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Pyne, Susan and Adams, David R and Pyne, Nigel J. (2016) Sphingosine 1-phosphate and sphingosine kinases in health and disease : recent advances. Progress in Lipid Research, 62. pp. 93-106. ISSN 0163-7827 , http://dx.doi.org/10.1016/j.plipres.2016.03.001

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Sphingosine 1-phosphate and sphingosine kinases in health and disease: recent advances

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Key words: Sphingosine 1-phosphate, sphingosine kinase, S1P receptors, intracellular signaling, Sphingosine kinase inhibitors

1. Introduction
2. Sphingosine kinases
3. S1P receptors and S1P release
4. Intracellular targets of S1P and novel roles of sphingosine kinases
5. Sphingosine kinases in disease
- **5.1 Role of sphingosine kinases in cancer**
  - *5.1.1 Sphingosine kinase 1*
  - *5.1.2 Sphingosine kinase 2*
- **5.2 Role of sphingosine kinases in the cardiovascular system**
- **5.3 Role of sphingosine kinases in the central nervous system**
- **5.4 Role of sphingosine kinases in inflammation**
- **5.5 Role of sphingosine kinases in diabetes**

6. Sphingosine kinase inhibitors
7. Summary
8. References

Abstract

Sphingosine kinases (isoforms SK1 and SK2) catalyse the formation of a bioactive lipid, sphingosine 1-phosphate (S1P). S1P is a well-established ligand of a family of five S1P-specific G protein coupled receptors but also has intracellular signaling roles. There is substantial evidence to support a role for sphingosine kinases and S1P in health and disease. This review summarises recent advances in the area in relation to receptor-mediated signaling by S1P and novel intracellular targets of this lipid. New evidence for a role of each sphingosine kinase isoform in cancer, the cardiovascular system, central nervous system, inflammation and diabetes is discussed. There is continued research to develop isoform selective SK inhibitors, summarised here. Analysis of the crystal structure of SK1 with the SK1-selective inhibitor, PF-543, is used to identify residues that could be exploited to improve selectivity in SK inhibitor development for future therapeutic application.

1. Introduction

Sphingosine 1-phosphate (S1P) is a pleiotropic lipid that has a wide variety of physiological and pathophysiological roles [1-3]. It is one of a multitude of sphingolipids and glycosphingolipids that are readily synthesised and/or inter-converted in a spatial and temporal manner in response to environmental change and stimuli [4, 5]. These, in turn, are integrated with the wider cellular metabolic network [6]. S1P is synthesised by two distinct isoforms of sphingosine kinase (SK1 and SK2) and elicits cellular responses through well-established receptor-mediated mechanisms and by affecting a number of intracellular target proteins. In general, the effects of S1P (proliferation, migration, cell survival etc.) are largely opposed to those of ceramide (apoptosis, senescence, growth arrest etc.) and the concept of the ‘sphingolipid rheostat’ was proposed, whereby the inter-conversion of ceramide, via sphingosine, to intracellular S1P contributes to cellular fate [7, 8]. However, it is now apparent that the situation is far more complex and a more advanced model incorporates the autocrine and paracrine effects of S1P

(acting via its receptors), amplification loops whereby S1P activates pathways that enhance its own formation and signaling as well as the intracellular effects of S1P, mediated by its target proteins [4, 9]. Furthermore, it is recognised that ceramides of differing acyl chain and sphingoid base composition may have distinct roles [10] and act independently of S1P in a membrane- and target-specific manner. For example, ceramide-enriched microdomains affect mitochondrial function [11] whereas ceramide-activated target molecules include protein phosphatases (PP1, PP2A and PP2C), protein kinase Cζ and AKT [12]. Moreover, ceramides with different fatty acid chain length can exert opposing cellular effects in a given cell type (e.g. C16 ceramide promotes proliferation whereas C18 ceramide mediates cell death) [13]. Recent advances in lipidomics and cell surface analysis of lipids is likely to progress our understanding here [14, 15] but will need to be coupled with the development of biosensors for S1P and for specific molecular species of ceramide. Importantly, other sphingolipids (such as dihydroceramides) and sphingolipid derivatives (such as *trans*-2-hexadecenal, a breakdown product of the irreversible cleavage of S1P by S1P lyase [16]) that were previously believed to be biologically inactive are also now recognised signaling molecules, which require further investigation [17, 18] (Fig. 1). However, the focus of this article is on some of the more recent advances in relation to S1P and, particularly, the function of SK1 and SK2 in health and disease.

2. Sphingosine kinases

S1P is produced by the ATP-dependent phosphorylation of sphingosine, catalysed by SK1 and SK2. Recent comprehensive reviews on these enzymes are available [19, 20]. Therefore, only key features are included here. The two enzymes exhibit partial redundancy since $Sk1^{-/-}$ or $Sk2^{-/-}$ mice are phenotypically normal whereas elimination of both genes is embryonic lethal due to neurological and vascular defects [21]. SK1 and SK2 contain five conserved domains (C1–C5) with the catalytic domain formed within C1-C3 and the ATP binding domain located in the C2 region [22]. These well characterised enzymes, which differ in their biochemical properties, sub-cellular distribution and physiological roles, are regulated in a spatial and temporal manner

by post-translational modification and interaction with specific proteins and lipids (for review see [4]). For example, while both enzymes can be phosphorylated by extracellular signal-regulated kinases (ERK-1/2) in response to agonists [23, 24], the activation of SK1 is more pronounced and coupled with its translocation, in a calcium and integrin-binding protein 1 (CIB1)-dependent manner [25] from the cytoplasm to the plasma membrane (where S1P could be available for export). In contrast, SK2, which can localise to the endoplasmic reticulum or is associated with mitochondria [26] also contains both nuclear localisation and nuclear export sequences and shuttles in and out of the nucleus, being exported upon phosphorylation by protein kinase D [27]. S1P generated in the nucleus has the potential to regulate gene expression (see 4 *Intracellular targets of S1P and novel roles of sphingosine kinases*).

Both enzymes are expressed as multiple spliced variant forms although the functional significance of this is yet to be fully established. In general, studies of SK employ the shortest isoforms (SK1a, NM_001142601, 384 amino acids and SK2a, AF245447, 654 amino acids) and it should be borne in mind that these might not necessarily be the most physiologically relevant forms in a particular cell system studied. Interestingly, the 36 amino acid N-terminally extended SK2b isoform has higher catalytic activity, using FTY720 and sphingosine as substrates, compared with the SK2a isoform, indicating that the N terminus may contribute to a conformation with improved catalytic activity [28]. Moreover, SK1b (which contains an additional N-terminal 86 amino-acids) is more resistant to removal from cells via the proteasome (compared with SK1a). For example, the treatment of androgen-sensitive LNCaP cells with a catalytic inhibitor of SK1, SKi (SKI-II, 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole) [29], induced the proteasomal degradation of SK1a and SK1b, accompanied by a reduction in S1P and an increase in sphingosine and C22:0 and C24:0 ceramides, and induction of apoptosis [30]. A similar proteasomal degradation of SK1a was observed in androgen-independent LNCaP-AI cells whereas SK1b was resistant (possibly due to a compensatory increase in SK1b mRNA levels), associated with a lack of increase in C22:0 and C24:0 ceramides and resistance to apoptosis [30, 31]. These studies suggest distinct functional roles for SK1a and SK1b and

support the concept that SK1b might play a more prevalent role in the acquisition of chemo-resistance in cancers. Sensitivity to apoptosis can be restored to these cells by either using the allosteric inhibitor, (*S*)-FTY720 vinylphosphonate [32] or by combined treatment with SK1 siRNA (to prevent mRNA translation of SK1a and significantly, SK1b) and SKi [30]. Stabilisation of SK1b might also be afforded by competing acetylation at a GK rich motif (GGKGK), thereby preventing its ubiquitination and thus, evasion from the proteasome [33]. If this was the case, the N-terminal extension of SK1b would have to render the GGKGK motif more accessible in SK1b as it is common to both SK1a and SK1b isoforms. It is also possible that other potential post-translational modifications of the N terminal extension of SK1b provide protein stabilisation. Alternatively, the N terminal extension of SK1b could provide distinct protein/protein interaction sites that allow it to evade the proteasome and/or to regulate non-overlapping signaling pathways compared with SK1a. SK1 interacts with a number of proteins [19] and recent studies have demonstrated that SK1a and SK1b can form complexes with different proteins to potentially affect distinct cell biology. For instance, protein phosphatase 2A was identified as a SK1a-interacting protein whereas allograft inflammatory factor 1-like protein, the latent-transforming growth factor β-binding protein, and dipeptidyl peptidase 2 were found to associate exclusively with SK1b [34].

