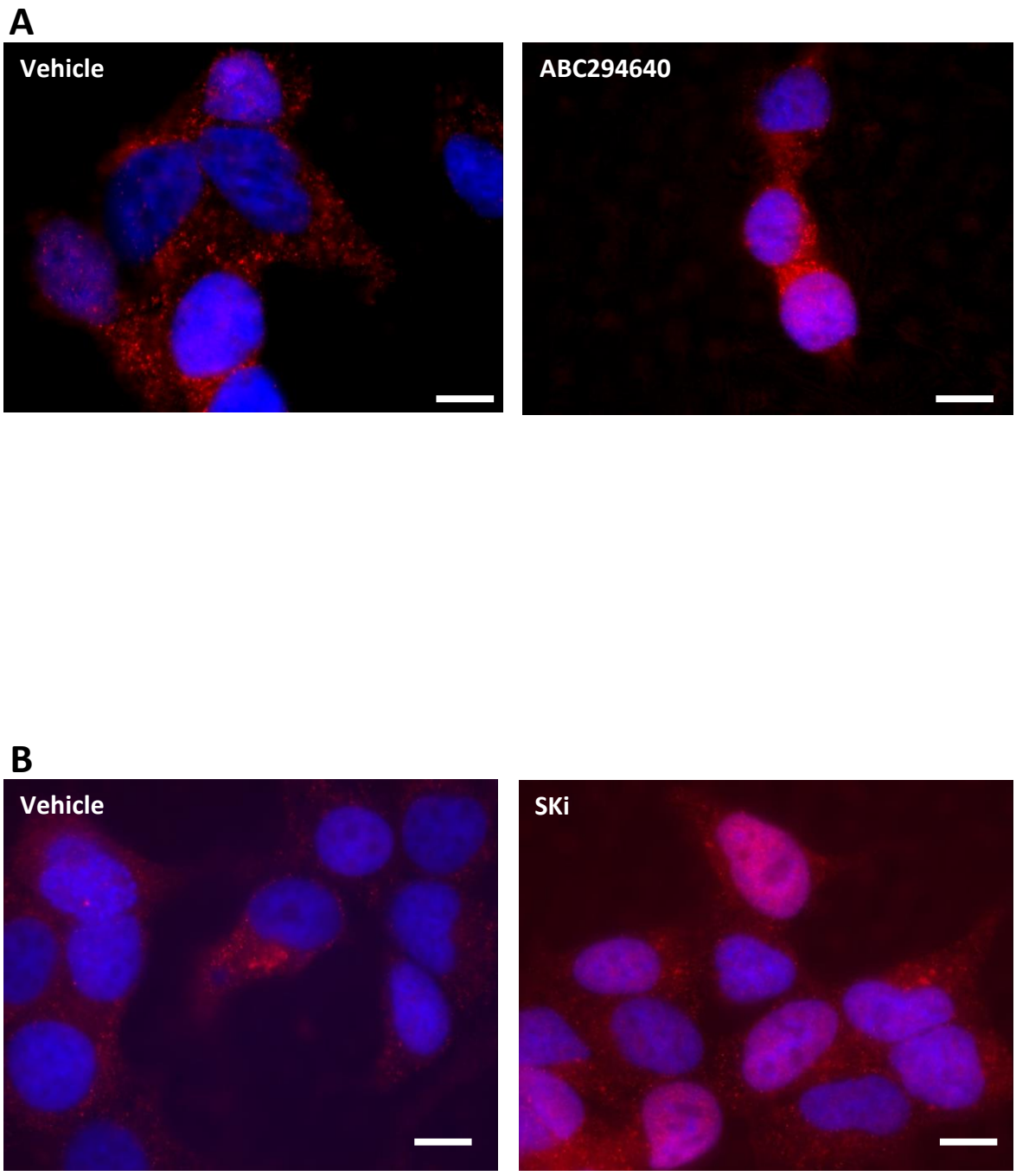


Figure 4.10 ***Immunofluorescence microscopy images showing the effect of ABC294640 and SKi on p53 localisation in LNCaP AI cells.*** *Cells were treated with (A) ABC294640 (25µM, 14hrs), (B) SKi (25µM, 14hrs) or vehicle (DMSO 0.1% v/v) as indicated and processed for immunofluorescence using anti-p53 antibody and FITC secondary antibody*

(red signal) and nuclear stain DAPI (blue signal). Results are representative of data from multiple fields and samples from three separate experiments.

4.2.11 The effect of nutlin on senescence of LNCaP AI cells.

The E3 ligase MDM2 catalyses ubiquitination of p53 and thereby regulates p53 stability at the proteasome. MDM2 has a structural RING domain which is known to play a key role in protein ubiquitination. A RING domain is also found in TRAF2 which has previously been shown to be activated by S1P (Alvarez et al., 2007). Thus, if S1P is able to similarly activate MDM2 at its RING domain it is possible that dual SK1/SK2 inhibition leads to very low intracellular S1P levels which in turn leads to low MDM2 activity and thus less p53 degradation, thereby increasing p21 expression.

In order to establish whether the phenotype induced by ABC294640 and SKi was recapitulated by MDM2 inhibition, a small molecule inhibitor of the p53 and MDM2 interaction, nutlin, was used. Nutlin is currently in phase 1 clinical trials for the treatment of retinoblastoma due to its ability to induce the non-genotoxic stabilisation of p53 (Secchiero et al., 2011). Nutlin was tested for its ability to inhibit DNA synthesis (measured by [^{3}H] thymidine uptake), up-regulate p53/p21 protein and finally for its ability to translocate p53 to the nucleus. Indeed, the senescent phenotype induced by ABC294640 and SKi was fully recapitulated by nutlin in a concentration dependant manner (Figure 4.11). Nutlin treatment resulted in (A) an accumulation of p53 and p21, (B) induced growth arrest and (C) resulted in nuclear redistribution of p53 (Figure 4.11). This suggests that MDM2 could be indicated in the mechanism of SKi and ABC294640 action. However, more experiments are needed to confirm this, for example investigating the effect of MDM2 siRNA or by transfecting cells with an MDM2 construct. Several attempts were made to transfect LNCaP AI cells with MDM2 however these were unsuccessful (data not shown).

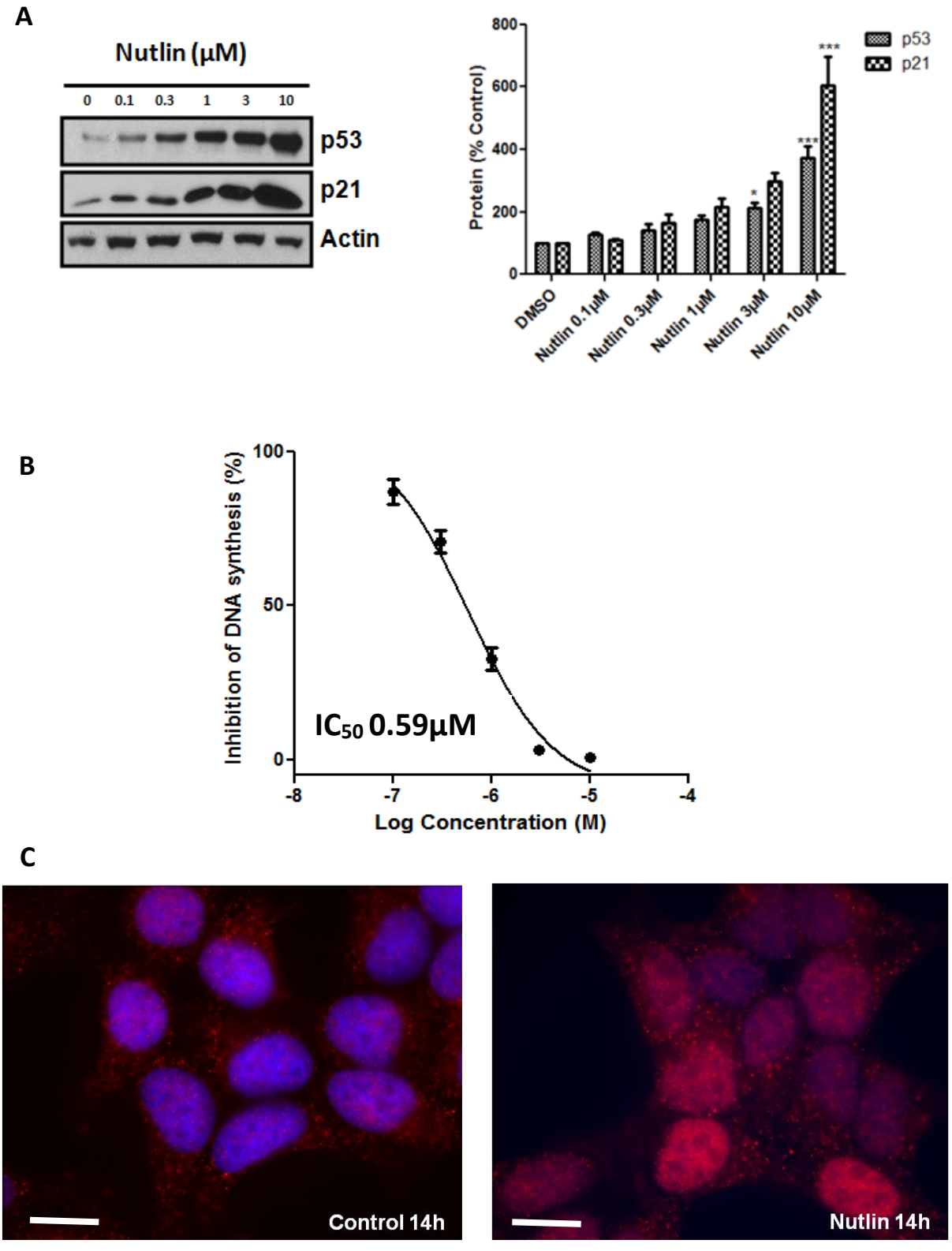


Figure 4.11 ***Effect of nutlin in LNCaP AI cells.*** *Cells were treated with nutlin (24 hours) and (A) p53 and p21 levels determined by western blot. Also shown is quantification of the effect expressed as mean +/- S.E.M, n=3 experiments, normalised to actin. (B) DNA synthesis was measured as descrbed previously, n=3 (C) p53 localisation was measured by immunofluorescence microscopy. Blue signal is DAPI and red signal is p53.*

4.2.12 The role of protein synthesis in regulating p53 expression in LNCaP AI cells.

To assess whether ABC294640 and SKi up-regulate p53 by modulating TP53 gene expression, cells were pre-treated with cycloheximide (CHX), an inhibitor of protein translation (Mastrandrea et al.,2005), prior to the addition of ABC294640 or SKi. Cyclohexemide blocked the ABC294640 or SKi-induced up-regulation of p53 expression in LNCaP AI cells (Figure 4.12). This data suggests that the effect of ABC294640 and SKi is at least partially mediated by increased p53 protein synthesis. It is important to consider that because p53 is constantly subject to rapid turnover under basal conditions it is possible that treatment of LNCaP AI cells using CHX could rapidly deplete p53 entirely from these cells if it were not replaced. However, CHX treatment did not decrease basal p53 (figure 4.12). These findings therefore suggests that ABC294640 and SKi increase p53 protein transcription and synthesis.

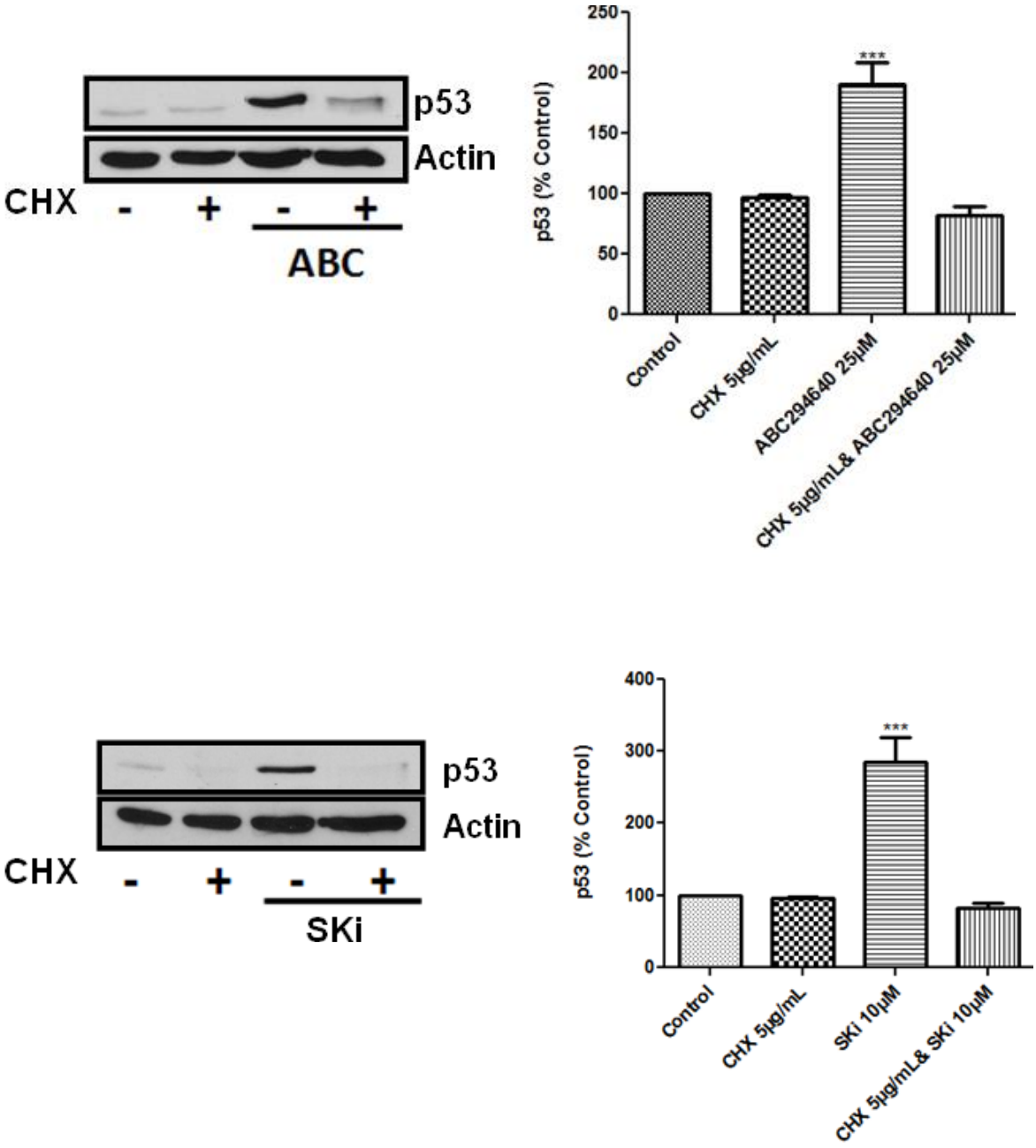


Figure 4.12 ***Effect of CHX on p53 in response to ABC294640 and SKi.*** *Cells were pre-treated with CHX (5µg/mL, 30 minutes) before (A) ABC294640 (25µM) or (B) SKi (10µM) or vehicle (DMSO 0.1% v/v). Results are expressed as means +/− S.E.M for n = 3 experiments and analysed by one-way ANOVA ***p < 0.001 versus control.*

4.2.13 The role of autophagy in regulating senescence of LNCaP AI cells.

There are links between autophagy and both ABC294640 and SKi in the literature. For example, has been shown by several groups that ABC294640 inhibits autophagy (Belijanski et al., 2010; Xun et al., 2015; Ding et al., 2016). On the contrary, it has previously been shown that SKi stimulates autophagy (Tonelli et al., 2013). Because of the conflicting reports regarding the relationship between ABC294640, SKi and autophagy, it was of interest to determine whether an autophagic effect of these compounds was linked to the induction of senescence observed upon ABC294640 or SKi treatment. To investigate this, several pharmacological compounds that affect autophagy were used. These included inhibitors of autophagy, baflomycin and chloroquine, and an inducer of autophagy, rapamycin. These modulators of autophagy were tested for their ability to (i) alter SK1 protein levels and (ii) induce senescence (assessed using the senescent marker protein p21).

It appears that inhibition of autophagy rather than its stimulation is linked to the up-regulation of p21 protein: inhibitors baflomycin and chloroquine caused an increase in p21 whilst rapamycin had no effect (figure 4.13). However no clear relationship was observed between autophagy and the degradation of SK1 protein in LNCaP AI cells, as both inhibitors of autophagy yielded opposite effects on SK1 protein levels. Furthermore, the autophagy stimulator rapamycin reduced SK1 expression in a similar fashion as inhibitor baflomycin (Figure 4.13). Thus, it is unlikely that autophagy is implicated in the induction of senescence in LNCaP AI cells.

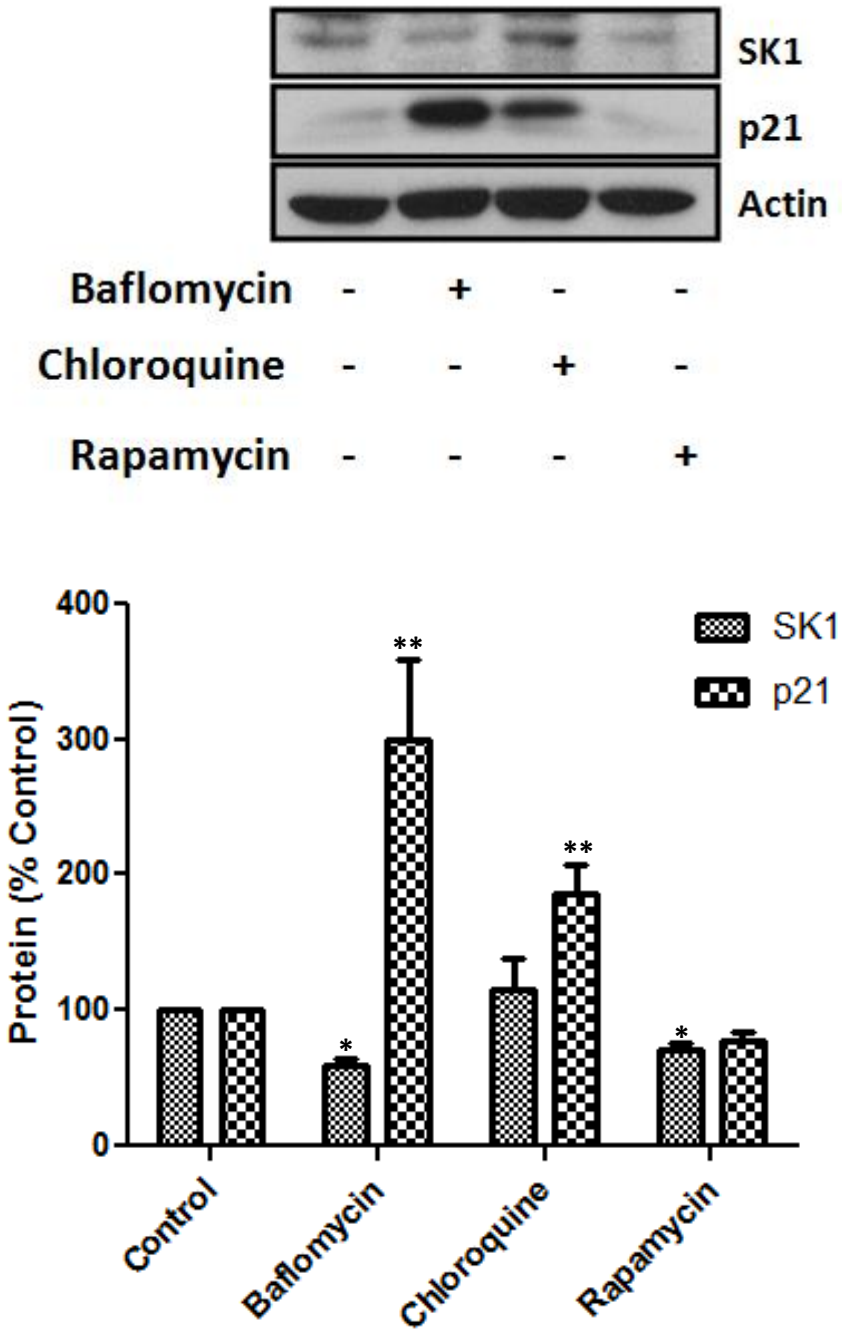


Figure 4.13 ***Effect of autophagy mediators on p21 and SK1 protein levels in LNCaP AI cells.*** *Cells were treated with baflomycin (0.5 nM, 24 hours), chloroquine (15 μM, 24 hours), rapamycin (100 nM, 24 hours) or vehicle alone (DMSO 0.1% v/v) as indicated. Cell lysates were collected and protein levels determined using SDS PAGE and Western blotting. Blots were stripped and re-probed for actin to verify similar protein loading. Also shown are bar graphs of the quantification of the effect of compounds on p21 and SK1 protein levels. Results are expressed as means +/− S.E.M for n = 3 experiments, and analysed by one-way ANOVA *p < 0.05, **p<0.01 versus control.*

4.2.14 Dual SK1/SK2 inhibition does not induce senescence in LNCaP AI cells.

In order to affirm the finding from siRNA experiments that dually targetting SK1 and SK2 does not induce senescence, a combination of SK1 and SK2 selective inhibitors were tested. The SK1 selective inhibitor PF543 (100nM) was used in combination with SK2 selective inhibitor ROMe (10μM) in order to determine the effects of dual SK1 and SK2 inhibition on p53 protein levels.