3. S1P receptors and S1P release

S1P-specific G protein-coupled receptors, $S1P_1$-$S1P_5$ [35, 36] have specific physiological roles, such as in the cardiovascular, immune and nervous systems in the modulation of vascular barrier integrity, vascular tone and trafficking of lymphocytes, respectively [37]. However, S1P receptors also participate in the pathophysiology of autoimmunity, inflammatory diseases and cancer. Therefore, the S1P receptor family is of significant therapeutic interest. Indeed, a sphingosine-like molecule, FTY720 (or fingolimod, formulated as Gilenya™), is being exploited therapeutically as the first oral treatment for relapsing and remitting multiple sclerosis. This pro-drug is phosphorylated by SK2 and the resulting FTY720-phosphate is released from cells to agonise S1P receptors ($S1P_2$ being an exception). Chronic exposure to FTY720 (acting as

FTY720-phosphate) down-regulates $S1P_1$, thereby limiting $S1P_1$-mediated inflammatory T cell invasion of the CNS and ameliorating symptoms of multiple sclerosis. FTY720 also reduces astrogliosis and supports nerve remyelination and recovery [38].

There is intense interest in developing receptor selective agonists/antagonists and an S1P-sequestering monoclonal antibody, some of which are in clinical trials [39]. The design of receptor-selective small molecules has been spurred on by elucidation of the crystal structure of $S1P_1$, in complex with an antagonist, which suggests that S1P accesses the binding pocket of the receptor by lateral movement within the plane of the lipid bilayer and between two transmembrane helices [40]. Sources of extracellular S1P in plasma include platelets (which lack S1P lyase), erythrocytes and vascular endothelial cells. The manner in which S1P is released differs between these cell types. For example, platelets require activation and employ distinct calcium-dependent and ATP-dependent transporters of S1P whereas erythrocytes, which are a major source of S1P, constitutively release S1P in an ATP-dependent manner, likely involving an ABC type transporter [41]. In contrast, the Spns2 transporter passively exports S1P from vascular endothelial cells [42] (reviewed in [43]). Indeed, Spns2 knockout mice exhibit protection from inflammation in a number of disease models including airway inflammation, colitis, arthritis and experimental autoimmune encephalopathy (EAE), suggesting that inhibitors of Spns2 may be useful in the treatment of inflammatory diseases [44]. S1P is associated with carrier proteins, such as albumin and high density lipoprotein (HDL) (Fig. 2). Interestingly, $Apom^{-/-}$ mice have been used to demonstrate that the proportion of circulating S1P that is bound to ApoM in high density lipoprotein, rather than to albumin, is dispensable for lymphocyte trafficking yet restrains lymphopoiesis through a $S1P_1$-mediated effect on bone marrow lymphocyte precursor cells [45]. Additionally, $S1P_1$ signaling in endothelial cells is more sustained in response to HDL-bound S1P compared to that of albumin-bound S1P [46]. This appears to be associated with entrapment of the HDL-bound S1P-$S1P_1$ receptor at the plasma-membrane with selective and distinct signaling, resulting in attenuation of TNFα-induced activation of NF-κB and ICAM-1 expression. In contrast, albumin-bound S1P-$S1P_1$ receptor is

internalised and operates via endocytosed G_i-mediated signaling [47]. The entrapment of HDL-bound S1P-$S1P_1$ receptor at the plasma membrane suggests that this ligand bound form of the receptor might be associated with an accessory protein that prevents its endocytosis and therefore originates a plasma membrane-directed $S1P_1$ receptor signaling programme, which is anti-inflammatory. Therefore, distinct pools of plasma S1P exist, with differing physiological roles. This raises the possibility of targeting these distinct ligand-bound $S1P_1$ receptor pools for therapeutics. Indeed, it has been shown previously that the signaling from $S1P_1$ can be specified by its formation with other proteins in a complex, such as the platelet-derived growth factor receptor β (PDGFRβ) [48]. $S1P_1$-PDGFβ receptor complex function involves, G_i, β-arrestin and PDGFRβ tyrosine kinase activity as multipliers of signal output from the complex and these signalosomes are retained in endosomes to regulate the ERK pathway in the cytoplasm to promote cell migration [49].

Further insight into the role of S1P pools and its specific receptor-mediated signaling will be facilitated by the recent development of two types of transgenic $S1P_1$ reporter mice. One of these employs a GFP expression reporter following activation of a $S1P_1$/transcription factor fusion protein that is cleaved by a β-arrestin/protease fusion protein [50]. The other makes use of the differential internalisation of a competent $S1P_1$/GFP fusion protein *versus* a non-binding $S1P_1$:RFP fusion protein [51]. It is anticipated that these $S1P_1$ reporter mice will allow the tissue-specific interrogation of $S1P_1$ activation, including in disease models, and assessment of the relative amounts of local extracellular signaling S1P and whether this becomes deregulated in, for instance, vascular disease. Additional S1P receptor sub-type-specific transgenic reporter mice could prove to be powerful tools in furthering our understanding of the different S1P receptors in health and disease.

4. Intracellular targets of S1P and novel roles of sphingosine kinases

There are a number of intracellular target proteins of S1P which are differentially regulated by SK1 and SK2; likely due to the differential subcellular localisation of the two SK isoforms (see Table 1). For example, SK1-derived cytoplasmic S1P acts as a cofactor for the E3 ligase activity of TNF receptor associated factor 2 (TRAF2) (which associates with and enhances SK1 activity [52]), which catalyses the Lys63-polyubiquitination of the protein kinase RIP1. RIP1 is a signaling platform in the NF-κB pathway [53] and regulates cell survival, inflammatory and immune responses. In support of this, the interaction of TRAF2 with TRAF-interacting protein (TRIP) (which attenuates the E3 ligase activity of TRAF2 and thereby reduces pro-inflammatory cytokine production) reduces the binding of S1P to the TRAF2 RING domain [54]. However, the role of SK1 in TRAF2-NFκB signaling is controversial as others have obtained contrasting results. For example, TNFα-mediated activation of NF-κB and cytokine production is unaffected in macrophages lacking both SK1 and SK2 [55]. In addition, TNFα-mediated NF-κB signaling was disrupted in keratinocytes devoid of TRAF2 but unaffected in the absence of SK1 [56] and siRNA knockdown of SK1 was without effect on TNFα-stimulated activation of NF-κB, nuclear translocation of p65/RelA or NF-κB-mediated transcription in HeLa cells [57].

Recently, a novel role for SK1/S1P in regulating correct endosomal processing/endocytic signaling and neurotransmission has been reported [58]. Artificially altering the cholesterol/sphingomyelin balance in the plasma membrane induces the formation of clusters of narrow endocytic tubular invaginations that are positive for N-BAR proteins. SK1 is co-localised in these tubules (and, physiologically to early endosomes and enriched in exocytotic and endocytotic compartments in nerve terminals) by interaction of a hydrophobic patch on the enzyme surface in a curvature-sensitive manner with the lipid bilayer. Moreover, knockdown of SK1 produces endocytic recycling defects and only wild type SK1, but not a hydrophobic patch mutant V268Q-SK1, rescued loss-of-function mutant neurotransmission defects [58]. The role of SK1 in regulating endosomal signaling might impact currently held views concerning 'inside-

out' signaling [59]. Thus, internalisation of constitutively active S1P receptor (i.e. in the absence of bound S1P ligand) might be facilitated by curvature-sensitive SK1 endosomal regulation without necessity for the release of S1P from cells or binding of S1P to S1P receptors at the cell surface.