Interestingly, pharmacologically targeting both SK1 and SK2 using a combination of inhibitors did not recapitulate the effects of ABC294640 or SKi on p53 protein levels in LNCaP AI cells (Figure 4.14) and was consistent with siRNA findings which showed that silencing SK1 and/or SK2 expression did not increase p53 or p21 (Figure 4.7). Taken together, these findings evidence the fact that dually targeting both SK1 and SK2 is not responsible for the induction of senescence by ABC294640 or SKi.

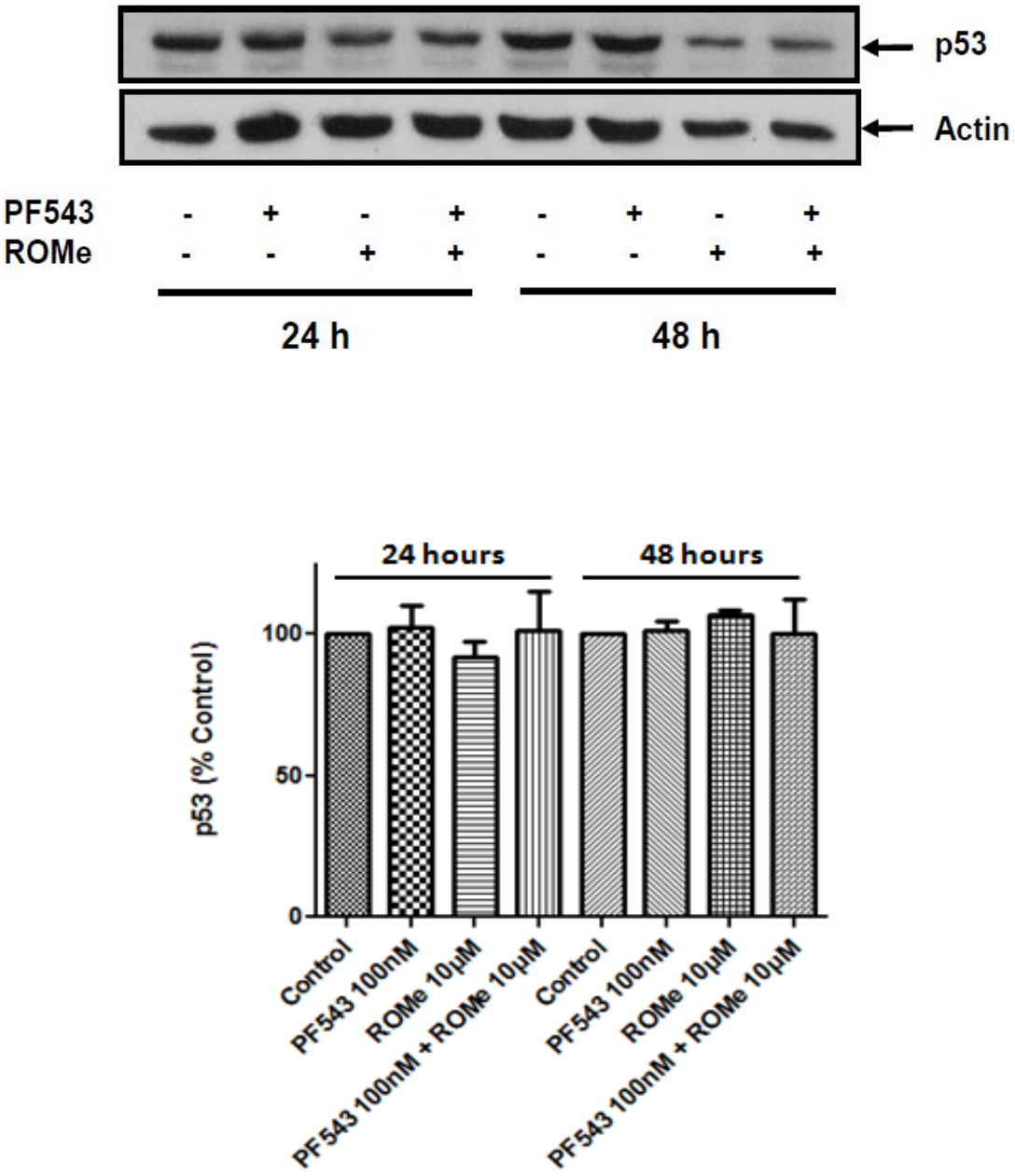


Figure 4.14 ***Effect of PF543 and ROMe on p53 protein levels in LNCaP AI cells.*** *Cells were treated with PF543 (100nM) or ROMe (10 μM) or a combination of PF543 and ROMe or with vehicle alone (DMSO 0.1% v/v) for 24 or 48 hours, as indicated. Cell lysates were collected and protein levels determined using SDS PAGE and Western blotting. Blots were stripped and re-probed for actin to verify similar protein loading. Also shown are bar graphs of the quantification of the effect of SK inhibitors on p53 protein levels. Results are expressed as means +/− S.E.M for n = 3 experiments.*

4.3 Discussion

4.3.1 SK inhibition reduces DNA synthesis and induces growth arrest in LNCaP AI cells

[^{3}H] Thymidine experiments demonstrated that all of the SK inhibitors (SK1 selective, SK2 selective and non-selective SK1/2 inhibitors) tested (except PF543) can inhibit DNA synthesis of LNCaP AI cells at concentrations which have been shown to inhibit SK (Figure 4.1). This suggests that both SK1 and SK2 have a role in maintaining proliferation of LNCaP AI cells.

Similar to cell viability experiments in chapter 3 (Figure 3.1), PF543 was unable to inhibit DNA synthesis in LNCaP AI cells (Figure 4.1). This finding is consistent with that of Schnute and colleagues who first characterised PF543 in 1483 head and neck carcinoma cells. They reported that inhibition of SK1 had no effect on the proliferation or survival of 1483 cells, despite a dramatic change in the cellular S1P/sphingosine ratio. Of note, however, as discussed previously is the fact that PF543, whilst reducing S1P levels, failed to increase ceramide levels until concentrations >3μM (Schnute *et al*., 2012). Therefore, it is perhaps necessary to both decrease S1P *and* increase ceramide to evoke an anti-proliferative response (See chapter 3 discussion).

ABC294640 has previously been shown to cause growth arrest in A498 kidney adenocarcinoma cancer cells with an increase in the proportion of cells in G1/G0 phase and a decrease in the proportion of cells in G2/M and S phase (Gao et al., 2012). Similarly, SKi caused growth arrest in A498 cells, but at S phase with a decrease in the proportion of A498 cells in G2/M phase (Gao et al., 2012). As previously discussed, ROMe has also been shown to promote growth arrest in MCF-7 breast cancer cells and to reduce [^{3}H]-thymidine incorporation into cells in a concentration-dependent manner (Lim et al., 2012). This data is supported by previously published data which shows that SK2 knockdown resulted in growth arrest of MCF-7 cells (Van Brocklyn et al., 2005).

Cell cycle analysis using FACS supported findings from DNA synthesis experiments that ABC294640 and SKi induce growth arrest in LNCaP AI cells and also elucidated the cell cycle stage at which each compound induces growth arrest. Cell cycle analysis revealed that ABC294640 and SKi halt the cell cycle at the same stage in LNCaP AI cells, with both

causing growth arrest at G1/0 with a reduced proportion of cells in S phase (Figure 4.5). For ABC294640, this is consistent with findings in A549 cells and also with studies demonstrating that ABC294640 induces significant inhibition of growth, proliferation, and cell-cycle progression in prostate cancer cells although this study does not include cell cycle staging experiments (Schrecengost et al., 2015). Conversely, SKi induces growth arrest at S phase in A549 cells with a decrease in the proportion of G2/M phase (Gao et al., 2012). These differences could reflect cell type differences between A549 and LNCaP AI cells and might reflect different efficacies of ABC294640 and SKi of modulating each of their respective targets in both LNCaP AI and A498 cells (Gao et al., 2010).

p21 induces p53 dependant growth arrest at G1 phase (Abbas and Dutta, 2009). It would be useful to definitively link the induction of growth arrest by ABC294640 and SKi to p21/p53 up regulation. In order to achieve this the effects of each compound could be tested following p53 and/or p21 removal using siRNA. It would also be interesting to perform cell cycle analysis to determine growth arrest profiles for each of the SK inhibitors included in the current study or indeed using siRNA. This may be useful to elucidate whether isoform selectivity affects the stage of the cell cycle at which growth arrest occurs. Indeed, it has previously been shown that SK inhibitors with different isoform selectivity induce different phenotypes. For example, in Jurkat and CEM-R cells T lymphocyte leukaemia cells it was shown that the SK2 inhibitor ROMe induced an autophagic cell death without activation of ER stress and UPR pathway whilst SKi induced apoptotic cell death concomitant with ER stress and URP activation (Evangelisti et al., 2014).

4.3.2 ABC294640 and SKi induce senescence of LNCaP AI cells

Of the panel of SK inhibitors tested, only ABC294640 and SKi were able to up regulate p53 and p21 in LNCaP AI cells (Figure 4.3). Because only these two inhibitors, which affect both SK1 and SK2, were able to induce a senescence phenotype whereas SK1 selective or SK2 selective inhibitors did not (PF543 and RB-005 or ROMe) it appeared that targeting both SK1 and SK2 was required to induce p53 and p21 up regulation in LNCaP AI cells.

Western blotting data for markers p53 and p21 was supported by [^{3}H] thymidine uptake experiments in which concentration dependence for the ability of ABC294640 and SKi to reduce cell proliferation was established. IC_{50}s measured here of 1.29μM for SKi (Figure 4.8) correlates well with the IC_{50} for SK inhibiton for this compound which is 0.5μM (French et al., 2003). Similarly the IC_{50} for ABC294640 was shown to be 19.3μM and this is similar to

the IC_{50} reported for ABC294640 and S1P reduction in cells (IC_{50} 26μM) (French et al., 2010), which could be seen as an indicator of causality. A role for SK1 in the induction of p53 and p21 could be suggested by time course data in which it appears that p21 and p53 induction occurs after SK1 down regulation by both ABC294640 and SKi (Figure 4.3). However, it is possible that this could be a consequence of antibody sensitivity and additional experiments (for example measurement of transcript by PCR) would be required to strengthen this data. Indeed, it is shown that solely removing SK1 from cells does not recapitulate this as indicated by siRNA experiments (Figure 4.7) as well as experiments using SK1 selective compounds (Figure 4.2 and Figure 4.14). What can be concluded from this data is that SKi appears to remove SK1 protein completely between 4 and 8 hours whereas ABC294640 takes around 24 hours and induces only a partial response (Figure 4.3). This could be a consequence of the fact that SKi has a primary catalytic effect on SK1 protein as well as a secondary effect via up regulation of the proteasome whereas ABC294640 lacks any catalytic activity against SK1 (See Chapter 3).

Interestingly, a link between p53 and SK1 has previously been demonstrated. However, contrary to the current study, SK1 was reported to be a down-stream target of p53 (Hefferan-Stroud et al., 2012). This study showed that SK1 was proteolysed in response to genotoxic stress in a p53 dependant manner in MEF cells cultured from either WT or p53 KO mice. SK1 protein was removed in response to UV radiation in WT mice whereas SK1 removal is absent in response to UV radiation in p53 KO counterparts. These effects were mediated via caspase 2 and were reversed using a caspase-2 inhibitor (Heffernan-Stroud et al., 2012). Beta galactosidase (SA-β-gal) was also used to measure senescence in thymus tissue from mice of four genotypes – WT, SK1 KO, p53 KO or SK1/p53 KO. The induction of sensescence appeared to correlate with restoration of ceramide and sph to basal levels, with staining increased in SK1 KO thymic tissue and interestingly most intensely increased in SK1/p53 KO tissue. The study also found increases in p21 and to a lesser extent p16 cells cultured from SK1 KO mice (Heffenan-Stroud et al., 2012). Attempts were made to quantify senescence using p16 antibodies and SA-β-gal in the current study but were unsuccessful (data not shown). Although there are contrasting findings regarding the sequence of events in both of these studies they nevertheless support the notion that SK1 and p53 are metabolically linked and that the proteasome plays a pivotal role in facilitating this. It would be interesting to investigate the role of caspase 2 in LNCaP AI cells. Interestingly, caspase 2 inhibition has recently been demonstrated to significantly alter the sphingolipid metabolome in breast

cancer cells. Furthermore, p53 mediated activation of caspase 2 was required for induction of SK1 proteolysis. In triple negative breast cancer cells which lacked caspase 2, SK1 was not degraded in response to chemotherapeutic drug doxorubicin (Carroll et al., 2017).

SKs have been further linked to p21 throughout the literature. In contrast to the current study, Gao and colleagues reported a decrease in p21 and p53 protein in response to ABC294640 in kidney adenocarcinoma cells but also report growth arrest with a marked increase in the proportion of cells in G1 phase and a reduction in cells in G2/M phase (Gao et al., 2012). This contrasts with the widely accepted actions of p53 and p21 as mediators of cellular senescence. Furthermore, ABC294640 has been shown to increase p21 in three different pancreatic cancer cell lines (Lewis et al., 2016) as well as prostate cancer cells (Figure 4.3). It does seem apparent that cell type differences exist with respect to the relationship between SKs, p53 and p21 with some studies reporting a negative relationship between these proteins (Gao et al., 2012) whereas others report a role for p53 up-stream of SK1 (Heffernan-stroud et al,. 2012) whilst this study suggests that p53 is down stream of SK inhibitor treatment (Figure 4.3).

4.3.3 The role of ROS in regulating SK1, p53 and p21 in LNCaP AI cells

There are multiple accounts of a relationship existing between ROS and SKs in the litreature. It has previously been shown that SKi induces an oxidative stress response in androgen-independent LNCaP-AI cells as evidenced by the accumulation of oxidised glutathione and switching from aerobic glycolysis to the pentose phosphate pathway, which may be a means to counteract oxidative stress by production of NADPH (Watson et al., 2013). Significantly, LNCaP and LNCaP AI cells treated with SKi show an elevated ratio of oxidised glutathione (GSSG) to reduced glutathione (GSH), indicative of oxidative stress. Furthermore, SKi treated LNCaP and LNCaP AI cells display elevated NADPH, which is consistently indicative of an oxidative stress response. Furthermore, it has been demonstrated in various human carcinoma cell lines (colon HCT-116, breast MDA-MB-231, lung NCI-H358) that targeting SK1, either pharmacologically using SKi or molecularly using shRNA, increased levels of ROS. In this study, the authors measured ROS by fluorescence using fluorogenic dye, 2’7’-dichlorofluorescin diacetate (DCFDA) and noted an increased amount of ROS in response to SK1 knockdown using shRNA. This ROS induction was associated with deranged PKB/Akt signalling as well as a sensitisation to chemotheraputic agent doxorubicin (Huwiler et al., 2011). Furthermore, the effects of SKi on AR protein are partially reversed by

NAC pre-treatment in LNCaP AI cells (Tonelli et al., 2013). It has also previously been demonstrated that SKi up regulates Nrf2, a redox-sensitive transcription factor, through the formation of Keap1 dimers which stabilise Nrf2 by inhibiting its proteasomal degradation (Mercado et al., 2014). Furthermore, p53 has previously been shown to mediate ROS generation in mammalian cells (reviewed in Liu et al., 2008). However, data presented herein places ROS generation up stream of p53 accumulation in response to SKi or ABC294640 as is the change in p53 is reversed using anti oxidant NAC (Figure 4.6).

The aforementioned effect of ABC294640 or SKi treatment observed on p53 and p21 expression was reversed by the reactive oxygen species scavenger, N-acetyl cysteine (NAC, Figure 4.6). However, the effect of ABC294640 or SKi on the proteasomal degradation of SK1 was not modulated by NAC (Figure 4.8) or other antioxidants, GSH and Tiron (Figure 4.9). This suggests that whilst p53 and p21 expression are regulated by ROS formation, the proteasomal degradation of SK1 in response to treatment of LNCaP AI cells with ABC294640 and SKi is not. These findings place SK1 degradation up-stream of ROS and p53/p21 up-regulation or alternatively place SK1 and p53/ p21 on separate pathways. The latter notion is supported by siRNA experiments in which knockdown of SK1 and SK2 did not increase p53 or p21 levels (figure 4.7).

It is possible that multiple mechanisms are involved in the degradation of SK1 in response to SK inhibitors – one that degrades the enzyme by involving ROS generation and proteasome activation as with ABC294640 and SKi and one which involves target engagement (binding of SK1 inhibitor to SK1) and subsequent susceptibility to ubiquitination. However, one would expect that if ABC294640 activates only the ROS dependant pathway (as there is no target engagement with SK1) that its effects on SK1 protein should be reversed by NAC, which they are not. It is also possible that the down regulation of SK1 by these inhibitors is too profound an effect to be overcome by anti-oxidant pre treatment.

Some of the effects of SKi are reversed using antioxidant NAC such as AR down regulation in LNCaP cells (Tonelli et al., 2013) whereas others such as SK1 degradation are not (see chapter 4). To draw a definitive conclusion about this the ability of ABC294640 and SKi to module AR expression and the role of NAC in this would need to be tested in LNCaP AI cells. However, the face that different proteins exhibit different sensitivities to NAC suggests that divergent or convergent signalling sequences exist rather than one linear one. It could

alternatively represent the fact that some actions are up stream of ROS generation (e.g. SK1 degradation) whereas other are downstream of ROS generation (e.g. AR down regulation).