Intracellular S1P can affect gene expression. For example, nuclear SK2 is in a repressor complex with histone H3 and histone deacetylase 1 and 2 (HDAC 1/2) at the promoters of genes such as the cyclin-dependent kinase inhibitor p21 and the transcriptional regulator c-fos. The resulting S1P inhibits HDAC 1/2, thereby sustaining histone acetylation at specific lysine residues and enhancing transcription of, for example, p21 and c-fos [60]. Nuclear SK2-phosphorylated FTY720 is reported to similarly inhibit HDAC and, acting independently of S1P receptors, can suppress breast cancer development, restore oestrogen receptor alpha (ERα) expression and increase therapeutic sensitivity to tamoxifen in mouse models of breast cancer [61]. Recently, cytoplasmic S1P has been suggested to bind to the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ), thereby recruiting the PPARγ co-activator (PGC1β) and enhancing expression of PPARγ target genes in endothelial cells [62]. Thus, addition of exogenous S1P enhanced the expression of plasminogen-activated inhibitor-1 and PGC1β in a receptor-independent manner. In addition, S1P was shown to directly bind to PPARγ *in vitro* and mutation of His323 (predicted to hydrogen bond with the phosphate head group of S1P by *in silico* docking with the ligand binding domain of PPARγ) reduced binding of PPARγ to a S1P-affinity matrix and decreased S1P-induced PPARγ activation when expressed in cells. S1P-regulation of PPARγ was suggested to be involved in vascular development, which is reduced in $Sk1^{-/-}/Sk2^{+/-}$ mice, and may be targeted therapeutically to manipulate neovascularisation. However, the identity of the SK isoform responsible for the S1P-dependent regulation of PPARγ remains to be identified.

At the mitochondria, SK2-derived S1P binds to the predominantly inner mitochondrial membrane protein, prohibitin 2 (PHB2), which regulates mitochondrial assembly and function. Depletion of SK2 or of PHB2 results in dysfunctional mitochondrial respiration at the level of cytochrome-c oxidase (complex IV of the electron transport chain) [63]. Moreover, the hearts of *Sk2*$^{-/-}$ mice are not protected from ischaemic injury by preconditioning, unlike wild type mice, and knockdown of SK2 or PHB2 or cytochrome c oxidase in cardiomyocytes similarly abolished cytoprotection by preconditioning [64]. These data suggest that interaction of mitochondrial S1P with homomeric PHB2 is important for cytochrome-c oxidase assembly, mitochondrial respiration and cytoprotection. In contrast, SK2-derived S1P has been reported to cooperate with the mitochondrial protein, BAK, to affect mitochondrial outer membrane potential and cytochrome c release during apoptosis [65]. Therefore, the role of mitochondrial S1P may be cell context dependent.

Interestingly, the phosphorylation of ezrin (of the ezrin-radixin-moesin family of adapter molecules, required for cancer cell invasion) in response to epidermal growth factor (EGF), requires SK2 and intracellular $S1P_2$. This intracrine action of intracellular S1P is supported by the failure of EGF to stimulate S1P release from cells and the inability of the S1P antibody sphingomab to inhibit phosphorylation of ezrin in response to EGF. However, there is a requirement for SK2 and Spns2 [66] and it is possible that close proximity localization of Spns2 with the $S1P_2$ receptor might enable delivery of S1P to the receptor via lateral diffusion through the lipid membrane in endosomes. This model raises the possibility that other S1P receptors may be similarly activated in endosomes and identifies new targets for therapeutic intervention in cancer.

An additional role of SK2-derived S1P is the stabilisation of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, which maintains telomeres and is often enhanced in activity in cancer cells (Fig. 3). S1P, formed by SK2, binds to hTERT at the nuclear periphery in human and mouse fibroblasts. Computer modelling and mutagenesis

demonstrated that the C'3-OH of S1P binds with D684 in hTERT and that mutation of this residue or depletion of SK2 decreased hTERT stability, reduced telomere integrity and promoted senescence. The binding of S1P to hTERT prevents the interaction of hTERT with makorin ring finger protein 1 (MKRN1), an E3 ubiquitin ligase that tags hTERT for degradation. Importantly, wild type hTERT, but not S1P-binding deficient hTERT restores tumor growth when SK2 was pharmacologically inhibited. S1P binding to hTERT was suggested to mimic its phosphorylation, which normally stabilises telomerase to enhance cell proliferation and tumor growth [67]. Therefore, targeting SK2 with inhibitors may be effective in cancer therapeutics to eliminate replicative immortality.

The β-site amyloid precursor protein (APP) cleaving enzyme-1 (BACE1), which is the rate-limiting enzyme for amyloid-β peptide (Ab) production, is also a target of intracellular S1P [68]. S1P specifically binds to BACE1 and increases its proteolytic activity. Moreover, BACE1 activity was decreased by either pharmacological inhibition of SK1/SK2 or knockdown of SK1 or SK2 whereas overexpression of S1P degrading enzymes had the opposite effect. Notably, SK2 activity was upregulated in the brains of patients with Alzheimer's disease and may be a potential therapeutic target in this disease.

5. Sphingosine kinases in disease

5.1 Role of sphingosine kinases in cancer

5.1.1 Sphingosine kinase 1

SK1 is functionally linked with some of the hallmarks of cancer. For instance, the over-expression of SK1 enhances the Ras-dependent transformation of fibroblasts into fibrosarcoma [69]. Indeed, K-RasG12V is a common mutation in cancer and, through SK1, increases the production of S1P and decreases ceramide levels. Over-expression of the K-RasG12V oncogene signaling promotes translocation of SK1 from the cytoplasm to the plasma membrane

via Raf/MEK/ERK signaling. Indeed, constitutively active B-Raf or MEK1 activate SK1 [70]. Therefore, SK1 can function within the context of oncogenic transformation. SK1 activation and localization to the plasma membrane and subsequent activation of $S1P_2$ by released S1P ('inside-out' signaling) also regulates transferrin receptor 1 (TFR1) expression [71]. This is important as inhibition of TFR1 prevents SK1-induced cell proliferation, survival and neoplastic transformation of NIH3T3 fibroblasts. Knockout of *Sk1* or *Sk2* also reduces tumor progression and high expression of SK1 and SK2 in tumors is associated with poor clinical prognosis in cancer patients (reviewed in [72]).

SK1 is also functionally linked with inflammation and the subsequent development of cancer. Thus, S1P enhances colitis associated cancer via a malicious amplification loop involving SK1, $S1P_1$, NFκB, STAT3 and IL-6 [73, 74]. Furthermore, inhibition/down-regulation of SK1 blocks the Warburg effect; a phenomenon where cancer cells are addicted to high rates of aerobic glycolysis for ATP production and anabolic metabolism [75]. SK1 is also be involved in the neovascularisation of tumors involving *paracrine* angiogenesis and lymphangiogenesis. Thus, siRNA knockdown of SK1 in breast and glioma cancer cells reduced migration and tube formation in an experimental system where these cancer cells are co-cultured with vascular or lymphatic endothelial cells. S1P also induces endothelial cell sprouting in 3-dimensional collagen matrices [76]. Finally, cancer stem cells are recognised as being important in initiating cancer progression and SK1 via $S1P_3$ and Notch signaling promotes cancer stem cell proliferation to increase tumorigenesis in nude mice [77].

There are many examples which provide additional evidence for a role of SK1 in cancer. For instance, SK1 is overexpressed head and neck squamous cell carcinoma (HNSCC) (stages I-IV). The knockout of SK1 reduces S1P generation and decreases tumor incidence, multiplicity, and volume in 4-NQO-induced HNSCC carcinogenesis. This was associated with reduced cell proliferation, increased apoptosis and reduction in phosphorylated AKT levels [78]. SK1/S1P also prevents proteasomal degradation of Bcr-Abl1 protein to increase its stability. Thus, siRNA

knockdown of SK1 in imatinib-resistant K562/IMA-3 cells, or *Sk1*$^{-/-}$ MEFs exhibit reduced Bcr-Abl1 stability. S1P formed by SK1 is released to act on the $S1P_2$ receptor to inactivate PP2A and prevent dephosphorylation of Bcr-Abl. Pharmacological intervention with the sphingosine like compound, FTY720 which inhibits SK1 and reactivates PP2A restores sensitivity to imatinib or nilotinib in primary $CD34^+$ mononuclear cells obtained from chronic phase and blast crisis CML patients [79]. In addition, certain myeloproliferative neoplasms are characterized by the expression of the Jak2(V617F) oncogene, which inactivates PP2A. Thus, reactivation of PP2A (and possibly inhibition of SK1) by FTY720 reduces Jak2(V617F) activity and decreases leukemic allelic burden and splenomegaly and increases Jak2(V617F) leukemic mice survival. The effects of FTY720 require a PP2A interacting protein SET K209. These findings are important in establishing an interplay between the S1P signaling pathway, SET2-PP2A and Jak2 in driving in myeloproliferative neoplasm [80]. SK1 is also involved in tumor-induced hemangiogenesis and lymphangiogenesis. Treatment of a murine model of breast cancer metastasis with the selective SK1 inhibitor SK1-I ((2*R*,3*S*,4*E*)-N-methyl-5-(4-pentylphenyl)-2-aminopent-4-ene-1,3-diol (BML-258)) suppresses S1P levels, reduces metastases to lymph nodes and lungs, and decreases overall tumor burden. Moreover, hemangiogenesis and lymphangiogenesis is inhibited by this SK1-I in the primary tumor and lymph nodes [81].

We have also shown that the siRNA knockdown of SK1 reduces $S1P_3$ expression and ERK-1/2 activation in response to S1P in MCF-7 breast cancer cells. These findings indicate that SK1 and $S1P_3$ function in an amplification loop to promote ER positive breast cancer progression [82]. S1P binding to the $S1P_3$ receptor also increases translocation of SK1 from the cytoplasm to plasma-membrane of MCF-7 cells, suggesting that there is a balance between intracellular and extracellular S1P that requires SK1 to function as a sensor to coordinate a tightly regulated cell migration response [82]. Recent evidence also provides a strong case for S1P functioning with the context of metastasis, which is responsible for mortality in cancer patients. S1P formed systemically by SK1 rather than from tumor-derived S1P promotes metastasis. Thus, reduced systemic, but not tumor SK1 prevents S1P elevation, and inhibits TRAMP-induced prostate

cancer growth in *TRAMP*$^{+/+}$ *Sk1*$^{-/-}$ mice, or lung metastasis of multiple cancer cells in *Sk1*$^{-/-}$ mice. The S1P formed by SK1 binds to the $S1P_2$ receptor and prevents induction of the metastasis suppressor, Brms1 (breast carcinoma metastasis suppressor 1). Thus, sequestration of systemic S1P with the anti-S1P monoclonal antibody, Sphingomab, attenuated lung metastasis and this was reversed by Brms1 knockdown [83]. In addition, the migration of melanoma cells is dependent of SK1 expressed in fibroblasts that are found in the stroma surrounding tumors. Indeed, local tumor growth and dissemination is enhanced more efficiently by co-injection of wild-type skin fibroblasts compared with fibroblasts from *Sk1*$^{-/-}$ mice [84, 85]. SK1 is also released as a catalytically active enzyme in vesicles shed by human breast carcinoma 8701-BC cells. The enzyme substrate sphingosine is present in shed vesicles where it is produced by neutral ceramidase. Shed vesicles are therefore a site for S1P production in the extracellular medium that can potentially drive metastatic conversion of tumor cells [86]. TGF-β also induces an increase in SK1 expression and this can be correlated with metastasis and increased viability of MDA-MB-231 cells, suggesting that TGF-β and the SK1/S1P axis might have a critical role in promoting metastasis [87]. SK1 is also involved in the acquisition of chemotherapeutic resistance. For instance, osteoblastic-derived S1P induces resistance of prostate cancer cells to therapeutics including chemotherapy and radiotherapy. Bone metastases from prostate cancer cells are associated with osteoblastic differentiation resulting in abnormal bone formation. S1P/$S1P_3$ signaling is important during differentiation to mature osteoblasts and regulates expression of Runx2; a key transcription factor involved in osteoblastic maturation [88].

In addition to promoting cancer progression, SK1 might also be associated with the development of oncogene tolerance dependent on the genetic background of the cancer. In this context, SK1 functions in a protective role to reduce cancer cell migration. For instance, human EGF receptor 2 (HER2) increases SK1 expression in estrogen receptor-positive (ER^+) MCF-7 HER2 cells. SK1, in turn, limits HER2 expression in a negative-feedback manner. The

HER2-dependent increase in SK1 expression reduces p21-activated protein kinase 1 (p65 PAK1) and ERK-1/2 signaling via a desensitisation/HER2 tolerance mechanism (Fig. 4). This is correlated with improved prognosis in patients who have a low HER1-3/SK1 expression ratio in their tumors compared to patients that have a high HER1-3/SK1 expression ratio [82]. Therefore, therapeutic targeting of SK1 in cancer should be informed by stratification of patient populations in order to establish whether the nature of the biochemical functioning of the enzyme is conducive to intervention. This contrasts with the interaction of HER2 with the $S1P_4$ receptor where there is a positive functional interaction to increase signaling gain resulting in enhanced activation in response to S1P [89]. This is of importance as HER2/ERK signaling has been linked with metastasis.

5.1.2 Sphingosine kinase 2

Treatment of various cancer cell lines with SK1 or SK2 siRNA elicited differential effects on p53, p21, ERK1, ERK2, FAK, and VCAM1. These findings indicate that SK1 and SK2 exhibit non-overlapping and non-redundant functions in tumor cells. Moreover, loss of SK2 from these cells produced stronger anti-cancer effects compared with the loss of SK1 [90]. ABC294640 (3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide) is a SK2 selective inhibitor (K_i = 10 μM [91]) and exhibits no inhibition of SK1 activity at concentrations as high as 100 μM (see 6 *Sphingosine kinase inhibitors* and Table 2). ABC294640 treatment of early stage and advanced prostate cancer cells induces a reduction in Myc and androgen receptor (AR) expression. This corresponds with significant inhibition of growth, proliferation, and cell cycle progression. Oral treatment of mice with ABC294640 also reduced xenograft tumor growth [92]. However, there are recognised off-target effects of ABC294640 (see 6 *Sphingosine kinase inhibitors*), including promoting the ubiquitin-proteasomal degradation of SK1 [93].

The influence of SK2 on Myc is a common regulatory mechanism in a number of different cancers. For instance SK2 has a role in B-cell acute lymphoblastic leukemia (B-ALL) by

influencing expression of Myc. Knockout of *Sk2* reduced leukemia development in a mouse model of ALL and pharmacologic inhibition extends survival of mice in xenograft models of human disease. The mechanism by which SK2 regulates Myc expression in leukemic cells may involve S1P-dependent inhibition of HDAC1/2 activity. Thus, decreased levels of acetylated histone H3 within the Myc gene promoter were detected; significant as Myc is a prognostic marker of B-ALL disease progression and severity [94].