The mechanism for the induction of ROS and its relationship to SK inhibition is yet to be established, but it is possibly due to an interruption of mitochondrial oxidative phosphorylation. Indeed, p53 up-regulation (the current study) as well as AR down regulation (Tonelli et al., 2013) by SKi are reversed by free radical scavenger NAC. It has previously been demonstrated that 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). Therefore, SKi could possibly activate NADPH oxidase, which utilizes NADPH to produce superoxide. However, NADPH oxidase inhibitor diphenyleneiodium (DPI) failed to reverse these effects in LNCaP cells, ruling out a role of NADPH oxidase in these cells (Tonelli et al., 2013). Various attempts were made in the current study to measure ROS levels in response to treatment of LNCaP AI cells with SK inhibitors. For example, the cell permeable fluorogenic DCFDA was employed in LNCaP AI cells as previously described (Huwiler et al., 2011). However, although this assay was attempted reliable data with appropriate control values could not be generated (data not shown).

4.3.4 ABC294640 and SKi alter p53 localisation in LNCaP AI cells

Dynamic distribution of p53 between the nucleus and cytoplasm is one of the control mechanisms of p53 function. Indeed, p53 can localise to the cytoplasm, the nucleus or the mitochondria and the sub-cellular distribution of p53 is pivotal in determining its function. Furthermore, it is becoming increasingly evident that dysregulation of this process is involved in promoting abnormal and oncogenic cell signalling (Conforti et al., 2015). Nuclear sequestration of p53 is a cell stress response which could result in cancer cell death or growth arrest. This is because p53 functions in the nucleus as a transcription factor which regulates cell cycle arrest and apoptosis (O'Brate and Giannakakou, 2003). This is supported by the notion that tumours in which p53 is 'trapped' in the cytoplasm due to a mutation, which prevents its nuclear/cytoplasmic shuttling, are less responsive to chemotherapy and generally have poorer prognosis than tumours in which p53 retains the ability to move between the nucleus and the cytoplasm (Moll et al., 1995; Ueda et al., 1995; Sembritzki et al., 2002).

In light of these facts, the finding that ABC294640 and SKi are able to cause nuclear redistribution of p53 (Figure 4.10) has clear clinical relevance and could be an important

facet of the anti-oncogenic actions of ABC294640 and SKi. Indeed, attempts have been made to synthesise compounds which reverse aberrant nuclear shuttling of p53, for example KPT-330 (Selinexor), which is an inhibitor of p53 nuclear export currently in Phase I/II clinical trials (Conforti et al., 2015).

Because DNA damage leads to p53 activation (O'Brate and Giannakakou, 2003), it is possible that ABC294640 and SKi elicit a DNA damage response perhaps involving ROS generation and mitochondrial dysfunction which in turn leads to p53 activation via its nuclear sequestration and inhibition of proteasomal degradation. Indeed, it was previously demonstrated in oligodendroglia that hydrogen peroxide treatment resulted in nuclear translocation of p53 and subsequent induction of apoptosis (Uberti et al., 1999). Further experiments are required to establish the exact mechanism of p53 nuclear sequestration in response to ABC294640 and SKi and to establish if this indeed allows p53 to evade the proteasome and elicit anti-cancer effects.

4.3.5 The role of MDM2 and nutlin in regulating p53, p21 and SK1 in LNCaP AI cells

Tumour-necrosis factor (TNF) receptor-associated factor 2 (TRAF2) is a key component of the oncogenic NF_KB signalling cascade which is activated by TNFα binding to TNF receptors. It has been demonstrated that S1P binds to TRAF2 at the N-terminal RING domain and stimulates its E3 ligase activity (Alvarez et al., 2010). MDM2, which negatively regulates p53 expression by mediating continuous p53 proteasomal degradation, also contains a RING domain (Sherr and Weber, 2000). In light of this, it was tempting to speculate that S1P could also activate MDM2 and that combined inhibition of SK1 and SK2 by ABC294640 and SKi could reduce S1P levels below a certain threshold which would in turn halt S1P activation of MDM2, thereby reducing p53 ubiquitination and leading to an accumulation of p53. Both a pharmacological and molecular approach were attempted to assess this theory although the successful transfection of the MDM2 plasmid construct could not be verified (data not shown) which limited the study to the use of nutlin.

Nutlin is a small molecule inhibitor of the interaction between p53 and MDM2 and has been shown to induce p53 stabilization and cell cycle arrest. Indeed, nutlin has been investigated as a chemotherapeutic drug in wild type p53 tumours and is in phase I clinical trials for retinoblastoma (Secchiero et al., 2011). Interestingly, it has also been demonstrated that androgen deprivation – although tumour suppressive in the short term – ultimately confers tumour promoting adaptation. This can facilitate the advent of senescence-resistant malignant

cell populations which notably harbour chemo-resistance and enhanced pro-survival mechanisms such as inhibition of p53-mediated cell death (Dominick et al., 2013). Such chemo-resistance has previously been demonstrated by our group in that LNCaP cells undergo apoptosis in response to SKi treatment (characterised by PARP cleavage) mediated by a reduction in SK1a and SK1b whereas LNCaP AI cells resist apoptosis due to SK1b evading the proteasome (Loveridge et al. 2010). In the study by Dominick and colleagues, pharmacological p53 reactivation using nutlin was sufficient to overcome the survival response and trigger apoptosis of the resistant cell population. This highlights potential therapeutic promise of exploiting p53 up-regulation to treat chemotherapy resistant cancers.

LNCaP AI cells carry no p53 mutations which could potentially confer resistance to nutlin. Thus, as expected, nutlin inhibited DNA synthesis with an IC_{50} of 0.59μM and induced a senescent phenotype with increase expression of p53 and p21. In the literature, nutlin is reported to inhibit the interaction of p53 and MDM2 with an IC_{50} of 0.09 μM in a non cell based assay (Vassilev et al., 2004). These IC_{50}s are close enough to indicate causality although it would be better to generate an IC_{50} for the inhibition of MDM2 by nutlin in LNCaP AI cells. Findings from western blotting also correlate well with published data – nutlin was able to induce an up-regulation of p53 protein at concentration as low as 0.1μM. Interestingly, nutlin also increased p21 expression at a similar concentration to that which increased p53 expression indicating that nutlin activates growth arrest via the p53 dependant p21 pathway (Figure 4.11). As expected, nutlin also induced the nuclear sequestration of p53 protein (Figure 4.11). Nutlin shares these three properties with ABC294640 and SKi, which provided some indication that these compounds may be acting to indirectly inhibit MDM2 in order to induce the senescent phenotype. However, a molecular approach using either siRNA to remove MDM2 or to over express MDM2 would be required to further investigate whether the effects of ABC294640 and SKi were mediated through MDM2.

4.3.6 Autophagy and senescence in LNCaP AI cells

ABC294640 is an inducer of autophagy in pancreatic and kidney cancer cells (Beljanski et al., 2010). SKi however inhibits autophagy in LNCaP AI cells as evidenced by the fact that SKi induces the accumulation of the non-lipidated inactive form LC3 (LC3-I) rather than lipidated LC3-II (autophagy marker protein) in LNCaP AI cells. (Tonelli et al., 2013). Because these compounds appear to have opposing effects on autophagy in these different

cell studies, one cannot assume a link between ABC294640 and autophagy. Several modulators of autophagy were used to test the presence of any links between inhibition or stimulation of autophagy and senescence of LNCaP AI cells. Indeed, we confirm here that modulation of autophagy in LNCaP AI cells did not induce the senescence marker, p21 (Figure 4.13).

The fact that SKi inhibits autophagy in LNCaP AI cells could contribute to the oxidative stress response observed upon SKi treatment in these cells. This is because mitochondria are the main endogenous producers of cellular ROS and are subject to turnover via the autophagic pathway as they become faulty. Thus, inhibition of autophagy could lead to an accumulation of faulty mitochondria, which could in turn result in aberrant ROS production (Jin and White, 2008). Although attempts were made to measure ROS generation in response to SK inhibitors in LNCaP AI cells using DCFDA and also using a HEK293T cell line stably transfected with an inducible MitoTracker® construct (a reporter of mitochondrial health), reliable data was unable to be obtained (data not shown). Furthermore, modulation of autophagy by each of these compounds using, for example by western blotting for LC3 and LC3-II would need to be measured in addition to ROS data to draw any reliable conclusions about this.

4.3.7 Dually targeting SK1 and SK2 does not induce senescence in LNCaP AI cells

Surprisingly, siRNA experiments demonstrated that dually targeting SK1 and SK2 did not recapitulate the effects of ABC294640 or SKi on p53 or p21 expression in LNCaP AI cells (Figure 4.7). SK2 knockdown was unable to be assessed in these experiments due to the lack of a commercially available antibody sensitive enough to measure SK2 expression in LNCaP AI cells. Importantly, data generated using a combination of an SK1 inhibitor and an SK2 inhibitor (PF543 and ROMe, respectively) supported the findings of molecular data generated using siRNA (Figure 4.14). This ruled out the possibility that ABC294640 and SKi's action on SK1 and SK2 was the sole cause of the induction of senescence in LNCaP AI cells. Therefore, this implies that another previously unidentified target was responsible for the observed phenotype.

Loveridge et al. (Loveridge et al., 2010) previously showed an increase in several species of dhCers in LNCaP cells in response to SKi treatment and suggested that this compound may

inhibit dihydroceramide desaturase. Similar increases in the levels of dihydroceramides has been observed in LNCaP AI cells (Tonelli, unpublished) and ovarian cancer cells (Illuzzi et al 2010). Moreover, it was recently reported that SKi inhibits dihydroceramide desaturase, the last enzyme in the *de novo* synthesis of ceramide (Cingolani et al., 2014). In the latter study, SKi was predicted through computational modelling to inhibit NADH-cytochrome b5 reductase and to thereby indirectly inhibit dihydroceramide desaturase in a non competitive fashion with a Ki of 0.3μM in both intact cells and cell lysates from HGC27, HeLa and T98G cells. This resulted in a marked accumulation of dhCers in cells with concomitant changes in cell cycle activity and accumulation of cells in G0/G1. Due to the similarities between ABC294640 and SKi reported herein and the data suggesting that a target other than SKs is involved in the induction of senescence, dihydroceramide desaturase was investigated as a target of SKi and ABC29460 in LNCaP AI cells.

CHAPTER 5:

THE ROLE OF DIHYDROCERAMIDE DESATURASE 1 IN SENESCENCE IN LNCAP AI CELLS

CHAPTER 5 – Des1 and senescence in LNCaP AI cells.

5.1 Introduction

This study has established that ABC294640 and SKi induce senescence of LNCaP AI cells (Chapter 4). It was also shown that the SK2 inhibitor ABC294640 induces the proteasomal degradation of SK1 (Chapter 3) in a similar manner to the SK1 and SK2 inhibitor SKi. Importantly, siRNA experiments knocking down SK1, SK2 or both SK1 and SK2 together suggest that the absence of these proteins is not responsible for the induction of senescence by ABC294640 and SKi (See Chapter 4). This implies that an alternative target/mechanism could underlie the action of these compounds to induce senescence in these cells.

Original studies suggesting that SK inhibitors could affect dhCer levels, and therefore potentially Des1, showed dhCer accumulation upon SKi treatment (Illuzzi et al., 2010; Loveridge et al., 2010). Initially, this was proposed to be due to relief of the S1P inhibition of dihydroceramide synthase (CerS), based on the finding that over-expression of SK1 in HEK293 cells resulted in a marked increase in dihydrosphingosine levels with an accompanying decrease in ceramide levels (Maceyka et al., 2005). However, this idea was challenged when it was shown that resveratrol, which is similar in structure to SKi, acts as an inhibitor of Des1 (Signorelli et al., 2009).

Since then, it has been suggested that SKi inhibits Des1 (Cingolani et al., 2014). However, it remains unknown whether inhibition of Des1 by SKi is direct or indirect. Several different opinions exist surrounding this. It was proposed by Hannun and colleagues that the inhibition of Des1 and accumulation of dihydroceramides occurs as a result of oxidative stress in various human cell lines (Idkowiak-baldys et al., 2010). Indeed, it has been shown that treatment of LNCaP AI cells with SKi results in an oxidative stress response (Watson et al., 2013). Results presented in Chapter 4 suggest that SKi mediates at least some of its effects due to ROS generation. However, it has been suggested by Fabrias and colleagues that SKi decreases Des1 activity via inhibition of NADH-cytochrome b5 reductase (supported by molecular modelling) (Cingolani et al., 2014).

However, in light of the effect of SKi on dhCer levels in LNCaP AI cells (Loveridge et al., 2010), the role of Des1 was investigated in order to determine if it is a common target responsible for the induction of the senescent phenotype observed upon ABC294640 or SKi treatment in these cells.

There are currently a limited number of reports which investigate the role of Des1 and the product(s) of its catalysis – dihydroceramide(s) – in cells and the physiological function of Des1/dhCers are still poorly defined. As previously discussed (Chapter 1), dhCers were originally thought to be biologically inactive molecules. Exogenous addition of dhCer to cells failed to induce growth arrest or apoptosis, leading to the belief that dhCers were simply precursors for ceramide (Bielawska et al., 1993; Sugiki et al., 2000; Ahn et al., 2000). However, in the first Des1 knockdown study, conducted in SMS-KCNR human neuroblastoma cells, Des1 siRNA treatment resulted in an accumulation of endogenous dhCer, inhibited cell growth and caused G1/0 growth arrest, which was accompanied by de-phosphorylation of pRB (Kraveka et al., 2007). Therefore, a potential role for dihydroceramides in the senescent phenotype induced by ABC294640 and SKi is a possibility.

The latter study was also the first to identify Des1 as an *in vitro* target for the synthetic retinoid fenretinide (*N*-(4-hydroxyphenyl)-retinamide (4-HPR) (Kraveka et al., 2007). This was confirmed when Des1 was shown to be a direct *in vitro* target of fenretinide, as characterised in SMS-KCNR cells (Rahmaniyan et al., 2011). These studies were supported by data from another group who reported Des1 inhibition by fenretinide and showed through this a role of dhCers in regulating autophagy (Zheng et al., 2006). Fenretinide inhibits Des1 in a dose dependent manner and inhibition of Des1 by fenretinide is competitive at 20 minutes. However, with longer incubations, inhibition is irreversible. Fenretinide is reported to inhibit Des1 with an IC_{50} of 2.32μM (Rahmaniyan et al., 2011).

In the current study, fenretinide has been chosen as a tool compound to investigate the role on Des1 and dhCers in regulating senescence in LNCaP AI cells. Fenretinide is a clinical candidate compound which is cytotoxic in various cancers (Wang et al., 2008) and has been in clinical trials for ovarian cancer, neuroblastoma, lymphoma and leukemia (Maurer et al., 2000). Interestingly, fenretinide was shown to impair DU145 and PC3 prostate cancer cell proliferation, migration and invasion (Benelli et al., 2012).

Several studies conducted *in vitro* have also indicated a role for Des1 in regulating proliferation and survival. In HCT116 and HCG27 cells, inhibition of Des1 using the inhibitor XM462 or the addition of exogenous short chain dhCer analogues resulted in delayed G1/S cell cycle transition and increased autophagy. Interestingly, these effects were blocked using an autophagy inhibitor 3-methyladenine (3MA) (Gagliostro et al., 2010).

Taken together, these findings indicate that Des1 and dhCer may have a role in maintaining prostate cancer cell survival and proliferation. Therefore, there is strong rationale for investigating Des1 and dhCer in relation to the effects of ABC294640 and SKi in LNCaP AI cells.

5.2 Results

5.2.1 ABC294640 induces the proteasomal degradation of Des1

It has previously been shown that accumulation of various species of dihydroceramide occurs upon treatment with SKi in LNCaP and LNCaP AI cells (Loveridge et al., 2010). This has also been observed in various other cell systems (Illuzzi et al., 2010; Cingolani et al., 2014). Furthermore, SKi is a known inhibitor of Des1 (Cingolani et al., 2014). As previously discussed (see Chapter 3), it has been established that SK1 selective inhibitors induce the proteasomal degradation of SK1 (Loveridge et al., 2010, Tonelli et al., 2010; Tonelli et al., 2013; MacRitchie et al., 2016). Similarly, SKi, which inhibits both SK1 and SK2, and ABC294640, an SK2 inhibitor, induce the removal of SK1 from cells. It was therefore of interest to see whether treatment with ABC294640 similarly removes Des1 from LNCaP AI cells. Thus, a series of concentrations of ABC294640 (1-25μM) were tested in the presence or absence of the proteasomal inhibitor MG132 (10μM) to test this hypothesis.

Remarkably, ABC294640 (5-25μM) removed Des1 from cells and this was by 70-80% at 25μM of the inhibitor. This effect was fully reversed for 5μM ABC294640 but only partially reversed for 25μM ABC294640 by pre-treatment of cells with the proteasomal inhibitor MG132 (10μM). However, it is unknown if the apparent removal of Des1 from LNCaP AI cells is due to an elevation of proteasomal activity by ABC294640 (see Chapter 3) or if this is an indication of target engagement between ABC294640 and Des1.

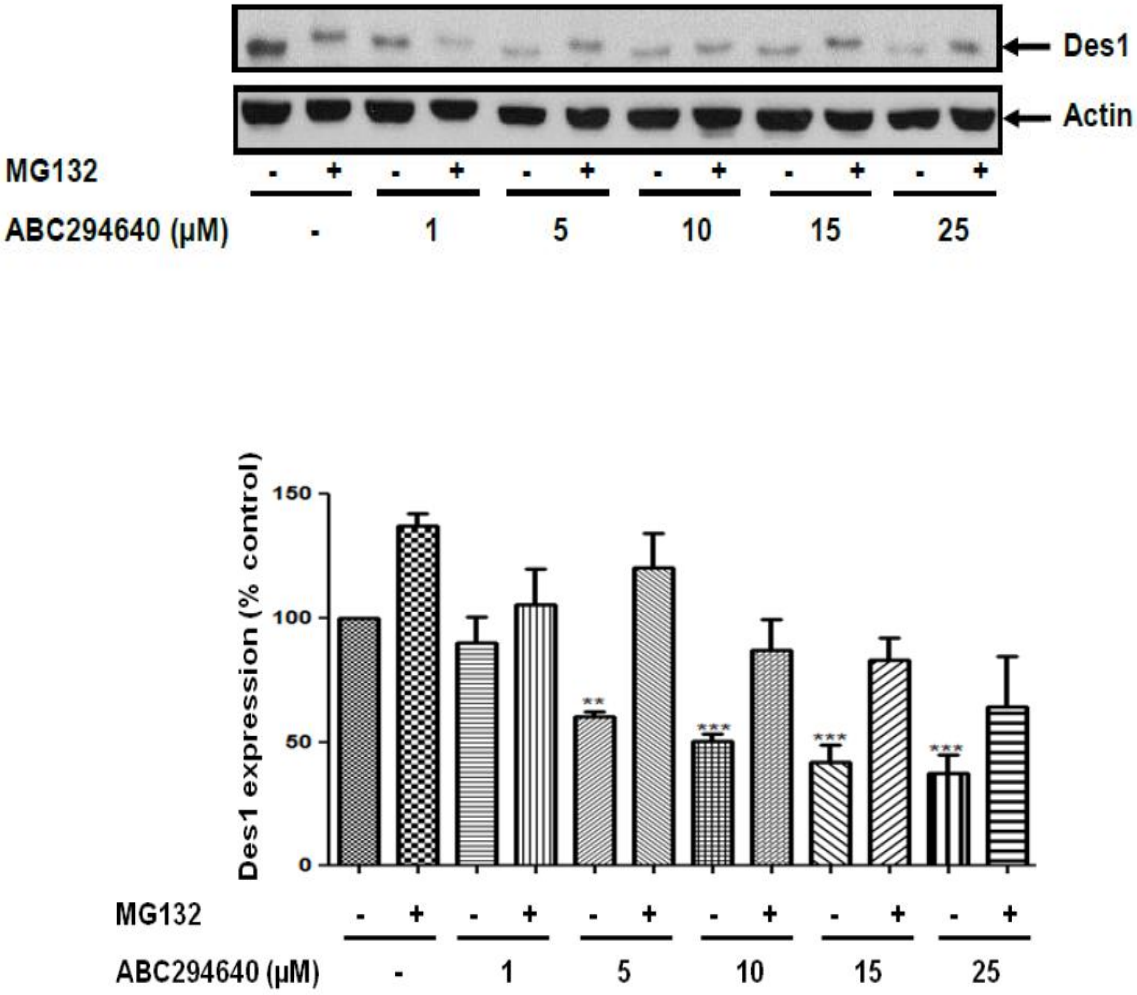


Figure 5.1 ***Effect of ABC294640 on Des1 expression in LNCaP AI cells.*** *Western blot demonstrating the effect of ABC294640 (1-25 μM) for 24 h in the presence and absence of MG132 (10 μM) on Des1 expression. Actin was used a protein loading control. Results are representative of at least three independent experiments. Also shown is a bar graph of the quantification of the effect of ABC294640 on Des1 expression. Results are expressed as means +/− SD for n = 3 experiments. **p < 0.01 versus control; ***p < 0.001 versus control.*

5.2.2 Effect of ABC294640 on sphingolipidome in LNCaP AI cells.

Having established that treatment of LNCaP AI cells with ABC294640 reduced levels of Des1 protein, it was of interest to determine its effects upon sphingolipids. In particular, whether ABC294640 was able to raise dhCers, analogous to the effects of SKi reported previously (Illuzzi et al., 2010, Loveridge et al., 2010). Therefore, the sphingolipidomics profile was investigated by LC-MS in collaboration with Michael Wakelam and Qifeng Zhang (Babraham Institute, Cambridge, UK) (see Appendix).

As expected, treatment of LNCaP AI cells for 24 hours with ABC294640 (25μM) resulted in a statistically significant accumulation of several species of dihydroceramides (C14, C16, C22 and C24dhCers). Increases in the other dhCer species analysed were also observed but were not statistically significant (Figure S.1). This provides additional evidence that ABC294640 could be acting upon Des1 in LNCaP AI cells thereby causing an accumulation of dhCer species.

In addition, the levels of ceramide and other sphingolipids was examined in order to determine if the accumulation of dhCer is at the expense of another sphingolipid.

Unlike dhCers which exhibited a universal increase in response to ABC294640 treatment, different chain lengths of ceramide exhibited differential effects. This resulted in no overall net gain or loss of ceramide. Interestingly, short chain ceramide species appear to be reduced by treatment of LNCaP AI cells with ABC294640. Significant decreases were detected in C16:0, C16:2, C18:0, C20:0 and C20:1 ceramides (Figure S.2). Conversely, longer chain length ceramide species were increased significantly for C22:0, C24:0, C24:1, C26:0 and C26:1 ceramide species (Figure S.2).

Surprisingly, given the widely accepted action of ABC294640 as a SK inhibitor, a statistically significant reduction in S1P was not observed. A downward trend in S1P was exhibited; however this data was not statistically significant (Figure S.3).

5.2.3 ABC294640 and SKi inhibit Des1 in Jurkat cells.

In light of the fact that treatment of LNCaP AI cells with ABC294640 resulted in accumulation of various species of dhCer, the ability of this compound to inhibit Des1 activity was tested in collaboration with Stuart Pitson and Melissa Pitman (University of South Australia, Adelaide). Intact Jurkat cells, labelled with DhCer-C6-NBD (a fluorescently tagged form of C6 dhCer) were treated with various concentrations of ABC294640 or SKi and Des1 activity measured by the formation of Cer-C6-NBD, which was identified by HPLC analysis.

Both ABC294640 and SKi were shown to inhibit Des1 in these cells. Inhibition of Des1 by ABC294640 exhibits an IC_{50} = 10.2 +/− 3.7 μM (*n* = 3) and SKi exhibited an IC_{50} = 0.63–0.69 μM (*n* = 2) (McNaughton et al., 2016). These values are similar to the IC_{50} values for inhibition of DNA synthesis of 19.3 μM and 1.29 μM for ABC294640 and SKi respectively in LNCaP AI cells (Figure 4.4).

Collectively, this data supports a role of Des1 and dhCer as mediators of growth arrest in LNCaP AI cells.

5.2.4 Des1 degradation by ABC294640 is up stream of oxidative stress.

It is known that the effects of ABC294640 on p53 and p21 protein are mediated by oxidative stress and are reversed by the anti-oxidant scavenger NAC (Figure 4.6). Conversely, the degradation of SK1 by ABC294640 and SKi is not reversed by NAC (Figure 4.8), suggesting that different pathways or mechanisms exist which appear to be independent of ROS and oxidative stress generation by these compounds. It is also known that Des1 is inhibited by ROS (Idkowiak-Baldys et al., 2010) and Des1 has been suggested as a cellular redox sensor (Rodriguez-Cuenca et al., 2015).

In light of these facts, it was important to test whether the degradation of Des1 by ABC294640 was contingent upon oxidative stress. To this end, LNCaP AI cells were pretreated with NAC (10 mM) prior to treatment with ABC294640 (25 μM).

Interestingly, NAC had no effect on the ABC294640 induced proteasomal degradation of Des1 (Figure 5.2), suggesting that the oxidative stress response is down stream of Des1 degradation (i.e. an oxidative stress response is not driving the degradation of Des1).

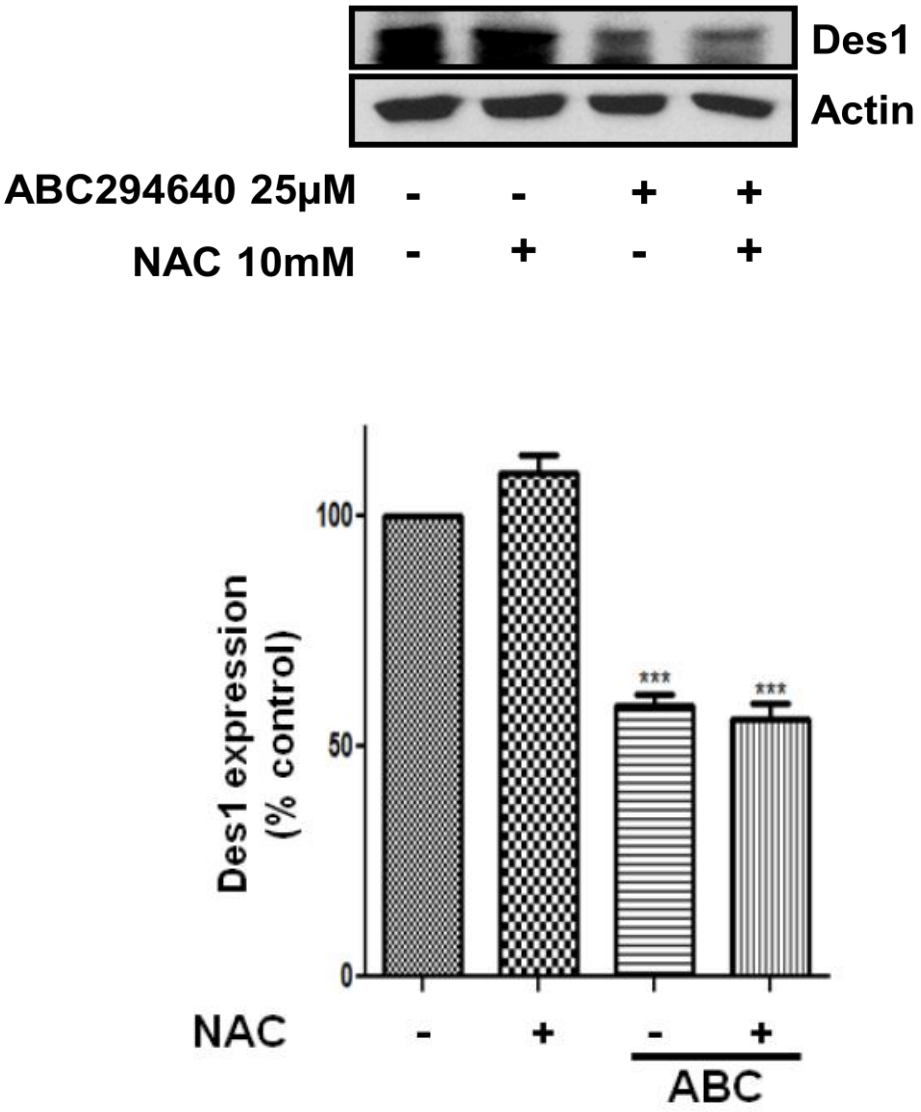


Figure 5.2 ***Effect of NAC on ABC294640 induced reduction in Des1 protein levels in LNCaP AI cells.*** *Western blot demonstrating the effect of ABC294640 (25 μM) for 24 h in the presence and absence of NAC (10 mM) on Des1 expression. Actin was used a protein loading control. Results are representative of at least three independent experiments. Also shown is a bar graph of the quantification of the effect of ABC294640 +/- NAC on Des1 expression. Results are expressed as means +/− SD for n = 3 experiments. ***p < 0.001 versus control.*

5.2.5 Effect of the Des1 inhibitor fenretinide on DNA synthesis.

It has previously been established that SKi affects Des1 activity (Cingolani et al., 2014) and it has now been established that ABC294640 also targets Des1, both via inhibition (Figure S.1 and section 5.2.3) and degradation (Figure 5.1). In light of these findings, it was of interest to determine whether these effects on Des1 are responsible for the induction of senescence by these compounds (Chapter 4). In order to investigate this, the Des1 inhibitor fenretinide (see section 5.1) was first tested for its ability to inhibit DNA synthesis (measured as a reduction in [^{3}H] thymidine uptake into newly synthesised DNA).

Indeed, fenretinide treatment causes a reduction in DNA synthesis in LNCaP AI cells with an IC_{50} of 0.58μM (Figure 5.3). This indicates that Des1 inhibition could be responsible for the induction of growth arrest by ABC294640 or SKi, although further experiments are required to confirm this.

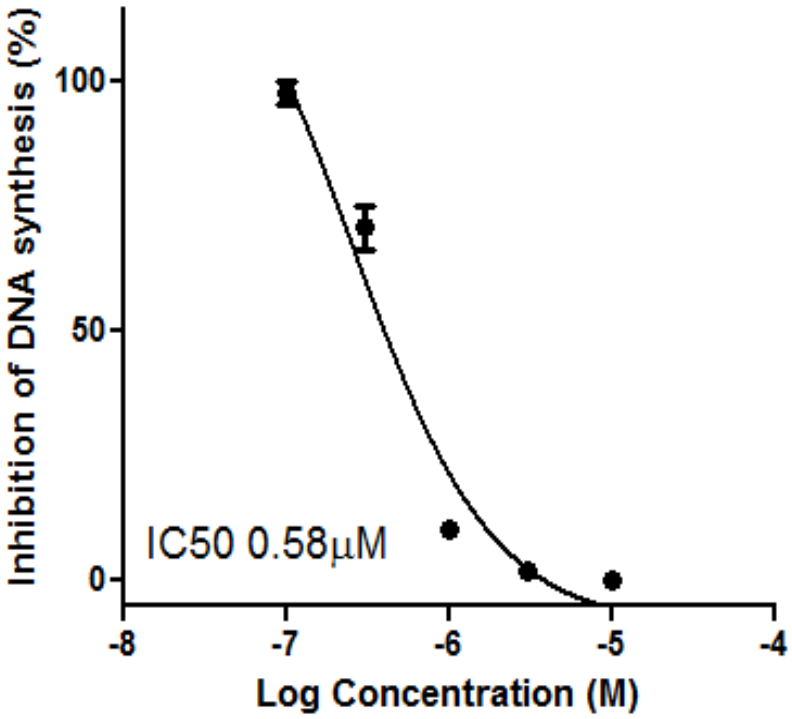


Figure 5.3 ***Effect of fenretinide on DNA synthesis in LNCaP AI cells****. The effect of a range of concentrations of fenretinide (0.1μM-10μM, 24hours) on [*^{3}*H]thymidine uptake into newly synthesised DNA. Results are representative of at least three independent experiments. Results are expressed as a percentage of [*^{3}*H]thymidine incorporation in the absence of fenretinide (means +/− SD for n = 3 experiments).*

5.2.6 Effect of the Des1 inhibitor fenretinide on SK1, p21 and p53 expression.

Having established that the Des1 inhibitor fenretinide inhibits DNA synthesis in LNCaP AI cells (Figure 5.3), it was necessary to establish its effects on (i) markers of senescence p53 and p21 and (ii) SK1 protein levels. Analysis of the effect of fenretinide on p53 and p21 should help to more firmly establish whether the induction of senescence ABC294640 and SKi in LNCaP AI cells (Chapter 4) could involve Des1 inhibition. To this end, a range of concentrations of fenretinide were assessed for their effects on senescent markers p53 and p21 as well as SK1 protein levels.

Treatment of LNCaP AI cells with Des1 inhibitor fenretinide (1-8μM) recapitulates the effects of ABC294640 and SKi on p53 and p21. The expression of both proteins was significantly increased by concentrations as low as 1μM fenretinide (Figure 5.4). Interestingly, fenretinide treatment also significantly induced the degradation of SK1 in these cells at concentrations of 1 μM or more. This caveat must be considered when interpreting these results; however, SK1 has already been excluded as a mediator of senescence in these cells using siRNA (Figure 4.7).

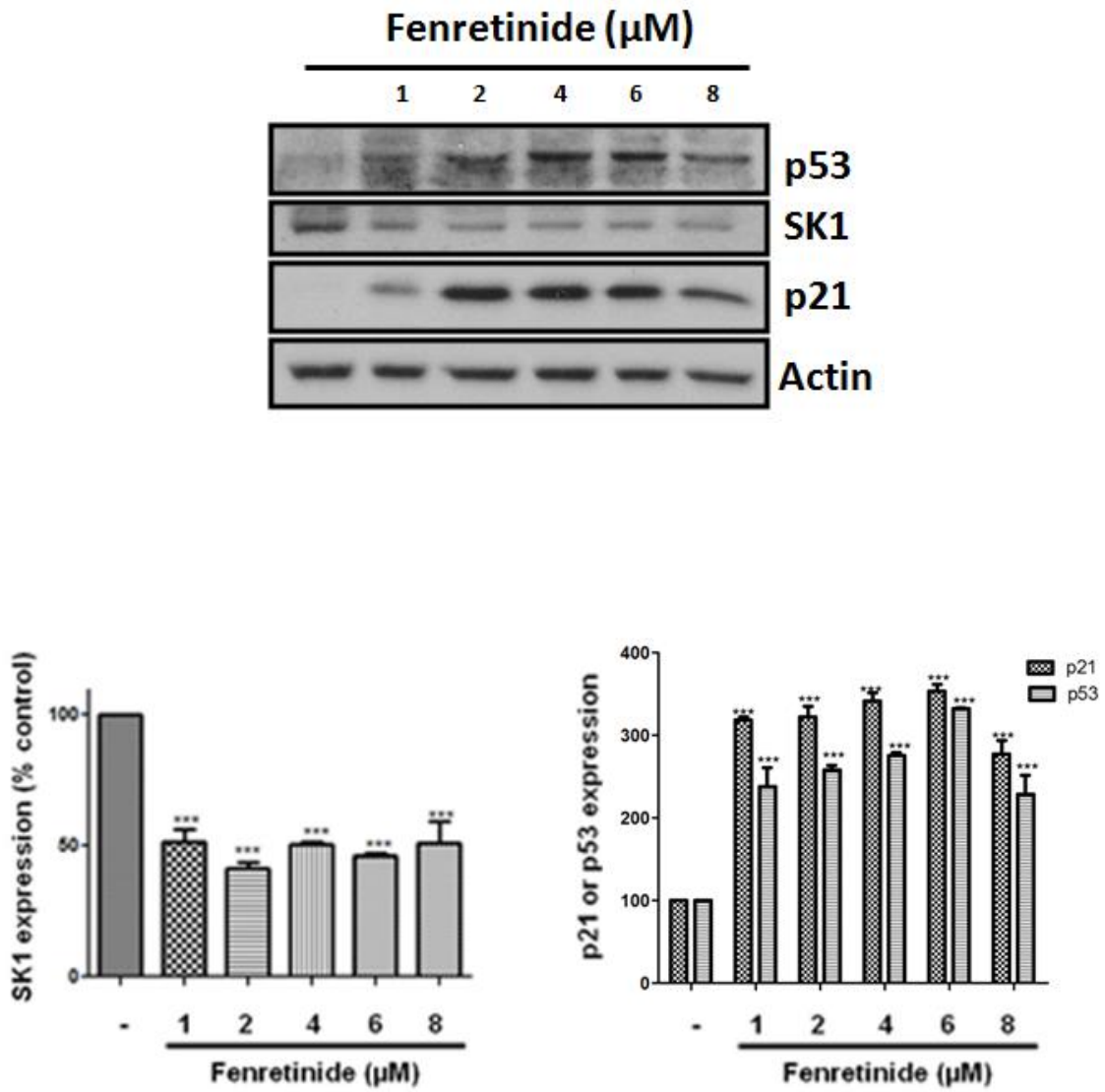


Figure 5.4 ***Effect of fenretinide on p53, p21 and SK1 expression***. *Western blots demonstrating the effect of fenretinide (1-8μM, 24 h) on SK1 protein levels or p21 and p53 protein levels. Actin was used a protein loading control. Results are representative of at least three independent experiments. Also shown are bar graphs of the quantification of the effect of fenretinide on SK1, p21 and p53 protein levels. Results are expressed as means +/− SD for n = 3 experiments. ***p < 0.001 versus control.*

5.2.7 Fenretinide does not inhibit SK1 or SK2.

In light of the finding that fenretinide degrades SK1 protein (Figure 5.4), it was necessary to test fenretinide for its ability to inhibit the catalytic activity of SK1, as degradation of SK1 is an indication of target engagement of SK1 inhibitors and the protein itself (Chapter 3; Pyne et al., 2016). It was also of interest to determine whether fenretinide affected SK2 activity. Therefore, fenretinide was assayed for both (A) SK1 and (B) SK2 inhibition in a radiometric assay employing the phosphorylation of sphingosine by ^{32}P-γ-ATP in the presence of purified recombinant SK1 or purified recombinant SK2 (Delon et al., 2004). This assay (performed in collaboration with S Pyne) uses a saturating concentration of ATP (250 μM) and sphingosine at the Km for each enzyme (3 μM for SK1 (Lim et a., 2011) and 10 μM for SK2 (Lim et al., 2011b)) and has been used successfully to identify and characterise novel sphingosine-competitive SK1 and SK2 inhibitors (Baek et al., 2013; Liu et al., 2013; Byun et al., 2013). The SK1 inhibitor RB005 (10μM) (Byun et al., 2013) and SK2 inhibitor F-02 (50μM) (Baek et al., 2013) were used in these experiments as positive controls for SK1 and SK2 inhibition, respectively.

Fenretinide does not inhibit purified recombinant SK1 at 1μM but is a very weak inhibitor at 10μM (Figure 5.5, upper panel). Fenretinide does not inhibit purified recombinant SK2 (Figure 5.5, lower panel) at either low (1μM) or supra maximal (10μM) concentrations . As fenretinide is only a very week inhibitor of purified recombinant SK1 at concentrations which are above the IC_{50} for inhibition of DNA synthesis (0.58μM – Figure 5.3) and induction of p53 and p21 protein (<1μM - Figure 5.4), it is unlikely that the effects of fenretinide are mediated through a direct effect on SK1.