Other SK2 inhibitors have been used to interrogate the role of SK2 in cancer. For instance, (*R*)-FTY720 methylether (ROMe) has a K_i = 16 μM [95] for SK2 inhibition and lacks activity against SK1 at concentrations as high as 100 μM [95]. ROMe induces the autophagic death of T-ALL cell lines (reversed by autophagy inhibitors but not apoptotic inhibitors) as evidenced by the accumulation of lipidated LC3-II) and patient lymphoblasts [96]. Significantly, ROMe also reduces phosphorylated AKT and c-Myc levels in T-ALL cells, which are prognostic markers for T-ALL disease progression [96]. These findings provide a rationale for targeting SK2 in T-ALL. This is further supported by the finding that SK2 inhibitor genetic signatures are correlated with publicly available gene expression datasets derived from paediatric ALL patients [94].

SK2 is also involved in enabling tumor cells to evade the immune system. SK2 deficient MCF-7 breast cancer cells display retarded growth *in vivo* and tumor associated macrophages are directed toward an anti-tumor phenotype exhibiting increased expression of pro-inflammatory mediators such as NO, TNFα, IL-12 and MHCII and a low expression of anti-inflammatory IL-10 and CD206 [97]. These findings indicate that tumor SK2 might direct polarisation of macrophages toward an M2 phenotype, thereby allowing the cancer cells to evade M1 driven pro-inflammatory responses that impede cancer progression.

5.2 Role of sphingosine kinases in the cardiovascular system

Hypoxia which induces pulmonary arterial hypertension is associated with increased expression of SK1 in human arterial pulmonary smooth muscle cells [98] and this might therefore underlie the mechanism for vascular remodelling in pulmonary arterial hypertension (PAH). Indeed, SK1 expression is increased in lungs from patients with PAH and *Sk*1$^{-/-}$ mice are protected from hypoxia-induced pulmonary hypertension [99]. The S1P formed by SK1 binds to the $S1P_2$ receptor to promote pulmonary arterial smooth muscle proliferation [99] (see Fig. 5). A variety of cardiovascular diseases such as pulmonary hypertension lead to a compensatory adaptive increase in cardiac muscle mass e.g. hypertrophy. However, this results in dysfunctional hypertrophy and the extensive apoptosis of cardiomyocytes, which results in heart failure and death [100]. Indeed, preventing apoptosis of cardiomyocytes is a major objective for the treatment of heart failure. Other studies have shown that the SK1 inhibitor, PF-543 reduces post-myocardial infarction (MI) cardiac remodelling and dysfunction [101]. Moreover, inhibition of S1P lyase enhances cardiac remodelling and dysfunction. In addition, FTY720 (which down-regulates $S1P_1$ and also inhibits SK1 [32]) reduces cardiac SK1/S1P/$S1P_1$ signalling and ameliorates chronic cardiac inflammation and cardiac remodelling and dysfunction *in vivo* post-MI [101] (see Fig. 5). Deletion of the *Sk*2 gene is also associated with a considerable increase in ischaemic reperfusion-induced injury and a reduction in the cardio-protective effect of ischemic preconditioning [102]. These findings suggest that SK2 exerts a beneficial function against heart failure. Moreover, heart failure is associated with neurological deficits caused by cerebral vasoconstriction. In this regard, TNFα via a SK1/S1P/$S1P_2$ receptor-mediated mechanism enhances myogenic tone [103].

SK1 also functions in the context of hyperoxia-dependent pathology. For instance, SK1 deficiency reduces hyperoxia-induced IL-6 accumulation and NADPH oxidase (NOX) 2 and NOX4 protein expression in lung. Moreover, S1P stimulates ROS generation which is essential for the development of bronchopulmonary dysplasia [104]. SK1 is also involved in fibrotic disease. Thus, S1P and SK1 expression levels are increased in patients with Idiopathic

pulmonary fibrosis (IPF) and S1P induces epithelial-mesenchymal transition via a mechanism involving the TGFβ-dependent regulation of $S1P_2/S1P_3$ and SMAD/RhoA signaling and which contributes to fibrosis [105]

SK2 is also involved in thrombosis. Thus, S1P levels are dramatically reduced in platelets from *Sk2*$^{-/-}$ mice and these platelets fail to secrete S1P and are less responsive to platelet aggregating agents. Interestingly, *Sk2*$^{-/-}$ mice are protected from arterial thrombosis after vascular injury and significantly, exhibit normal bleeding times. However, other studies have reported that the loss of the *Sk2* gene results in defective intravascular pro-platelet shedding at the final stage of thrombopoiesis and leading to thrombocytopenia [106]

5.3 Role of sphingosine kinases in the central nervous system

SK2/S1P has an important role in regulating the survival of the dopaminergic neurons, which is of relevance to the clinical development of Parkinson's disease. SK2 expression is markedly reduced in the *substantia nigra* region in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease mouse model. Interestingly, SK2 is localised predominantly in the mitochondria. This might be significant as mitochondrial dysfunction is a feature of Parkinson's disease. Indeed, the inhibition of SK2 activity reduces the expression of PGC-1a (the coactivator of PPARγ), NRF-1 and mitochondrial transcription factor A (TFAM)-key genes involved in regulating mitochondrial function. Inhibition of SK2 is associated with an oxidative stress response, which could be protected against by addition of exogenous S1P which acts via the $S1P_1$ receptor [107].

There is also evidence for a role for SK1 and SK2 in Alzheimer's disease (AD). Amyloid-β (Aβ) induces neuronal apoptosis, a key step in the pathogenesis of AD. Interestingly, beta-amyloid peptide fragment 25-35 (Aβ25-35) toxicity is associated with a marked down-regulation of SK1 expression. Toxicity is due to reduced expression of pro-apoptotic Bax and enhanced

expression of anti-apoptotic Bcl2 and is protected by over-expression of SK1 [108]. Moreover, others have reported that siRNA knockdown of SK1 expression increases Aβ load and worsens learning and memory ability in APP/PS1 mice [109]. In contrast, SK2 activity is up-regulated in brains from patients with AD and S1P formed by SK2 binds to full-length BACE1 and increases its proteolytic activity [68]. These findings suggest that intracellular S1P formed by SK2 directly modulates BACE1 activity and increases formation of toxic Aβ. SK activity is also reduced in Niemann-Pick type C disease (NP-C) patient fibroblasts and NP-C mouse Purkinje neurons due to defective vascular endothelial growth factor (VEGF) formation [110].

SK2 is also involved in nociception. Mice deficient in *Sk2* exhibit substantially lower spinal S1P levels compared to wild-type C57BL/6 mice. These mice demonstrate facilitation of nociceptive transmission during the late response in a formalin model of acute peripheral inflammatory pain. Chronic peripheral inflammation increased the relative mRNA expression of P2X4 receptor, brain-derived neurotrophic factor and inducible nitric oxide synthase in the ipsilateral spinal cord of wild-type but not in *Sk2*$^{-/-}$ mice [111].

5.4 Role of sphingosine kinases in inflammation

SK1 has a pro-inflammatory role in cancer [73] and rheumatoid arthritis [112] but exerts a protective role on neuro-inflammation [113]. We therefore consider that rather than initiating inflammatory responses, SK1 functions to exert a modulatory role in a disease-specific manner. SK1 has been linked with the TNFα-dependent regulation of the NFκB which induces pro-inflammatory mediators. For instance, SK1 bind to TRAF-2 (purported to be a ring finger E3 ligase) and S1P directly binds to and activates TRAF2 E3 ligase activity to catalyse the lysine-63-linked polyubiquitination of RIP1, which functions as a signaling platform for recruitment and phosphorylation of IκB kinase, IκB degradation and NF-kappaB activation. These findings lead to the proposal that the regulation of K63 polyubiquitination by SK1/S1P represents a novel signaling paradigm in inflammation [53]. Indeed, IRF1 (interferon-regulatory factor 1) undergoes