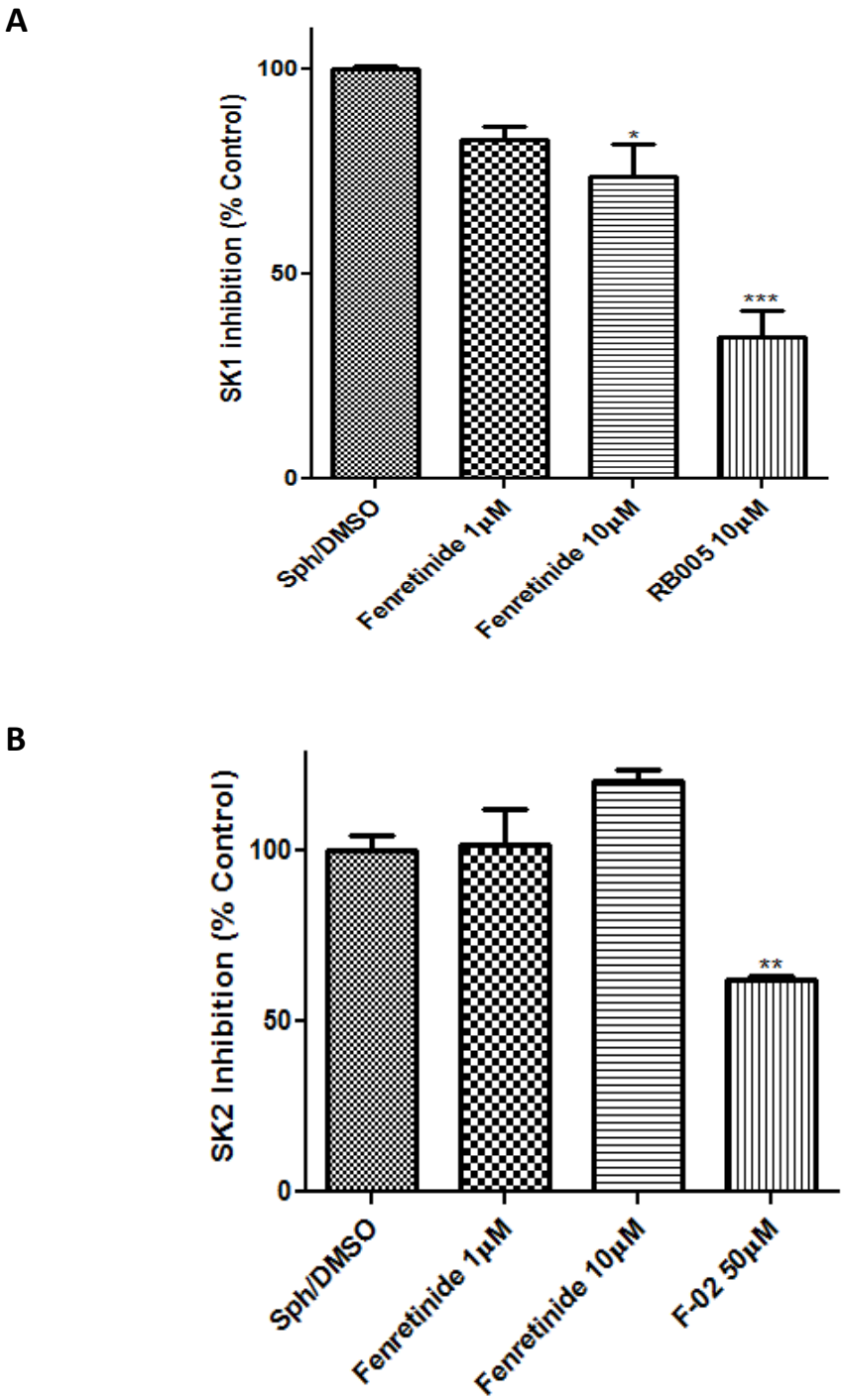


Figure 5.5 ***Effect of fenretinide on SK1 and SK2 activity.*** *Fenretinide (1µM or 10µM), RB005 (10µM) or F02 (50µM) as indicated were tested for the ability to inhibit purified recombinant (A) SK1 (using 3µM Sph and 2*50 *µ*M ^{32}P-ATP) *or (B) SK2 (using 3µM Sph and 2*50 *µ*M ^{32}P-ATP) *activity in vitro. Results are expressed as percentage of activity in the absence of inhibitor (mean +/− SD for n = 3 samples).*

5.2.8 Fenretinide is a proteasomal activator.

Previous data indicates that fenretinide does not inhibit SK1 (Figure 5.5) although it does induce the degradation of SK1 protein (Figure 5.4). Moreover, others have shown that there are two routes by which SK1 degradation is enhanced by small molecule SK1 inhibitors – (a) *via* a conformational change induced upon inhibitor binding or (b) via an increase in proteasomal flux which in turn increases SK1 degradation *via* this route (See Chapter 3). As it has been demonstrated here that fenretinide does not directly inhibit SK1, it is probable that fenretinide, like ABC294640 and SKi, is decreasing SK1 expression levels by increasing its turnover by the ubiquitin proteasomal system.

To assess whether fenretinide stimulates proteasome activity in LNCaP AI cells, the Proteasome-Glo™ Cell-Based Assay (as described in Chapter 3) was used. As shown in Figure 5.6A, treatment of LNCaP AI cells with fenretinide (5 μM) resulted in an increase in proteasomal activity in these cells. As an additional measure of effects on the proteasome, fenretinide was also tested for its ability to increase ubiquitination (measured by western blotting for ubiquitin protein). Indeed, a concentration dependence between fenretinide and overall protein ubiquitination has been established (Figure 5.6B), indicating that fenretinide also enhances ubiquitination of proteins.

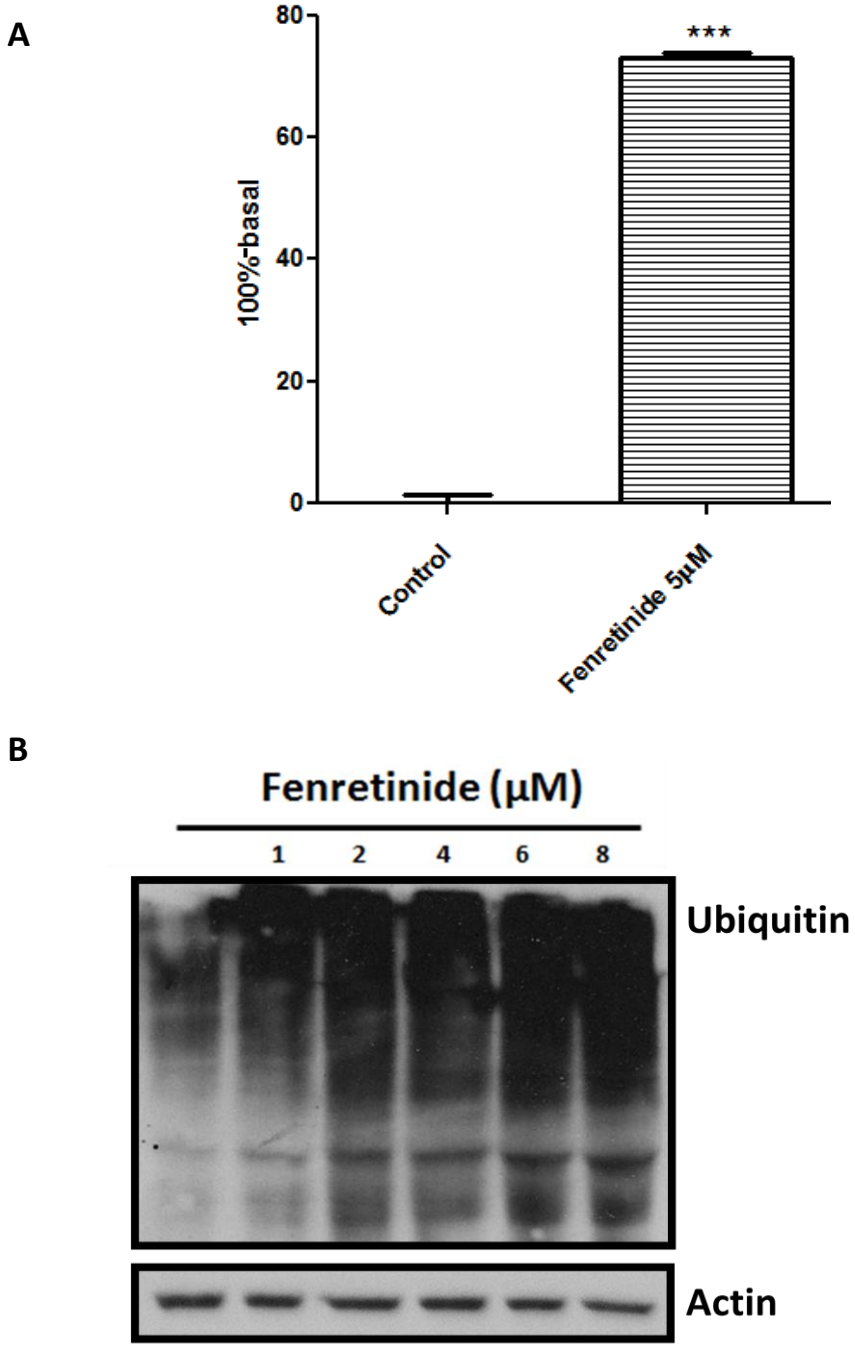


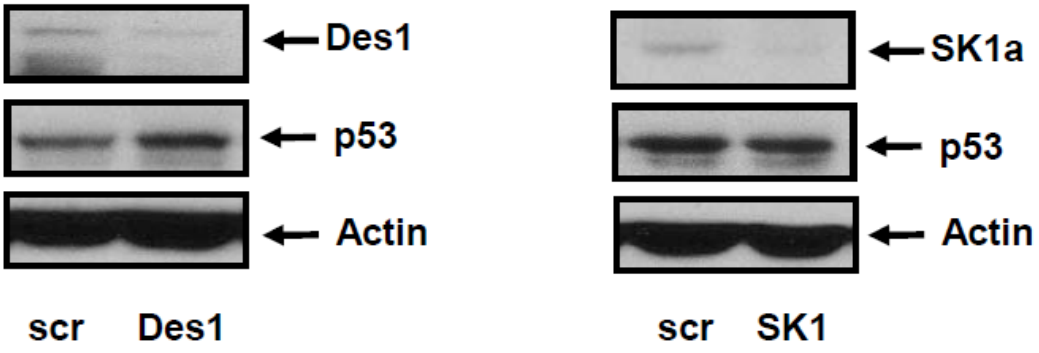
Figure 5.6 ***Effect of fenretinide on proteasomal activity in LNCaP AI cells***. *(A) Histogram demonstrating the effect of fenretinide (5μM) on proteasomal activity in LNCaP AI cells. (B) Western blots demonstrating the effect of fenretinide (1-8μM, 24 hours) on ubiquitination of proteins. Actin was used a protein loading control. (A) Results are expressed as means +/– S.E.M for n = 3 experiments. ***p < 0.001 versus control.*

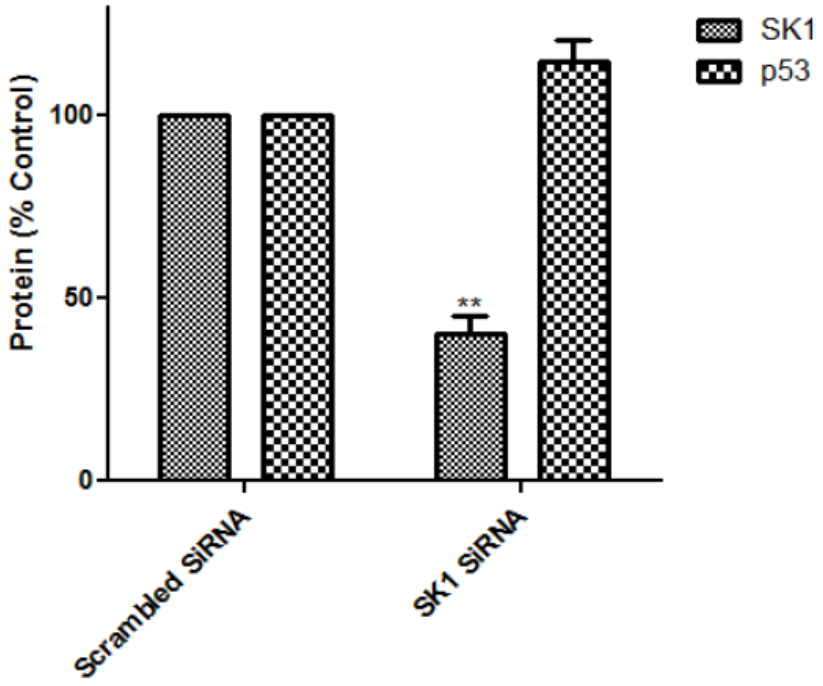
5.2.9 Effect of Des1 and SK1 siRNA on senescence in LNCaP AI cells.

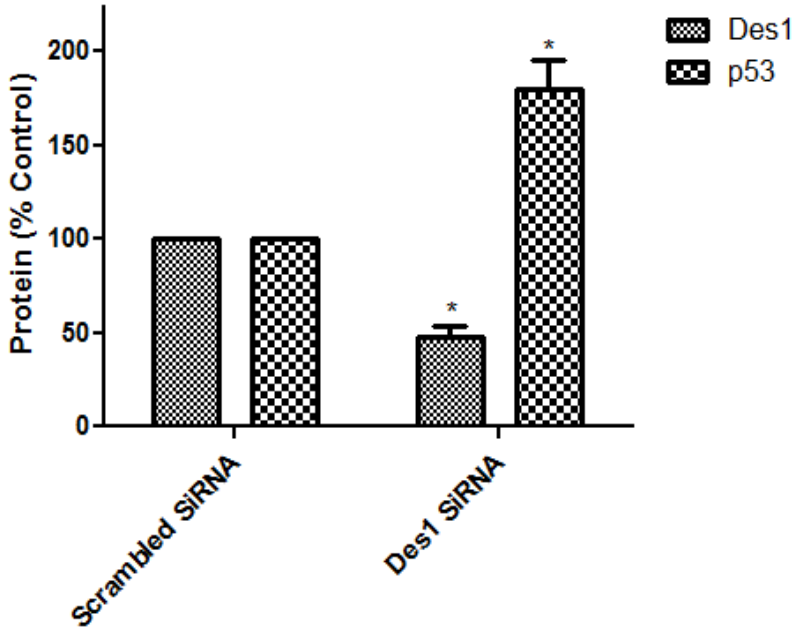
This study has established that treatment of LNCaP AI cells with the SK inhibitors, ABC294640 and SKi, increases expression levels of the senescent markers p53 and p21 (See Chapter 4). Also, it has been demonstrated that these compounds inhibit Des1 (Appendix Figure S.1 and section 5.2.3; Cingolani et al., 2014; Venant et al., 2015; McNaughton et al., 2016). Moreover, treatment of LNCaP AI cells with the Des1 inhibitor fenretinide similarly induces senescent markers p53 and p21 (Figure 5.4) and inhibits DNA synthesis (Figure 5.3). It is therefore possible that Des1 inhibition or removal is responsible for induction of senescence in LNCaP AI cells. In order to further investigate this theory, siRNA was used to specifically knock-down the expression of Des1 or Des1 together with SK1.

The transfection of LNCaP cells with Des1- or SK1-targeted siRNA resulted in a marked reduction in the respective protein levels compared to cells transfected with scrambled siRNA, as assessed by western blotting (Figure 5.7A and B). This confirmed that knockdown of the desired targets has been achieved. It is demonstrated here that siRNA knockdown of Des1 alone increased p53 expression (Figure 5.7A) whereas siRNA knockdown of SK1 alone had no effect (Figure 5.7A). In contrast, a combination of Des1/SK1 siRNA increased the expression of p21 (Figure 5.7B). Thus, targeting a combination of both Des1 and SK1 is able to induce both markers of senescence in LNCaP AI cells.

A


Des1
p53
Actin
scr
Des1
SK1a
p53
Actin
scr
SK1


SK1
p53
Protein (% Control)
100
50
0
**
Scrambled SiRNA
SK1 SiRNA


Des1
p53
Protein (% Control)
200
150
100
50
0
*
*
Scrambled SiRNA
Des1 SiRNA

B

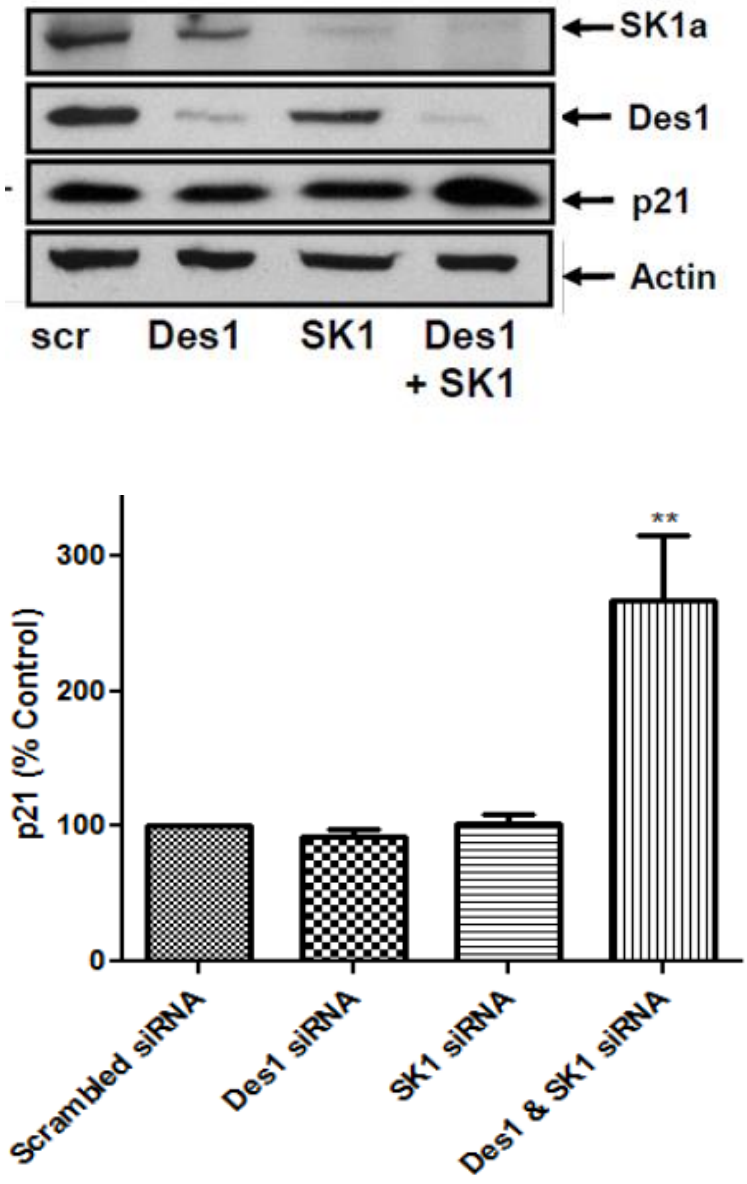


Figure 5.7 ***Effect of Des1- and SK1- targeted siRNA on p53 and p21 expression levels in LNCaP AI cells****. (A) Western blots demonstrating the effect of either Des1 siRNA or SK1 siRNA on p53, Des1 and SK1 levels (B) Western blots demonstrating the effect of Des1 siRNA, SK1 siRNA or their combination on p21 levels. Also shown are histograms of the quantification of western blotting data. Actin was used a protein loading control in all experiments. Results are expressed as means +/− SD for n = 3 experiments. *p<0.05, ** p<0.01 versus control.*

5.3 Discussion

5.3.1 Des1 is a target of ABC294640

Evidence presented herein highlights ABC294640 as a novel inhibitor of Des1. Indeed, ABC294640 treatment resulted in accumulation of several species of dhCer in LNCaP AI cells (Appendix Figure S1). Furthermore, experiments conducted in collaboration with the Piston group confirmed the status of ABC294640 as a Des1 inhibitor (IC_{50} 10.2 +/− 3.7 μM) in a cell based assay in Jurkat cells (McNaughton et al., 2016).

Interestingly, it is also shown herein that ABC294640 degrades Des1 protein in LNCaP AI cells (Figure 5.4). Therefore, it is possible that ABC294640 does not inhibit Des1 directly at all and the apparent inhibition observed is actually solely due to Des1 protein removal. As all of the assays which have been used to assess inhibition of Des1 by ABC94640 have been conducted in cells (Venant et al., 2015; McNaughton et al., 2016), it has not been possible to distinguish a direct effect from an indirect effect. Use of a non cell based assay to investigate the ability of ABC294640 to inhibit Des1 could resolve this issue.

Interestingly, it is also known that Des1 is inhibited by ROS (Idkowiak-Baldys et al., 2010; Fabrias et al., 2012). Moreover, some of the effects mediated by ABC294640 are reversed using antioxidant scavenger NAC; for example, effects on p53 (see Chapter 4). Thus, it is possible that ABC294640 indirectly inhibits Des1 via ROS production. Further experiments are required to discern this. Experiments conducted herein demonstrate that the degradation of Des1 by ABC294640 is up-stream of ROS because it is not reversed using ROS scavenger NAC. It is also possible however that the inhibition of Des1 and the degradation of Des1 by ABC294640 are not mutually exclusive and exist on separate signalling pathways.

Concomitant with the current study, ABC294640 was identified as an inhibitor of Des1 by another group (Venant et al., 2015). The finding of the current study that ABC294640 inhibits Des1 is consistent with the findings of Venant and colleagues. In addition to the inhibition of Des1, they reported that ABC294640 decreased prostate cancer cell viability and reduced cMyc expression, which is also consistent with reports herein (see Chapter 3). They also reported that ABC294640 decreased AR expression (Venant et al., 2015). Notably, these properties are common to the effects observed for SKi which also reduced cMyc, decreased

AR and decreased cell viability in prostate cancer cells (Watson et al., 2012; Tonelli et al., 2013). Moreover, Venant and colleagues reported that ABC294640 induced a >3-fold increase in dhCer species and this was associated with inhibition of Des1 activity. These findings are also supported by data presented herein (Supplementary data, Figure S1). Interestingly, Des1 inhibition by ABC294640 was shown to be independent of SK2 inhibition in the Venant study as increases in dhCers were still observed in samples from SK2 deficient mice. Furthermore, *in vivo* work demonstrated that ABC294640 slowed the growth rate of TRAMP-C2 xenographs in murine studies, parallel with increases in dhCers, indicating causality between dhCer accumulation and growth arrest of xenographs, although additional experiments would be needed to confirm this (Venant et al., 2015). This does however provide some early indication that dhCer accumulation can cause growth arrest *in vivo* and warrants further experiments. Importantly, findings of the current study taken together with the study of Venant and colleagues highlight a novel action of ABC294640 on Des1 *in vitro* and *in vivo*.

5.3.2 Effect of ABC294640 on sphingolipid levels in LNCaP AI cells

In light of the novel findings reported herein with respect to ABC294640, it was of interest to examine changes to the sphingolipidome in response to this compound. Analysis of the multiple components of the sphingolipid pathway is of great value because of the multifaceted and intertwined nature of the pathway and its complex metabolic fluxes. Furthermore, by examining the levels of key bioactive sphingolipids, one can hope to elucidate the particular metabolite or metabolites responsible for mediating the effects of of a pharmacological inhibitor. To this end, sphingolipidomic analysis was performed by LC-MS, which is currently the sole technology capable of producing results of adequate sensitivity and specificity to measure such small quantities of structurally similar analytes (Merrill et al., 2005).

Surprisingly, ABC294640, which degrades SK1 (Chapter 3) failed to significantly reduce S1P levels in LNCaP AI cells, although the trend was downwards (Appendix S1). Perhaps if more samples were analysed, a significant reduction in S1P would be observed; however, this was not possible in the current study. Indeed, Venant and colleagues observed a dose-dependent decrease in S1P levels in response to ABC294640 in TRAMP-C2 prostate cancer cells with the most marked decrease observed upon treatment with 20µM and 30µM

ABC294640, comparable with the 25μM used in the current study (Venant et al., 2015). Alternatively, ABC294640 may not inhibit or degrade SK substantially enough in LNCaP AI cells to produce a marked reduction in S1P levels. This would suggest that the primary mechanism by which ABC294640 elicits its senescent effects in LNCaP AI cells is not through targeting SK and altering the ceramide and S1P axis but by affecting another target. This is supported by the finding that ABC294640 produced similar effects in wild type MEF cells and in MEF cells cultured from SPHK-2 deficient mice, albeit at a higher ABC294640 concentration (Venant et al., 2015).

In support of the notion that the primary mechanism through which ABC294640 exerts its effects is through a target other than or in addition to SK2, lipidomic analysis revealed that ABC294640 treatment resulted in a significant increase in dhCer in LNCaP AI cells, suggesting that ABC294640 targets Des1 in these cells (Figure S1). This data is similar to that published for TRAMP-C2 cells in which it was shown that ABC294640 treatment resulted in an increase in dhCer both *in vitro* and *in vivo* (Venant et al., 2015). However, Venant and colleagues concluded that dhCer accumulation was due to inhibition of Des1 activity by ABC294640 rather than by a reduction in Des1 expression, which they did not assess. This conflicts with findings herein which show that ABC294640 induces a reduction in Des1 protein expression in LNCaP AI cells (Figure 5.1).

Interestingly, both studies concluded that ABC294640 induced an increase in several of the dhCer species measured. These were dhC14:0, dhC16:0, ,dhC22:0 and dhC24:0 in the current study versus dhC14:0, dhC16:0, dhC18:0 , dhC18:1, dhC20:0, dhC20:1 and dhC22:0 in the study by Venant and colleagues (Venant et al., 2015). The latter study also measured dhCer species in tumours from mice injected with TRAMP-C2 cancer cells and then treated with or without ABC294640. Consistent with *in vitro* studies, *in vivo* studies showed a marked increase in dhCer species in tumours resected from ABC294640 treated mice. Importantly, these mice did not exhibit any toxicity associated with ABC294640 treatment but did exhibit a measureable decrease in tumour progression, size and weight versus control mice (Venant et al., 2015). This provides some evidence that work conducted investigating ABC294640 *in vitro* has similar effects *in vivo*.

Interestingly, ABC294640 treatment resulted in differential distribution of ceramide species without causing an overall net gain or loss of total ceramides. Furthermore, ceramides with shorter chain lengths were decreased by ABC294640 treatment and this appears to be counter

balanced by longer chain ceramide species which were increased (Figure S2). These findings are in partial agreement with the findings in TRAMP-C2 cells where a similar flux in molecular species distribution but not total amount of ceramide in response to ABC294640 was observed (Venant et al., 2015). However, in TRAMP-C2 cells, there was an increase in short chain ceramides and a counter regulatory decrease in longer chain ceramides (Venant et al., 2015). It is difficult to rationally explain these conflicting findings in albeit different prostate cancer cell lines. However, these findings highlight is that complex, chain length specific metabolism with respect to ceramide and dhCer is relevant in prostate cancer cell metabolism in response to treatment with ABC294640. This is undoubtedly an area which warrants further study.

Various chain length specific actions of ceramide have been exemplified. For example, in HeLa cells, over expression of CerS1 had no effect with respect to apoptosis in response to ionising radiation. In the same study, over expression of CerS2 and a consequential rise in C24:0 and C24:1 ceramide was anti apoptotic whilst CerS5 and C16:0 increases were pro apoptotic (Mesicek et al., 2010). This study provides a good example of how differential expression of CerS isoforms can alter ceramide content of cells and how this can in turn affect cancer cell metabolism and pathology. Related to this study, a plethora of studies exist which link a rise in C16:0 ceramide to pro apoptotic actions in cancer cells including those conducted in haemopoetic cells (Siskind et al., 2010), leukaemia cells (Thomas et al., 1999; Panjarian et al., 2008), hepatocytes (Osawa et al., 2005), B cells (Kroesen et al., 2001), HCT-116 colon carcinoma cells (Schiffman et al., 2010), neutrophils (Seumois et al., 2007) and particularly relevantly in LNCaP prostate cancer cells (Eto et al., 2003). In another study in MCF-7 breast cancer cells ad HCT-116 colon cancer cells it is reported that over expression of CerS4 and CerS6 resulted in accumulation of short chain ceramides (C16:0, C18:0 and C20:0) whereas over expression of CerS2 resulted in accumulation of long and very long chain ceramides, but only after exogenous addition of long chain Acyl-CoAs for use as substrates. In both cell lines accumulation of short chain ceramides resulted in inhibition of proliferation and induction of apoptosis whereas accumulation of longer chain ceramides resulted in increased cell proliferation and survival (Hartmann et al., 2011) . This study demonstrates that ceramide disequilibrium occurs in oncogenesis and this contributes to cancer cell fate.

Conversely, data presented herein demonstrates a reduction in short chain ceramides and an increase in long chain ceramides in response to ABC294640 treatment in LNCaP AI cells

(Figure S2). This would appear to contradict the majority of published data where short chain ceramides are linked with pro apoptotic effects (Grösh et al., 2012) including studies conducted in LNCaP cells (Eto et al., 2003). However, it has previously been demonstrated that LNCaP AI cells are resistant to apoptosis in response to sphingolipid modulating compounds. For example, LNCaP AI cells are resistant to apoptosis in response to SKi treatment due to differential expression of SK1b which is able to evade proteasomal degradation and in turn confer apoptotic resistance (Loveridge et al., 2010). Perhaps LNCaP AI cells also undergo similar differences in ceramide disequilibrium which result in induction of an alternative, senescent phenotype in response to ABC294640 treatment. It would be of interest to do a direct comparison between LNCaP and LNCaP AI cells with respect to this. However, a comparison of the two cell lines was out of the remit of this study.

Overall, there is plentiful evidence in the literature alluding to the fact that chain length specific actions of sphingolipids exist. A review published by Grösh and colleagues examines the chain length specific properties of ceramides (Grösh et al., 2012). The relevant abundance of ceramides in a system is dictated by the expression of ceramide synthase (CerS) enzyme isoforms in that particular system, as six isoforms of these enzymes exist (CerS1-CerS6) which catalyse formation of ceramides of different chain lengths (Levy and Futerman, 2010). Tellingly, different CerS expression profiles exist in different mammalian tissues (Laviad et al., 2008). However, a caveat of this study was that the complement of ceramide species did not always correlate well with the expected expression of the CerS enzymes. This suggests that the enzymes may be regulated post transcriptionally, adding a layer of complexity to linking expression data with functional consequences.

There are fewer publications which investigate this phenomenon with respect to dhCer. Indeed, there are no published studies which specifically investigate the discrete roles of dhCers of differing chain lengths in cells. One study links high cell density of SMS-KCNR neuroblastoma cells with a reduction in Des1 activity and subsequent accumulation of all species of dhCer (Spassieva et al., 2012). Interestingly, these cells exhibited a G1/G0 growth cycle arrest in response to Des1 inhibition. Furthermore, the authors demonstrate that growth arrest can be recapitulated by conditioning low density cells with media taken from high density cells and also by adding reducing agents (Spassieva et al., 2012). It would perhaps be of interest to determine the effects of cell density on growth arrest and dhCer accumulation in LNCaP AI cells. Nevertheless, the Spassieva study is an example which links dhCer to growth arrest in cancer cells.

5.3.3 Des1 inhibitor fenretinide is a proteasomal activator

Evidence presented herein for the first time highlights fenretinide as a proteasomal activator (Figure 5.6A). This suggests that Des1 is a regulator of proteasomal activity.

Further experimental work to definitively discern the role of Des1 in the regulation of the proteasome would be of benefit to confirm this. This may involve, for example, further siRNA experiments to remove Des1 from cells and then measure proteasome activity. Alternatively, more potent and selective compounds which affect Des1 in the nano molar potency range for example XM642 (Munoz-Olaya et al., 2008) or GT11 (Triola et al., 2004) (discussed in 5.3.4), could be examined for their effect on the proteasome. These would of course first have to be examined for their ability to affect other targets of the sphingolipid signalling pathway.

The role of fenretinide as a proteasomal activator is supported by other studies. For example, it was shown that fenretinide induces the proteasomal degradation of stearoyl-CoA desaturase (SCD) in ARPE-19 human retinal pigment epithelial cells (Samuel et al., 2014). Like SK1, SCD has been shown to enhance cancer cell proliferation and survival through regulation of the balance of saturated to unsaturated fatty acids (Ford, 2010). SCD degradation in this study was fully reversed using the proteasome inhibitor MG132, which is similar to the reversal of SK1 and Des1 degradation by ABC294640 and SKi demonstrated herein (Figure 3.2 and Figure 5.1, respectively). Furthermore, SCD degradation by fenretinide was recapitulated by tunicamycin and thapsigargin, both of which are known inducers of ER stress. Interestingly, both ABC294640 and SKi have previously been linked to induction of ER stress and the unfolded protein response (UPR) (Belanjinski et al., 2010; Evangelesti et al., 2014). These findings, taken with the current study, suggest that Des1 may restrain proteasomal function under basal circumstances. Indeed, Des1 inhibition using fenretinide increased ubiquitination (as measured by western blotting) and also enhanced proteasome activity in a competition based luminogenic assay (Figure 5.6A).

Thus, these data could suggest that Des1 inhibition promotes ER stress and proteasomal activation, coupled to senescence with potentially functions to combat a build up of mis-folded proteins. Interestingly, ER stress and proteasomal activation were blocked using PYR-41, a specific inhibitor of ubiquitin activating enzyme E1 (UAE1) (Samuel et al., 2014). This finding, which suggests that UAE1 is a target of fenretinide, fits well with findings of the

current study, where ubiquitin blots show that fenretinide increases ubiquitination of proteins generally (Figure 5.6B). In light of the findings of Samuel and colleagues, PYR-41 was similarly tested in the current cell system for its potential to reverse Des1 and SK1 degradation; however, no reversal was observed for SK1 (data not shown). This is potentially due to differences in cell types or the fact that SK1 and Des1 could be coupled to alternative ubiquitin activating enzymes, of which there are at least ten (Schulman and Harper, 2009). Further experiments targetting each of the components of the ubiquitin proteasome pathway separately would be required to discern this.

5.3.4 Targeting Des1 induces senescence in LNCaP AI cells.

Data presented herein provides evidence to suggest that Des1 is a mediator of senescence in LNCaP AI cells. Indeed, treatment of LNCaP AI cells with Des1 inhibitor fenretinide inhibited DNA synthesis in LNCaP AI cells with an IC_{50} of 0.58μM (Figure 5.3), which correlates well with its IC_{50} for Des1 inhibition (2.32μM in rat liver microsomes (Illingworth et al., 2011). It would have been interesting to characterise the inhibition of Des1 by fenretinide in LNCaP AI cells in order to gain more specific insight, but this was out with the limitations of the current study. In addition to inhibiting DNA synthesis, fenretinide also increased expression of senescent marker proteins p21 and p53 (Figure 5.4) in a manner similar to ABC294640 and SKi. This is a good indicator that compounds affecting Des1 may be involved in the induction of senescence of LNCaP AI cells. It would also have been useful to assess the cell cycle stage at which fenretinide induced growth arrest in LNCaP AI cells.

Interestingly, fenretinide also decreased SK1 protein levels (Figure 5.4). Therefore, this limits the use of fenretinide as a tool to study Des1 exclusively. This also raised questions over the catalytic specificity of fenretinide for Des1 and against SK1. However, it was confirmed that fenretinide does not inhibit SK1 or SK2 using a recombinant protein/ radiometric assay system (Figure 5.5).

siRNA experiments were used to confirm the data generated using pharmacological inhibitors, i.e. that targetting Des1 lead to the up-regulation of p53 expression. Indeed, siRNA knockdown of Des1, but not SK1, recapitulated the effects of ABC294640, SKi or fenretinide in LNCaP AI cells (Figure 5.7A). Surpringly, siRNA knockdown of both Des1 and SK1 in combination was required to also induce induction of p21. This data suggests that it is the modulation of multiple facets of sphingolipid signalling including Des1 and SK1 by

ABC294640, SKi and fenretinide which leads to the senescent phenotype observed using these compounds. This contrasts with the effect of compounds which are solely selective for SKs, which do not induce p53 or p21 protein in LNCaP AI cells (Chapter 4). Although current data suggests that targeting Des1 plays a role in the induction of senescence, it would be possible to further investigate this using other Des1-specific inhibitor compounds such as XM642 (Munoz-Olaya et al., 2008) or GT11 (Triola et al., 2004). These would first need to be tested for their ability to inhibit SK1 or degrade SK1 protein, as previously mentioned.

GT11 was the first nano molar potency Des1 inhibitor to be synthesised. It exhibited an IC_{50} of 23nM in cells. At higher concentrations (>5μM), the compound provoked an accumulation of S1P and dhS1P and decreased *de novo* sphingolipid synthesis, which was reported to be due to a non specific action on S1P lyase (Triola et al., 2008). Reports using this inhibitor in cells are very limited, perhaps due to the non specific actions it exerts at higher concentrations. XM642 is another nanomolar potency Des1 inhibitor which inhibits Des1 both *in vitro* and *in vivo*, with IC_{50} values of 8.2μM and 780nM, respectively. XM642 was also shown to be a mixed inhibitor (Munoz-Olaya et al., 2008). Several studies have been conducted using XM642 which yielded results consistent with the findings of the current study. Interestingly XM642 was shown to induce dihydroceramide accumulation which resulted in induction of autophagy in the absence of apoptosis in HGC-27 gastric cancer cells (Signorelli et al., 2009). This is consistent with the actions of ABC294640, which also induces autophagy in A-549 kidney adenocarcinoma cells as well as PC-3 prostate cancer cells and MDA-MB-231 breast cancer cells (Belanjinski et al., 2010). This makes it likely that this is a result of dhCer accumulation in response to ABC294640 (Appendix S1). On the contrary, SKi has previously been shown to inhibit autophagy (Watson et al., 2013). This could be a reflection of different efficacies between the multiple targets of ABC294640 and SKi (SK1, SK2 and Des1) or could represent a difference in time profiles between the two compounds, possibly also for each of the different targets. Indeed, it was shown n HCG27 cells that XM642 induced a transient early increase in dhCers which were subsequently converted to other sphingolipids. This could, of course, change the phenotype observed at a given time point (Gagliostro et al., 2012). Further work is required to stratify the relative contribution of the individual targets of ABC294640, SKi and fenretinide to the phenotype observed in LNCaP AI cells, potentially over multiple time points (both early and late) to fully discern this. Furthermore, it would be interesting to conduct further experiments to investigate the links between autophagy, ER stress and senescence. Very preliminary work in

this area using autophagic mediator compounds failed to generate any observable trend in relation to senescence (Figure 4.11). It would be interesting to conduct a time course of ABC294640, SKi and fenretinide and examine these for autophagic markers, such as the balance between LC3-I and LC3-II, for instance.