a Lys63 (K63)-linked polyubiquitination mediated by the apoptosis inhibitor cIAP2 and S1P. This involves the formation of an activated complex between cIAP2, SK1 and IRF1 in response to IL-1 [114]. However, others have obtained results that dissociate SK1 from TRAF2-NFκB signaling. Thus, macrophages deficient in both *Sk1* and *Sk2* do not display defects in TNFα-mediated activation of NFκB or inflammatory responses [55]. Indeed, the only observable change is enhanced autophagic markers. Moreover, the loss of SK1 potentiates induction of the chemokine RANTES which is regulated by TNFα via the NFκB pathway. TNF-induced IKK phosphorylation, IκB degradation, nuclear translocation of NFκB subunits and transcriptional NFκB activity were not altered by the loss of SK1. Therefore, SK1 exhibits an anti-inflammatory role as loss of SK1 ablates TNF-induced phosphorylation of p38 MAPK and increases RANTES and multiple chemokines and cytokine levels [57]. In addition, administration of the SK1 inhibitor, PF-543 to mice increased disease progression in the EAE model of multiple sclerosis and this was associated with a considerable increase in the infiltration of $CD4^+$ T-cells, $CD11b^+$ monocytes and $F4/80^+$ macrophages in the spinal cord. These findings indicate that SK1 functions in an anti-inflammatory manner, rather than a pro-inflammatory context in EAE [115]. In contrast, *Sk1* deficient mice are protected against development of TNFα-induced arthritis indicating that SK1 functions in a pro-inflammatory manner in this disease [112]. Genetic elimination of *Sk2* did not significantly impact the severity or progression of inflammatory arthritis, while pharmacologic inhibition of SK2 with ABC294640 induced more severe arthritis. The authors concluded that SK2 functions in an anti-inflammatory role in this disease and therefore therapeutic approaches require isoform specific inhibitors in order to target SK1 [116].

SK2 also functions in the context of inflammation and graft injury after liver transplantation. Hepatic S1P levels are increased after liver transplantation and this can be abrogated by inhibition of SK2 activity with ABC294640. The anti-inflammatory mechanism of SK2 involves reduced TLR4 expression, NF-κB activation, pro-inflammatory cytokine/chemokine production, adhesion molecule expression, infiltration of monocytes/macrophages and neutrophils, focal

necrosis and apoptosis. ABC294640 also promotes survival from ~25% to ~85% and therefore this enzyme has been proposed to be a new therapeutic target for liver graft failure [117].

5.5 Role of sphingosine kinases in diabetes

Dysfunction of the endoplasmic reticulum (ER) leads to an unfolded protein response (UPR), aberrant lipid biosynthesis and insulin resistance. ER stress activators such as tunicamycin and lipopolysaccharides increase SK2 expression via an activation transcription factor 4 (ATF4)-dependent mechanism. Hepatic accumulation of lipid droplets by high fat diet is also reduced by the SK2-mediated up-regulation of fatty acid (FA) oxidizing genes and increased FA oxidation in liver. In addition, glucose intolerance and insulin resistance are reduced by improved hepatic insulin signaling in cells over-expressing SK2 [118]. On the other hand, lipotoxicity-induced loss of islet β-cells in type 2 diabetes is modulated by SK1. Thus, genetic loss of *Sk1* results in diabetes, with a 3-fold reduction in insulin levels compared with the WT mice and a 50% reduction in high fat fed *Sk1*$^{-/-}$ mice. The over-expression of a dominant negative form of SK1 also markedly promoted palmitate-induced cell death in MIN6 and INS-1 β-cell lines [119] Moreover, overexpression of WT SK1 in high fat fed mice exhibit increased SK1 expression in the skeletal muscle and this was associated with reduced ceramide levels. Ceramide has been shown to induce insulin resistance, and insulin sensitivity was improved in the skeletal muscle of mice over-expressing SK1. Therefore perturbation of the sphingolipid rheostat by SK2/SK1 appears to enhance fatty acid metabolism and protection against ceramide-induced β-cell death [120].

6. Sphingosine kinase inhibitors

As both SK1 and SK2 have been implicated in various diseases [3, 39]; see 5 *Sphingosine kinases in disease*), there has been a drive to generate small molecule inhibitors that could be developed as novel therapeutics (reviewed by [121]). To date, a number of isoform-selective

sphingosine competitive SK inhibitors have been identified whereas few ATP-competitive inhibitors have been reported (Table 2).

High potency (nanomolar) sphingosine competitive SK1-selective inhibitors include PF-543 [122] and Genzyme 51 [123]; although there are no reports of the *in vivo* effects of the latter. PF-543 induces the proteasomal degradation of SK1a *in vitro,* thereby demonstrating target engagement [124] but fails to inhibit DNA synthesis [124] or to reduce cell viability in cancer cells [122]. The latter may be due to a failure to increase apoptotic ceramide species despite an observed reduction in S1P levels [122]. However, PF-543 has been used to support a role for SK1 in sickle cell disease, where blood S1P levels are elevated [125] and erythrocyte SK1 activity is regulated by activation of the adenosine A2B receptor [126]. PF-543 reduced sickling of red blood cells *in vitro* and *in vivo* [125] and SK1 may therefore provide a potential therapeutic target in this disease. Additionally, PF-543 has been shown to ameliorate cardiac remodelling following myocardial infarction where SK1/S1P/S1P_1 participates in cardiac inflammation and dysfunction [101]. However, PF-543 exacerbates disease progression in an EAE mouse model of relapsing and remitting multiple sclerosis, indicating that SK1 serves a protective, anti-inflammatory role in this disease [115].

Sphingosine competitive SK2-selective inhibitors include ABC294640, K145, SLR080811 and ROMe, which all have micromolar potency and some of which exhibit limited selectivity for SK2 over SK1 (see Table 2). Of these, ABC294640, induces cell death through both apoptotic and autophagic pathways [91, 127] and has been employed in numerous disease models including cancer [91], rheumatoid arthritis [128] and ulcerative colitis [129]. Indeed, ABC294640 is in phase I clinical trials for pancreatic cancer, solid tumors and refractory/relapsed diffuse large B cell lymphoma. Although ABC294640 reduces S1P levels in plasma [130] and in tumors [91], thereby supporting target engagement, this compound also has anti-oestrogenic effects as it binds to the oestrogen receptor where it acts as a partial antagonist [131]. Moreover, we have found that ABC294640 induces the ubiquitin-proteasomal degradation of SK1 in androgen-

independent LNCaP-AI prostate cancer via a mechanism that is independent of SK2 inhibition and involving oxidative stress. Therefore, some of the effects of ABC294640 might in fact involve SK1 [93] and/or other off-target effects. K145 reduced S1P levels, inhibited growth and suppressed ERK/AKT signaling in U937 cells and inhibited tumor growth *in vivo* [132] but has not been further investigated. SLR080811 reduces S1P in leukemia cells *in vitro* yet increases blood S1P levels *in vivo* [133]. This is consistent with the elevated blood S1P levels of *Sk*2 knockout mice [21], which may be due to a compensatory increase in SK1 expression [73]. However, it was recently reported that i/p injection of SK2-selective inhibitors induced a rapid increase in blood S1P due to a decrease in the clearance of S1P [134]. *Sk*2 knockout mice also exhibited a reduced S1P clearance, suggesting that SK2 may have an additional function other than simply generating S1P in cells [134]. ROMe ((*R*)-FTY720-methyl-ether) inhibits DNA synthesis in breast cancer cells [95], induces the autophagic death of leukemic cell lines [96] and enhances endothelial barrier integrity [135] *in vitro* and prevents disease progression in an EAE mouse model of relapsing and remitting multiple sclerosis (unpublished), which supports a pro-inflammatory role of SK2 in this disease.

Dual SK1/SK2 sphingosine-competitive inhibitors include SKI-II [29] and Amgen 82 [136]. SKI-II inhibits human SK1 and SK2 with micromolar potency [137], induces the proteasomal degradation of SK1a in cells [30], indicative of target engagement, and has been used for co-crystallisation with SK1 [138]. *In vivo* actions include the reduction of tumor volume [139], attenuation of bronchial hyper-responsiveness [140], inhibition of cerebral preconditioning [141] and exacerbation of atherosclerosis in low-density lipoprotein receptor deficient (LDL-$R^{-/-}$) mice [142]. However, it is suggested to have off-target effects including the indirect inhibition of dihydroceramide desaturase activity, resulting in elevated levels of dihydroceramides [30, 143]. Amgen 82, developed using the crystal structure of SK1 with bound SKI-II [138], inhibits both human SK1 and human SK2 with nanomolar potency [136] yet failed to reduce tumor cell viability except at supramaximal concentrations, despite lowering cellular S1P levels [137]. In the absence of information on changes in other sphingolipids, including

ceramides/dihydroceramides, it is unclear what the relevance of the lack of cytotoxicity is with these compounds or how this relates to the well-defined importance of SK1 in regulating cancer cell survival. Indeed, off-target effects on other sphingolipid metabolising enzymes, thereby preventing increases in ceramides/dihydroceramides, cannot be excluded. Although reducing plasma S1P levels by ~70% in mice, Amgen 82 failed to reduce tumor volume in a mouse tumor xenograft model *in vivo* [137], suggesting that circulating S1P does not determine tumor growth. Notably, however, Amgen 82 does not inhibit mouse SK2 [136] and inhibition of both SK1 and SK2 may be required to elevate apoptotic ceramides and/or induce cell death.

Recent advances include the development of an ATP-competitive inhibitor, MP-A08, which inhibits both human and mouse SK1 and SK2 with low micromolar potencies (Table 2; [144]). This was developed using homology modelling of the predicted ATP-binding pocket of SK1, using the solved crystal structures of the related bacterial lipid kinases, DgkB [145] and YegS [146, 147] and *in silico* small molecule docking. MP-A08 reduces cellular S1P levels, elevates cellular ceramides, sphingosine and dihydrosphingolipids, induces apoptosis, and inhibits cell proliferation and colony formation *in vitro*. Significantly, MP-A08 has minimal effect on apoptosis in SK1/SK2 double knockout mouse embryonic fibroblasts, thereby validating an 'on-target' effect on SK1/2. MP-A08 also has no significant inhibitory activity against a panel of 140 kinases or against dihydroceramide desaturase. *In vivo* effects include the reduction of tumor burden, induction of tumor apoptosis, reduction in tumor S1P and inhibition of tumor angiogenesis [144]. Collectively, this data re-affirm the validity of targeting SK for therapeutic benefit in cancer. The effect of MP-A08 has yet to be established in other animal disease models but is likely to prove very interesting in the future.

To date, there are no high potency SK2-specific inhibitors. However, with the solved crystal structures of SK1 in the absence and presence of SK inhibitors (SKI-II [138], PF-543 [148] and Amgen 23 [136]) and with ADP [138], and the crystal structures of related bacterial lipid kinases DgkB [145] and YegS [146, 147], it has been possible to define the sphingosine substrate

binding site (named the ‘J-channel’ due to its shape), the nucleotide binding site and detail of the interaction of sphingosine-competitive inhibitors [136, 138, 148] and an ATP-competitive inhibitor [144]. Such analysis can be used to inform on the design of isoform-selective inhibitors by identifying and exploiting key differences between SK1 and SK2 as a crystal structure for SK2 has yet to be reported. For example, co-crystallisation of SK1 with the nanomolar SK1 selective inhibitor, PF-543, established it to be buried in the enclosed sphingosine binding ‘J-channel’ ([148], consistent with its relatively long K_{off} $t_{1/2}$ of 8.5 minutes [122]) which has a funnel-like opening that positions the primary alcohol of sphingosine proximal to the γ-phosphate of bound ATP. Lipid entry is thought to involve a tunnelling mechanism whereby substrate enters tail first [138]. A key aspartic acid (Asp178; Asp308 in SK2) contributed by helix-α7 and a water networked sub-pocket at the mouth of the J-channel engages the 2-amino-3-hydroxyalkan-1-ol head group of sphingosine — or the 2-(hydroxymethyl)pyrrolidine subunit of PF-543 — both in their protonated states.

The absence of a crystal structure for SK2 makes definitive rationalisation of the observed SK1/SK2-selectivity profiles of established Sph-competitive inhibitors challenging. Sequence comparison does hint at some likely differences between the enzymes that may contribute to discriminatory behaviour however. Inspection of the available crystal structures, illustrated in Fig. 6 for PF-543, reveals that some 20 residues contribute to the direct ligand binding surface of the J-channel. Of these, SK2 differs at only 3 locations in the direct hydrophobic contact surface of the binding site: these are Val304, Leu517 and Cys533 in SK2, corresponding to Ile174, Met272 and Phe288, respectively, in SK1. Ile174 and Met272 are located at a pinch point in the throat of the J-channel; their substitution by Val and Leu respectively may make this region of the J-channel slightly wider in SK2 than in SK1. Thus, inhibitors that exert steric demand in this locus may potentially exhibit SK2-over-SK1 selectivity. Phe288 stoppers the toe of the J-channel; its replacement by Cys in SK2 is likely to result in a longer J-channel than in SK1 and may potentially also confer greater surface plasticity in that region of SK2 due to

loosened packing against adjacent hydrophobic residues. Increased length and steric demand in subunits targeted to the toe of the J-channel may potentially also confer SK2-inhibitory selectivity therefore. Indeed, structure activity analysis based on the SLR080811 scaffold supports a larger lipophilic binding cavity in SK2 compared to SK1 [149]. A further residue difference — Met312 of SK1, corresponding to Phe557 in SK2 — does not contribute directly to the J-channel but is sufficiently close that its substitution (depending on side chain rotation) might lead to differences in the ligand contact surface proximal to the sulfone oxygens of SK1-bound PF-543. This raises the question of whether J-channel surface encroachment in SK2 at that site might contribute to the pronounced SK1-inhibitory selective of this compound. Two other residue differences, Ala175 and Ala339 (corresponding to Ser305 and Thr584 in SK2), are also likely to have some indirect impact on ligand binding. In the PF-543 co-crystal structure, the backbone carbonyl of Ala339 is seen to hydrogen bond to a key structural water, labelled W1 in Fig. 6 that bridges to the hydroxymethyl group of the inhibitor. This hydroxymethyl, in turn, hydrogen bonds to the aspartate (Asp178) on helix-α7 that, together with W1, normally serves to bind the 3-OH group of Sph substrate. The second alanine, Ala175, is located on helix-α7 one turn along from Asp178 and forms part of the packing surface of the helix against a β-sandwich core skeleton in the protein. Substitution of the two alanines by Ser305 and Thr584 in SK2 is likely to lead to some alteration in the positioning of helix-α7 and thence in the proximal surface of the J-channel and presentation of the Asp178-cognate residue (Asp308) in SK2. In principle, the side chain of Ser584 might be able to hydrogen bond to either Asp308 or to Thr584. Although difficult to predict the precise detail of these substitutions, it is noteworthy that a nearby phenylalanine (Phe173 in SK1, conserved as Phe303 in SK2) on helix-α7 contributes to the van der Waals contact surface for the methyl substituent attached to the central arene ring of PF-543. Thus, slight repositioning of helix-α7 might lead to surface encroachment in SK2 at the site occupied by the methyl group of the, which might also be a contributory factor in the observed SK1 selectivity of PF-543. These considerations suggest that a detailed understanding of the J-channel surface contour properties and plasticity will be

important for optimising the development of high potency isoform-selective SK inhibitors in the future, and the emergence of a crystal structure for SK2 would undoubtedly assist in this goal.