XM642 has also been shown to modulate the cell cycle *via* Des1 and dhCer, consistent with findings herein, where fenretinide induced growth arrest with an IC_{50} of 0.58μM (Figure 5.3) and induced cell cycle inhibitor p21 expression (Figure 5.4). It has previously been reported that inhibition of Des1 and dhCer accumulation using XM642 modulates Rb hyper-phosphorylation and induced growth arrest at G0/G1 phase in SMS-KCNR human neuroblastoma cells (Kraveka et al., 2007). dhCer accumulation was also shown to modulate cyclin D1 expression. This is reminiscent of the ability of SKi to modulate cyclin D1 expression (Tonelli et al., 2013). Under basal conditions, cyclin D1 (like cMyc) is subject to constant turnover by the ubiquitin proteasomal system. Therefore, activation of the proteasome via Des1 inhibition and dhCers accumulation by SKi, ABC294640 and fenretinide is the most likely cause of the down regulation of cyclin D1 observed upon SKi treatment (Tonelli et al., 2013). It is tempting to speculate that this is also the case for XM642 treatment, although this would need to be tested. Interestingly, cyclin D1 has previously been shown to be a gate keeper of G0–G1/S phase transition (Sherr and Roberts, 1999). Thus it is also possible that cyclin D1 removal in response to the compounds which down regulate Des1 could play a role in their induction of senescence. It would be interesting to test this by, for example, using cyclin D1 siRNA. It is likely however that these compounds target a variety of proteins subject to removal by the proteasome (including but not exclusively SK1, Des1, cMyc and cyclin D1) which results in the senescence phenotype observed upon treatment. Further work is required to stratify the relative contribution of each down-stream mediator of ABC294640, SKi and fenretinide to the induction of senescence and also to identify the particular mechanisms involved.

In HCG27 cells, XM642 delayed G1/S transition of the cell cycle (Gagliostro et al., 2012). Furthermore, XM642 induced autophagy in these cells. It is interesting to speculate that it is ABC294640's action on Des1 which elicits the autophagic response in A-498 kidney carcinoma cells (Beljanski et al., 2010). Further work is required to establish the apparently conflicting nature of the inhibiton of autophagy by SKi. As previously mentioned, this could be due to time scale differences. Indeed, the inhibition of autophagy observed upon SKi treatment was tested at 48 hours and it is possible that at earlier time points a stimulatory

effect could be observed (Watson et al., 2012). Interestingly, the autophagic effect observed in response to Des1 inhibition is reported to be a survival response - blocking autophagy using 3MA impaired cell survival in response to XM642 by 40-50% (Gagliostro et al., 2012). In the same study, X642 also induced an ER stress response. Thus, it is likely that Des1 inhibiton, followed by dhCers accumulation, leads to autophagy and ER stress which mediates a stress response via p53. It is tempting to speculate that if this is coupled to SK1 inhibition that a programme of cellular senescence is then initiated via p21. Indeed, siRNA data in the current study shows that only dually targetting SK1 and Des1, in combination, induces the sensescence marker, p21 (Figure 5.7).

Des1 ablation has been shown to reduce cell proliferation in MEFs as well as in an *in vivo* model. Thus, MEFs extracted from Des1 knockout mice proliferated more slowly than wild type counterparts. Furthermore, activation of autophagy was also observed. Sphingolipid dysregulation was also prominent with dihydro forms of the most common sphingolipids being abundant as opposed to monounsaturated forms (Siqqique et al., 2013). Interestingly, Des1 ablation also resulted in the activation of both anabolic and catabolic signalling pathways. This is paradoxical and suggests that perhaps Des1 and dhCers determine whether a cell undergoes a programme of catabolic activity or anabolic. This could translate to whether a cell adapts and survives in response to cellular stress or to succumb to stress and apoptose in the context of cancer.

5.3.5 Des1 as a target for cancer treatment

The first indication that Des1 may be a target for cancer treatment came when it was reported that Des1 expression regulates metastases in nude mice (Zhou et al., 2009). Since then, several studies have suggested potential of Des1 as a novel target for cancer therapy (Summers et al., 2015). Indeed, a plethora of anti tumour agents exhibit Des1 activity. These include curcumin, celecoxib, resveratrol and Δ9-tetrahydrocannabinol. It has been suggested that the anti-cancer activity of these compounds is at least, in part, a result of dhCer accumulation (Fabrias et al., 2012). However, it has yet to be ascertained how much of their anti-tumour activity is attributable to Des1 inhibition/dhCer accumulation and it is certain each of these compounds lack selectivity for Des1.

An important study was recently conducted by Pitson and colleagues which clearly highlights the potential of Des1 a chemotherapeutic target. This study attempted to differentiate the

actions of SKi based on its various known effects and side effects, which are SK1/SK2 inhibition (French et al., 2010), SK1 degradation (Loveridge et al., 2010; Tonelli et al., 2010) and finally indirect Des1 inhibition (Cingolani et al., 2014). This study aimed to correlate the anti-cancer efficacy of SKi with each of these targets individually. To this end, several novel compounds were synthesised which were either selective inhibitors of SK1, SK2 or Des1 (or combinations of multi selective inhibitors for these three proteins). Remarkably, this study reported that compounds which conferred selectivity solely for Des1 had anti-proliferative effects. In PC-3 prostate cancer cells, compounds which were selective for Des1 inhibited growth with IC_{50}s of 0.34μM and 0.7μM, whereas those which were selective for SK1 and/or SK2 showed no significant growth inhibition (Aurelio et al., 2016). Interestingly, there was a correlation between growth inhibition and compounds which could alter redox status. This overlaps with both the finding that Des1 is inhibited by oxidative stress (Idkowiak-Baldy et al., 2010) and also the finding that SKi is a known inducer of ROS (Watson et al., 2013).

Importantly, study of Aurelio and colleagues (Aurelio et al., 2016) provides an explanation for some of the conflicting reports surrounding SK1 and SK2 inhibitors as anti-cancer agents. ABC294640 and SKi exhibit favourable efficacy in anti-cancer models in many systems over other more selective inhibitors of SK1 and/or SK2. Furthermore, numerous studies have been conducted which have aimed to evaluate the therapeutic promise of targetting SKs in human cancer using these compounds. Other more potent inhibitors for SKs, including PF543 which exhibits nanomolar potency for SK1, (Schnute et al., 2012) have been synthesised which do not display anti-cancer properties. This has led to questions over the promise of targetting SKs and the sphingolipid signalling pathway in cancer. The Schnute study highlights the fact that extreme care must be taken when assigning the effects of each inhibitor in relation to disease when the full impact of each compound on the wider sphingolipid signalling network is not known. For example, even under circumstances of S1P removal, PF543 was unable to generate accumulation of ceramide (Schnute et al., 2012). This indicates that additional compensatory mechanisms or shifts in the pathway likely exist to mitigate metabolic perturbations in response to this compound. This again highlights that the sphingolipid signalling network is more complicated than first thought and that there are enzymes and metabolites (or species of metabolites) which are yet to be properly characterised in terms of their physiological importance (Pyne et al., 2016). This is evident in the finding that dihydroceramide, which was previously thought to be biologically inactive, has an important role in mediating cell fate (Siqqique et al., 2015). Moreover, *trans*-2-hexadecenal, a

breakdown product of S1P cleavage by S1P lyase, was also recently found to have previously undiscovered intracellular actions (Kumar et al., 2011; Aguilar and Saba, 2012). Recent advances in lipidomics should progress understanding of the complicated nature of sphingolipid signalling in the future.

A study in glioblastoma also highlights the promise of targetting Des1 and dhCer in cancer treatment (Noack et al., 2014). Thus, a combination of sub-lethal doses of SKi and chemotherapeutic drug temozolomide (TMZ) resulted in a reduction in cancer cell viability which, importantly, did not affect astrocyte viability. Such treatment triggered a caspase-3-dependent cell death that was preceded by accumulation of dhSph and dhCer, oxidative stress, ER stress, and autophagy. Interestingly, this study demonstrated that a build up of dihydro sphingolipids rather than their counterparts was responsible for treatment efficacy. Furthermore, the study demonstrated a negative correlation between expression of anti oxidant genes and sensitivity to treatment (Noack et al., 2014). This provides further evidence that Des1 is a gatekeeper of oxidative stress which perhaps acts as a molecular switch between an adaption to oxidative stress and or an apoptotic response.

In support of this, a study in leukaemia cells demonstrated changes in the cell transcriptome in response to fenretinide treatment (Wang et al., 2009). Numerous cell stress responses were initiated by fenretinide, including a redox response, an ER stress/unfolded protein response and proteasome activation, consistent with the findings of the current study. Interestingly, these events were orchestrated by transcription factors Nrf2 and heat shock factor 1 (HSF1) (Wang et al., 2009). It has also previously been reported that SKi activates and stabilises Nrf2 by formation of keap1 dimers in primary bronchial human epithelial cells in a manner which was independent of SK inhibition (Mercado et al., 2014). Thus, it is tempting to speculate that it is the inhibition of Des1 activity by SKi and fenretinide which is responsible for the stabilisation and activation of Nrf2 by these compounds, which would highlight a novel interaction between Des1 and Nrf2. It would also be interesting to determine if ABC294640 was similarly was able to activate Nrf2 and downstream target genes. This could be resolved using siRNA for Des1 to investigate keap1 dimerisation and Nrf2 protein levels. Alternatively, it is possible that Des1 and SK1 could act in concert to stabilise Nrf2. The answer to this question could be resolved using siRNA for either SK1 or Des1 and in combination, in line with the current study.

Further work is required to elucidate the mechanism of Des1 inhibition by ABC294640 and SKi which could, in turn, reveal a novel therapeutic target to be exploited. This is likely to be an enzyme involved in redox signalling or cellular response to oxidative stress. It will also be interesting to see how the anti cancer properties of the novel Des1 inhibitors synthesised by Pitson and colleagues (Aurelio et al., 2016) translate to *in vivo* and clinical studies in the future. Finally, in light of the knowledge presented herein and elsewhere, there is clear rationale for the development of nanomolar potency Des1 selective inhibitors to be used either as tool compounds to fully discern the function of this enzyme in cells or as novel chemotherapeutic agents in cancer or perhaps other diseases where Des1 may play a role.

5.4 Summary

The present study adds to knowledge regarding Des1 and provides evidence that Des1 is an important intracellular effector of cell fate in LNCaP AI cells. Furthermore, evidence presented herein suggests that Des1 and SK1 are two enzymes of the sphingolipid pathways which are pivotal for mediating growth arrest and senescence of LNCaP AI cells. Thus, these findings provide rationale for further investigation of the role of Des1 both alone and in concert with SK1 in cell fate control in prostate cancer and other cancers alike.

Findings herein also suggest that targeting both SK1 and Des1 in combination may represent a novel means of targeting advanced stage prostate cancer. Furthermore, this provides new knowledge which can be used as a foundation for development of novel small molecule inhibitors which similarly target SKs and/or Des1 in order to further investigate the potential of modulating multiple facets of sphingolipid signalling (both *de novo* synthesis and the sphingolipid rheostat) to induce growth arrest of prostate cancer cells which ultimately could lead to the development of novel therapeutics.

CHAPTER 6:

GENERAL DISCUSSION

6. CHAPTER 6 - General Discussion

6.1 Selectivity of SK inhibitors – is it possible to avoid cross reactivity with other enzymes in the sphingolipid pathway?

An obvious issue which the current study brings in to the spotlight is one surrounding the selectivity of lipid like SK inhibitors for use as study tools or as chemotherapeutic compounds. In light of this, it is important to discuss the issue of the specificity and potency of the SK inhibitors which are currently available. Indeed, the development of more specific and more potent inhibitors of classical SL targets as well as the development novel inhibitors of 'orphan' SL sphingolipid targets is seen as pivotal to the advancement of the field and there is continued research to achieve this.

This pressing issue is not one which is unique to SKs but which affects kinases universally. Indeed, both lipid and protein kinases are well studied therapeutic targets due to their pivotal role in regulating critical cellular functions.

The majority of inhibitors of SK1 and SK2 inhibitors which have been created thus far target the sphingosine binding site and are sphingosine like in nature. Conversely, few inhibitors which target the ATP binding site have been synthesised. This may be because targeting the ATP binding site may affect a similar site in other kinases (all of which use ATP). It is sensible to presume that an inhibitor which is similar to an endogenous lipid which undergoes rapid metabolism may also be metabolised in cells. This could ultimately lead to selectivity towards alternative targets or perhaps even redundancy in cells. The fact that the sphingolipid signalling pathway is also subject to temperospatial regulation adds an additional layer of complexity to this problem. Indeed, there are reports in the literature of SK modulating compounds which are altered by cellular metabolism. For example, sphingosine analogue FTY270 (Gilenya^{R}) is an anti inflammatory pro drug currently licensed for treatment of multiple sclerosis which acts as a substrate for SK2. When FTY720 enters cells it is phosphorylated by SK2. The phosphorylated form then acts as a potent agonist at four S1P receptors. (Brinkmann et al., 2004). Following the original publications, it was shown that FTY720 is also a functional antagonist of the $S1P_1$ receptor which causes $S1P_1$ proteolysis upon drug binding (Chun et al., 2010). It was originally thought that the anti inflammatory properties of this drug were exerted via $S1P_4$ agonism but was subsequently shown to be via $S1P_1$ receptor proteolysis (Chun et al., 2010). These findings highlight that additional effects

of compounds can exist which are the potentially therapeutic despite differing from the initial intended target or mechanism of action. Indeed, the metabolic conversion and/ or modification of inhibitors of SKs and other SLs mediators is something which has not yet been fully studied. This information would be extremely useful however would be immensely difficult to discern.

Several reportedly selective inhibitors of SKs have been synthesised to date (including ABC294640, ROMe and K145) and all of these exhibit micromolar potency. Of these ABC294640 is seen as the 'gold standard' SK2 inhibitor. Indeed, ABC294640 is the most advanced of any of the available SK inhibitors towards the clinic and is in phase I clinical trials for pancreatic cancer, solid tumours and refractory/relapsed diffuse large B cell lymphoma as well as phase IIa trials for cholangiocarcinoma, renal cancer and B-cell lymphoma. Although ABC294640 reduces S1P in plasma and tumours, it is in fact a relatively poor inhibitor of SK2. Indeed, in an assay using recombinant SK1 and SK2, ABC294640 inhibited SK2 with an IC_{50} of 60μM with no activity towards SK1 at concentrations <100μM and no activity towards a broad panel (n=20) of diverse kinase substrates including lipid kinases (French et al., 2010). ROMe on the other hand, inhibits SK2 with an IC_{50} of 27 ± 1.3 μM (Lim et al., 2011) and K145 inhibits SK2 with an IC_{50} of 4.30±0.06μM although is only selective for SK1 over SK2 at concentrations >10μM (Liu et al., 2013). However it must be taken into account that these inhibitors could also affect Des1 or other targets in a similar manner to ABC294640 and this has not yet been studied. In the study by French and colleagues it is noted that ABC294640 is not the most potent inhibitor of the series synthesised in the study although it is superior in solubility, oral absorption and *in vivo* activity. The superior *in vivo* activity of ABC294640 versus other more potent analogues is perhaps an indicator that other targets are being hit. Although the French study continues to characterise ABC294640 extensively *in vivo* and *in vitro* SK1 protein levels are not assessed. ABC294640 treatment on SK1 mRNA levels have been assessed in prostate cancer cells and are surprisingly increased by >300% (Gao et al., 2012). This is likely a compensatory mechanism in response to the post translational degradation of SK1 via the proteasome by ABC294640 (McNaughton et al., 2016).

ABC294640 has also been shown to inhibit Des1 (Venant et al., 2015; McNaughton et al., 2016). Remarkably, ABC294640 is a more potent inhibitor of Des1 than it is of SK2. ABC294640 was shown to inhibit Des1 with an IC_{50} of 10.2 +/− 3.7 μM (McNaughton et al., 2016). Interestingly, this correlates better with the intracellular actions of ABC294640,

although causality has not been stratified in uniform cell system. This discovery that ABC294640 inhibits Des1 was made five years after the synthesis and characterisation of ABC294640 as a first in class SK2 inhibitor (French et al, 2010). This is reminiscent of the discovery that SKi is selective for both SK1 and SK2, as it was originally thought that SKi was a selective inhibitor of SK1. However, it is important to note that when this inhibitor was characterised isoform selectivity measurements were not made. SKi was shown only to be a pan inhibitor of SK which reduced S1P formation in cells. Selectivity assessment against other kinases only included a small panel of protein kinases ((ERK2 and PKCα) and a lipid kinase (PI3K) and SKi was without effect (French et al., 2003). A more advanced kinase profiling system would be the gold standard of investigation currently for the development of novel therapeutic kinase inhibitors of which several are currently commercially available.

Although it is certain that the current panel of SK inhibitors being used are more promiscuous in their target selection than originally thought, it is fair to argue in light of the current study that this is not always a negative thing. Indeed, opportunities could arise due to either the identification of novel therapeutically relevant targets or the generation of a multi faceted attack on oncogenic targets. Indeed, ABC294640 and SKi employed in the current study have been shown to negatively regulate AR, cMyc, SK1, SK2 and Des1 which could be therapeutically advantageous as these each of these targets fuel prostate cancer survival and progression (Tonelli et al., 2013; Cingolani et al., 2014; Venant et al., 2015; McNaughton et al., 2016). Furthermore, the current study provides rationale for further investigation of Des1 as a therapeutic target either alone or in combination with SK1 (and likely other targets) as current data suggests that targetting both of these enzymes together is more efficacious than targetting each alone in prostate cancer cells.

Discoveries made herein highlight the need for further characterisation of SK1 and Des1 inhibitors and dictate that more care should be taken when attributing the effects of an inhibitor to a single target as being entirely causal for its pharmacological effect. In spite of this, it is not common practice to universally screen inhibitors against the other targets in the sphingolipid signalling pathway and currently no study exists which tests all available compounds against the entire sphingolipdome.

6.2 SK1 and Des1 – stress sensors in cells?

Data presented herein suggests that SK1 protein expression could be regulated by its own activity. Indeed, it appears that when SK activity drops below a certain threshold (for example upon inhibitor treatment) the enzyme itself is then degraded. This suggests that SK1 activity either propagates its own expression, inhibits its own removal or both. Furthermore, the current study suggests that additional mechanisms exist to regulate SK1 expression which do not involve target engagement but rather are due to a stress response (involving ROS) which activates the proteasome to remove SK1. This could highlight SK1 as the molecular determinant which signals for activation of a downstream stress cascade which facilitates senescence or apoptosis.

Throughout the literature there are multiple accounts of DNA damaging agents causing the down regulation of SK1 protein including those which induce p53 protein. Actinomycin D (Act D) caused the post-transcriptional degradation of SK1 in MCF-7 breast cancer cells and this effect was recapitulated using alternative DNA damaging agents such as etopside, doxorubicin and γ-irradiation (Taha et al., 2004). In support of this, it was further demonstrated that removal of SK1 increases sensitivity to doxorubicin induced DNA damage (Huwiler et al., 2011). Thus, SK1 knockdown triggered oxidative stress by increasing ROS formation. This was recapitulated by SK inhibitor SKi and ultimately resulted in cells being more sensitive to doxorubicin induced DNA damage (Huwlier et al., 2011). Findings of the current study place p53 accumulation down-stream of oxidative stress. Therefore these findings of Huwiler and colleagues are consistent with those of the current study which suggest that SK1 is up stream of ROS generation, which would explain the lack of ability of ROS inhibitors or anti-oxidants to reverse the degradation of SK1 protein.

It is tempting to speculate that these agents (via DNA damage) cause activation of the proteasome in a manner similar to ABC294640 and SKi, which in turn activates the proteasome. Indeed, it has previously been demonstrated that etopside induced apoptosis can be prevented by proteasome inhibitors in both rat thymocytes (Stefanelli et al., 1998) and human leukemic cells (Watanbe et al., 2000). Similar links between doxorubicin and the proteasome have been reported. It has been demonstrated that doxorubicin activates the proteasome via action on both the proteasomal machinery and the up-stream ubiquitin apparatus (Liu et al., 2008). Furthermore, the proteasome has also been shown to have a role

as a carrier which promotes the nuclear translocation of doxorubicin, ultimately enhancing its therapeutic efficacy (Kiyomiya et al., 1998). Interestingly PICT1, which is a nucleolar protein that negatively regulates p53 function via regulating MDM2 was shown to undergo ubiquitin proteasomal degradation in response to Act D and doxorubicin as well as 5-fluorouridine in human cancer cells including MCF-7, HeLa, U20S and H2119 cells which in turn lead to the accumulation of p53. These effects were blocked by three distinct proteasome inhibitors (MG132, epoxomycin and lactacystin) but not by lysosomal inhibitors CA047Me or Pepstatin A. The authors concluded a role of PICT1 as a sensor of nucleolar stress which undergoes atypical proteasomal degradation in response to stress (Maehama et al., 2014). These findings are incredibly relevant in relation to the current study and even more so in light of the induction of p53 accumulation in response to PICT1 degradation. Indeed, p53 has previously been shown to act up stream of SK1 and it is possible that SK1 and/or Des1, via an unidentified mediator, influence p53 protein expression or degradation.

In a study by Taha and colleagues, p53 was placed up-stream of SK1. Thus, when p53 up-regulation was inhibited prior to Act D treatment, the effect on SK1 was reversed, suggesting p53 dependence in that system. Furthermore, the cell death response was rescued by S1P treatment. Importantly, this highlights that down regulation of SK1 protein/activity by the DNA damaging agent, Act D, was necessary for them to elicit a chemotherapeutic response. These findings taken together could highlight a role of SK1 as a universal stress sensor and mediator of drug resistance in cancer cells or could suggest that there is an undiscovered link in which SK1 regulates p53 protein, potentially involving Des1. Indeed, high expression of SK1 has previously been proven to confer chemotherapeutic resistance in a number of cancer cells (Taha et al., 2004; Bonhoure et al., 2006; Baran et al., 2007; Guillermet-Guibert et al., 2009).

In support of this it was recently demonstrated that SK1 was a mediator of drug resistance in HNSCC. Transcriptomic profiling revealed SK1 as a potential mediator of E2F7-dependent resistance to anthracyclines. SK1 was also shown to be a downstream target of E2F7. Importantly, pharmacological inhibiton of SK1 using SKI-I or knockdown of SK1 using shRNA enhanced the sensitivity of HNSCC cells to doxorubicin both *in vitro* and *in vivo*. (Hazar-Rethinam et al., 2015)

Des1 has also been coined an ‘oxidative stress bio-sensor’ in cells. This is based on the amplitude and kinetics of dhCer accumulation as a result of Des1 inhibition in response to oxidative stress (hypoxia in the case of the study in question) (Devlin et al., 2011).

The aforementioned studies separately examine the role of Des1 and SK1 as stress sensors in cells but research into the possible synergistic nature of these two sphingolipid mediators as stress sensors has not yet been undertaken. Links between Des1 and DNA damaging chemotherapeutics, such as Act D, doxorubicin and etopside, has also yet to be studied. Both of these avenues of interest provide impetus for further study in this area.

6.3 Defining the chemotherapeutically relevant facet of ABC294640 and SKi action and potential for translation to the clinic – one or multiple targets?

There is compelling evidence which suggests that targetting SK1 is an effective means of treating cancer. These include *in vitro* studies which show that SK1 is pivotal for cancer growth and progression and that aberrant SK1 signalling is a hallmark of oncogenic transformation (reviewed in Pyne & Pyne, 2010; Pchejetski et al., 2008; Sukocheva et al., 2009; Brizuela et al., 2010). These data are backed up by clinical studies which demonstrate that high expression of SK1 correlates with acquisition of chemotherapeutic resistance, reduced survival and increased recurrence of disease (Van Brocklyn et al., 2005; Kawamori et al., 2006; Ruckäberle et al., 2008; Li et al., 2009; Watson et al., 2010; Long et al., 2010; Ohtoski et al., 2012). Studies conducted *in vivo* also support a role of SK1 as a therapeutic target. It has been proven in murine models of cancer that inhibiting SK1 reduces tumour growth and metastasis (French et al., 2006; Brizuela et al., 2010; Pchejetski et al., 2010).

In spite of this extensive groundwork which clearly establishes SK1 as an oncogenic mediator, drugs which target solely target SK1 have yet to see translation to the clinic. Indeed, ABC294640, developed as a SK2 inhibitor, is the only compound which targets SKs which has progressed to clinical trials for cancer treatment. Furthermore, PF543, which is a nanomolar potency SK1 inhibitor which removes S1P from cells, failed to exhibit any inhibition of proliferation in cancer cells (Schnute et al., 2012). This highlights the fact that the situation is far more complex than initially thought and rather than making SK1 redundant as an oncogenic mediator perhaps suggests that additional work is required to elucidate the role of SK in cancer taking the entire sphingolipdome into account. Indeed, ABC294640, which the current study has shown to affect multiple oncogenic mediators, is efficacious in the clinic. This links to the previous idea that a lack of selectivity can be seen as opportunistic in the correct context.

Studies examining the role of Des1 in cancer have only begun to emerge. Indeed, a recent study by Aurelio and colleagues demonstrated that Des1 inhibition by SKi and SKi analogues was primarily responsible for the anti proliferative effects of these compounds. Interestingly, they concluded that SK2 inhibition and Des1 inhibiton may act co-operatively but state that this remains to be verified (Aurelio et al., 2016). The aforementioned study lead to the

generation of several nanomolar potency Des1 inhibitors and future work which tests these inhibitors in cancer cells and murine models of cancer should yield interesting data.

In the current study, it is demonstrated that ABC294640 and SKi induce a similar phenotype, i.e. senescence. It is also demonstrated, here and elsewhere, that both of these compounds have effects on at least five targets including cMyc, AR, Des1, SK1 and SK2. It is therefore possible that the induction of a catastrophic collapse of cancer signalling networks by these compounds due to both on and off target effects is responsible for the induction of senescence in LNCaP AI cells by these compounds. Thus, it is perhaps more efficacious to affect multiple targets in order to induce an anti oncogenic phenotype in these cells and, potentially, in the clinical setting.

6.4 Studies using ABC294640 as a selective SK2 inhibitor – could SK1 or Des1 also be involved?

A literature search of publications using search terms 'ABC294640' and 'sphingosine kinase 2' generates a total of 48 publications ranging from 2010 until 2018. Many of these studies directly assign selective SK2 inhibition to the effects observed in the study. These include studies in breast cancer (Antoon et al., 2010, Antoon et al., 2011, Antoon et al., 2012), hepatocellular carcinoma (Beljanski et al., 2011), arthritis (Fitzpatrick et al., 2011a; Fitzpatrick et al. 2011b; Baker et al., 2013; Xu et al., 2014), ovarian cancer (White et al., 2013), multiple myeloma (Venkata et al., 2014; Sundaramoorthy et al., 2018), lung cancer (Yang et al., 2015; Guan et al., 2016), colorectal cancer (Xun et al., 2015; Ding et al., 2016), pancreatic cancer (Lewis et al,. 2016) and prostate cancer (Gestaut et al., 2014; Schrecengost et al., 2015; Venant et al., 2015). Although the findings of the current study do not invalidate the findings of these studies, they perhaps do provide rationale for further mechanistic investigation of ABC294640 in these disease settings and models in light of the multi faceted nature of ABC294640 action demonstrated in the current study.

For example, the study by Xu and colleagues highlights the controversial role of SK2 in osteoarthritis (OA) (Xu et al,, 2014). In two studies ABC294640 reduced disease burden in rodent OA models (Fitzpatrick et al., 2011a; Fitzpatrick et al., 2011b). ABC294640 was shown to attenuate collagen induced arthritis in mice and also reduce bone and cartilage degradation. Additionally, ABC294640 reduced adjuvant-induced arthritis in rats (Fitzpatrick et al., 2011a). In another study by the same group, ABC294640 treatment reduced knee joint tissue damage and pain in a rat model of monoiodoacetic acid induced OA (Fitzpatrick et al., 2011b). However, another study interestingly reported that whilst ABC294640 improved TNFα induced arthritis in one murine model, genetic ablation of SK2 worsened arthritis (Baker et al., 2012). Furthermore, knockdown of SK1 was shown to be protective of arthritis in this model (Baker et al., 2012). The authors do state that SK1 activity was not affected upon genetic ablation of SK2; however they do not consider other potential targets of ABC294640, such as Des1. Although these studies are not directly associated with cancer they provide examples which raise question over the actions of ABC294640. Of course experiments would need to be conducted to delineate the role of ABC294640 in OA discerning the actions of each of its targets separately using both pharmacological and molecular approaches.Indeed, the authors of the Baker study conclude the importance of using specific SK inhibitors as opposed to more promiscuous inhibitors which could affect

targets with potentially opposing affects in inflammatory conditions such as OA and cancer (Baker et al., 2012).

6.5 Conclusion

Findings of the present study add to current knowledge surrounding the function of SKs in prostate cancer cells. Furthermore, evidence presented herein for the first time highlights Des1 as an important regulator of prostate cancer cell fate. Importantly, this could highlight targeting Des1 either alone or in combination with SK1 as a potential novel therapeutic approach for the treatment of advanced stage prostate cancer and other cancers alike.

Additionally, knowledge presented herein highlights the need to design more selective inhibitors which target either SK1 or Des1 alone so that these can ultimately be tested as anti-cancer therapies in cell systems, *in vivo* systems and perhaps, eventually, in clinical trials.

CHAPTER 7:

REFERENCES

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APPENDIX:

SUPPLEMENTARY DATA

Appendix: Supplementary Data

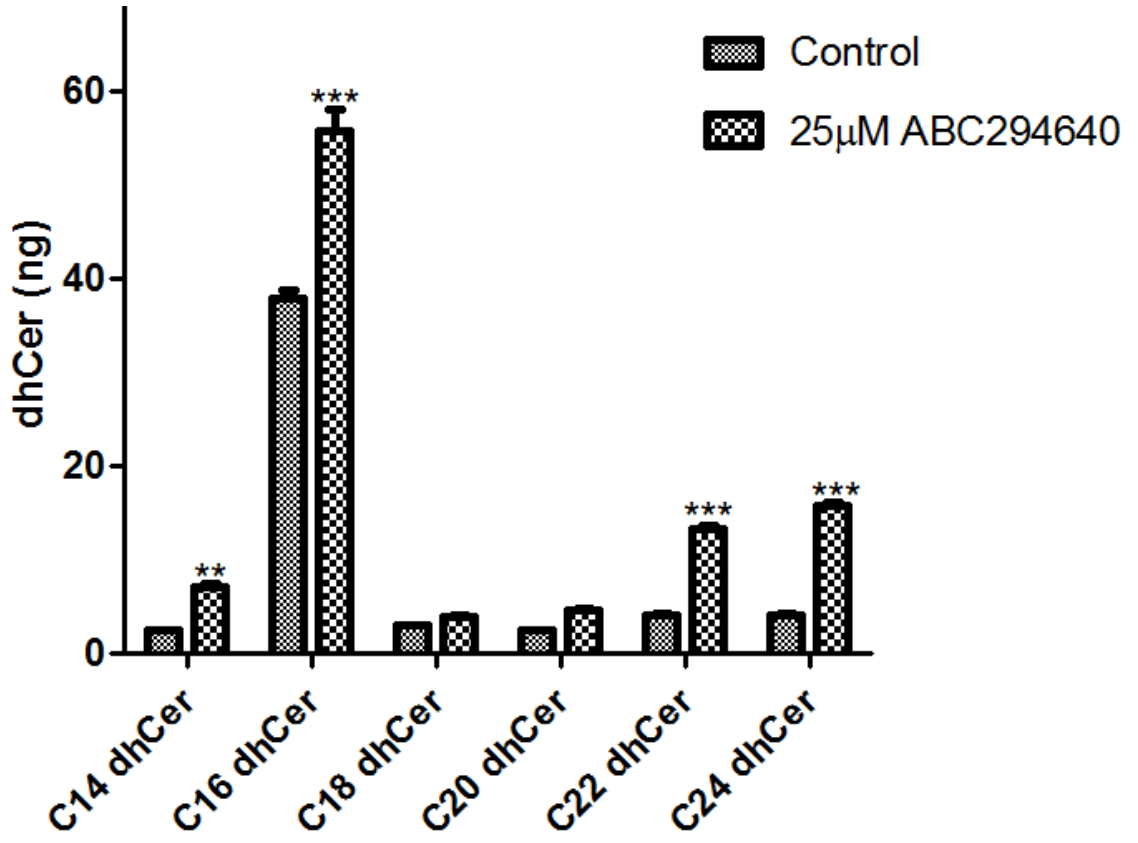

Figure S.1 ***Mass spectrometry analysis of dhCer levels in LNCaP AI cells treated with ABC294640(in collaboration with Michael Wakelam and Qifeng Zhang (Babraham Institute, Cambridge, UK)***. *Bar chart showing the effect of ABC294640 (25 μM, 24 hours) on dihydroceramide levels in LNCaPAI cells. Data are presented as ng per sample (mean with standard deviation of triplicate assays). ** p value <0.01, *** p value <0.001 for ABC294640-treated vs control cells. The sphingolipids analysed were dihydroceramides (DhCer-16:0-DhCer-24:0).*

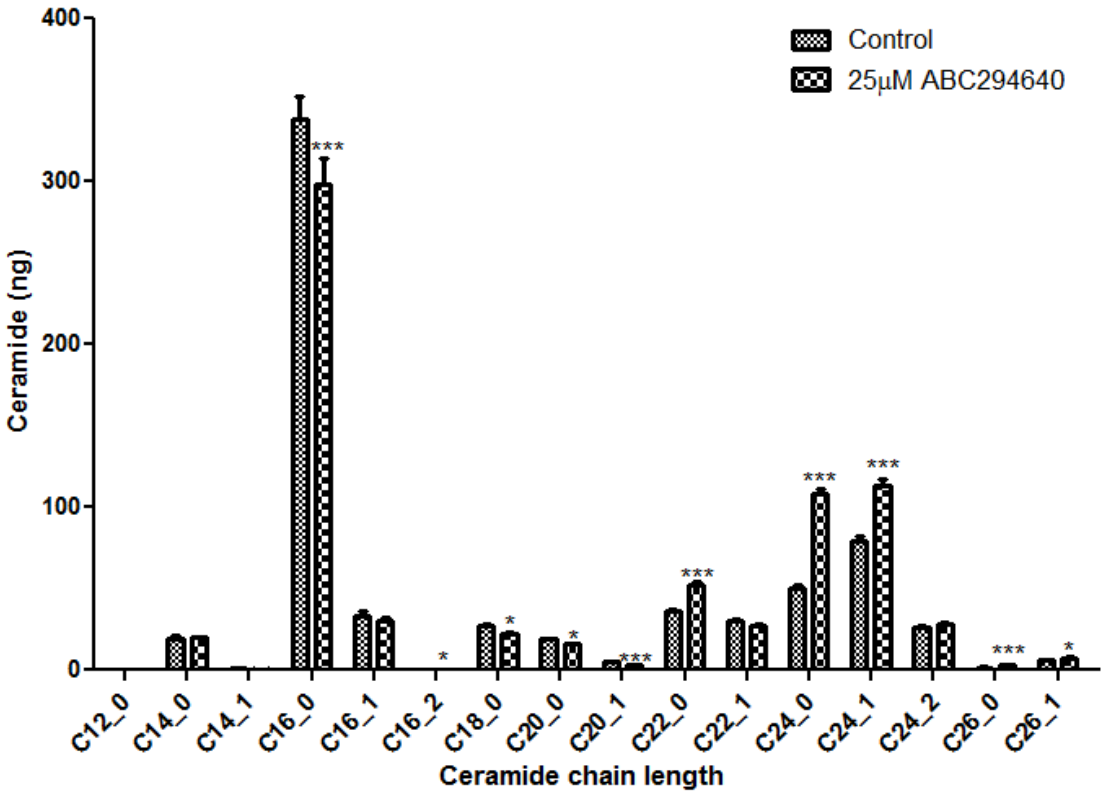

Figure S.2 ***Mass spectrometry analysis of ceramide levels in LNCaP AI cells treated with ABC294640 (in collaboration with Michael Wakelam and Qifeng Zhang (Babraham Institute, Cambridge, UK).*** *Bar chart showing the effect of ABC294640 (25 μM, 24 hours) on ceramide levels in LNCaP AI cells. Data are presented as ng per sample (mean with standard deviation of triplicate measurements). * p <0.05, ** p <0.01, *** p <0.001 for ABC294640-treated vs control cells. The sphingolipids analysed were ceramides (Cer-12:0-Cer-26:1).*

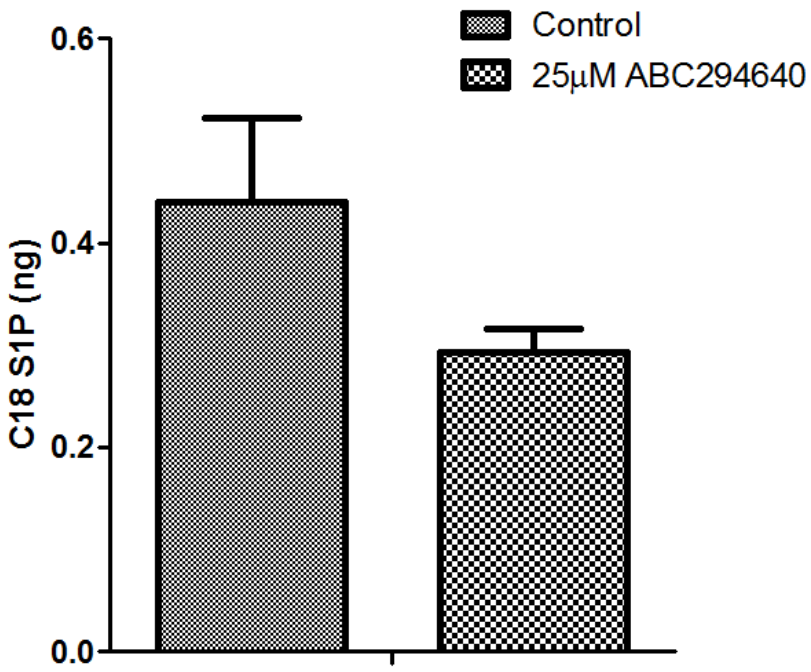

Figure S.3 ***Mass spectrometry analysis of S1P levels in LNCaP AI cells treated with ABC294640 (in collaboration with Michael Wakelam and Qifeng Zhang (Babraham Institute, Cambridge, UK)***. *Bar chart showing the effect of ABC294640 (25 µM, 24 hours) on S1P levels in LNCaP-AI cells. Data are presented as ng per sample (mean with standard deviation of triplicate measurements).*