7. Summary

In the last 5 years that have been major advances in understanding the role of S1P and sphingosine kinases in healthy and diseased cells. Major advances have included a realisation that the S1P receptors can exhibit biased signaling that might be altered by formation of complexes with other signaling proteins and chaperones of S1P. Sphingosine kinases play a key role in catalysing formation of S1P, which has also been shown to regulate essential cellular processes e.g. replicative immortality in cancer cells, by binding to intracellular target proteins such as tHERT. Key advances in the future will be the use of transgenic animals expressing fluorescent tagged S1P receptors (work in this area has already been initiated), sphingosine kinases and other sphingolipid metabolising enzymes, to establish mechanisms regulating these enzyme/receptor systems *in vivo*. In addition, resolution of the atomic structures of these receptors and enzymes will enable structure-activity directed optimisation of inhibitors/activators that can be developed as therapeutics. SK1 and SK2 exhibit some non-overlapping functions, and therefore there is a need to develop potent isoform selective inhibitors/activators. This is essential, as there is ample evidence to clearly demonstrate a role for S1P, SK1 and SK2 in many diseases, as highlighted in this review.

Figure Legends

Fig. 1. Sphingolipid metabolic pathways. Ceramides can be derived from *de novo* synthesis, via dihydroceramides, or from hydrolysis of sphingomyelin or breakdown of glycosphingolipids. Ceramide, sphingosine and S1P are interconverted and S1P irreversibly cleaved to hexadecenal and phosphoethanolamine. The biological activities (blue dotted arrows) of the various sphingolipids are summarised. Enzymes, which occur as multiple isoforms, are shown in red (Des, dihydroceramide desaturase; CDase, ceramidase; CerS,

ceramide synthase; SK, sphingosine kinase; S1PP, S1P phosphatase) and have specific subcellular localisations (not shown).

Fig. 2. ApoM-S1P regulation of anti-inflammatory signaling. HDL-ApoM containing S1P activates the $S1P_1$ receptor and, through β-arrestin-mediated signaling, induces a profound anti-inflammatory action against TNF-driven biology [47]. Interestingly, the HDL-S1P-$S1P_1$ receptor is retained at the plasma-membrane. However, S1P is known to drive G_i and β-arrestin-dependent endocytosis of the $S1P_1$ receptor leading to, for instance, activation of ERK-1/2 [49]. Therefore, we speculate that an anchor protein binds HDL-S1P-$S1P_1$ to entrap the receptor with β-arrestin at the plasma-membrane and to drive plasma-membrane directed anti-inflammatory signaling. These studies, along with previous identification of $S1P_1$-PDGFβ receptor complexes [49] (which direct migratory as opposed to growth effects, in response to PDGF) highlight the complexities of $S1P_1$ receptor signaling in directing different biological programmes in cells and identifies new avenues for future research and therapeutic targeting.

Fig. 3. Regulation of telomerase by SK2-derived S1P. S1P produced by nuclear SK2 interacts with Asp684 of the catalytic subunit of telomerase, hTERT. This mimics hTERT phosphorylation, prevents its interaction with MKRN1 and limits the proteasomal degradation of hTERT. The resulting telomerase activity maintains the telomere and cell proliferation.

***Fig. 4*. Functional interaction between HER2 and SK1 in estrogen receptor positive breast cancer cells.** HER2 substantially increases SK1 expression which, in turn, is associated with suppressed expression of HER2 and ablated migration of estrogen receptor positive breast cancer cells in response to S1P. $S1P_3$ regulation of PAK1 and localisation of activated ERK-1/2 in lamellipodia are associated with a migratory phenotype. However, the HER2-dependent increase in SK1 drives a putative S1P-dependent down-regulation of PAK1, thereby limiting the formation of actin enriched lamellipodia containing activated ERK-1/2 leading to ablated migration. We have described this phenomenon as oncogene tolerance to reflect how SK1 function in breast cancer cells can be altered in a HER2 oncogenic background.

Fig. 5. Role of SK1 in pulmonary arterial hypertension (PAH). SK1 functions to promote arterial smooth muscle proliferation via an $S1P_2$-dependent mechanism that involves STAT3 and ERK-1/2 signaling [99]. Indeed, inhibition of SK1 or *Sk1* knockout mice are protected from PAH and this can be recapitulated with an $S1P_2$ antagonist. We have also demonstrated that the SK1 inhibitor, PF-543, protects against dysfunctional right ventricular hypertrophy (N. MacRitchie, S. Pyne and N.J. Pyne (unpublished data)) in a hypoxia-induced mouse model of PAH. These findings are similar to the protective effect induced by PF-543 on post-MI cardiac remodelling [101].

Fig. 6. Binding mode detail for the SK1-selective inhibitor, PF-543. **(A)** The overall tertiary structure and binding site disposition is shown for the co-crystal structure (PDB: 4V24) of SK1 (ribbon) with bound PF-543 inhibitor (green surface) [148]. The binding site for Mg-ATP is defined (brown sphere / pink surface) by superimposition of Mg-ADP from its co-crystal structure (3VZD) with SK1 [138]. The binding site for the nucleotide lies in the N-terminal domain of the protein; the Sph substrate binding site, here occupied by PF-543, is hosted by the C-terminal domain. Mg marks the position of the catalytic centre at the junction of the two domains. The lipid substrate binding site comprises a J-shaped hydrophobic tube, the 'J-channel' that is formed by packing of three loops (cyan, yellow and salmon ribbon) against one face of a core β-sandwich substructure in the C-terminal domain. The reverse face of the β-sandwich is occupied by a fourth loop (light green) that fulfils a regulatory function. Access to the Sph binding site is thought to involve opening and closure of the cyan-coloured loop encompassing helices α-7/ α-8. **(B)** Detail is shown for SK1-bound PF-543 (green stick) with Mg (brown sphere) and ADP (pink stick) superimposed as in (A). The polar head group of the inhibitor is bound to Asp178 and a structural water (W1) that is networked by hydrogen bonds to Ser168, Ala339 and Gly342. The tail of the inhibitor is curved to fit the fully enclosed J-channel. Three key residues contributing to the direct ligand binding surface — Ile174, Met272 and Phe288 (red surface) — differ in SK2. Three others that are not in the direct binding surface — Ala175, Met312 and Ala339 — also differ. Collectively the residue substitutions alter the binding

site sufficiently in SK2 to abrogate binding, possibly through J-channel surface encroachment (green arrows) on the inhibitor proximal to the methyl and sulfone groups (see text).

8. References

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Table 1 Intracellular target proteins of S1P

Abbreviation	Name	Comments
BACE1	β-site amyloid precursor protein (APP) cleaving enzyme-1	S1P increases the proteolytic activity of BACE1 (the rate-limiting enzyme for amyloid-β peptide (Ab) production) [68]
HDAC1/2	Histone deacetylase 1/2	SK2-derived S1P enhances expression of genes which have SK2/HDAC1/2 associated with their promoter [60]
PHB2	prohibitin 2	SK2-derived S1P regulates mitochondrial assembly and function [63]
PPARγ	peroxisome proliferator-activated receptor gamma	S1P enhances expression of PPARγ target genes [62]
hTERT	human telomerase reverse transcriptase	SK2-derived S1P stabilises this catalytic subunit of telomerase by preventing its interaction with makorin ring finger protein 1 (MKRN1), an E3 ubiquitin ligase [67]
TRAF2	TNF receptor associated factor 2	S1P enhances TRAF2 E3-ligase activity [53] TNF receptor-associated factor (TRAF)-interacting protein (TRIP) suppresses the TRAF2-S1P interaction [54] But genetic knockout of SK1/SK2 [55] or SK1 [56] or siRNA knockdown of SK1 [57] reported to not affect TNF-α-mediated responses

Table 2 Properties of selected sphingosine kinase inhibitors

Inhibitor	Chemical name	K_i for SK1	K_i for SK2	Examples of *in vivo* use	Comments
Sphingosine competitive					
PF-543	(*R*)-(1-(4-((3-Methyl-5-(phenylsulfonylmethyl) phenoxy)methyl)benzyl)-pyrrolidin-2-yl)methanol	3.6 nM [122]	>100 fold selectivity over SK2	Reduces red blood cell sickling [125] and post MI cardiac remodelling [101]; exacerbates symptoms in EAE mouse model [115]	No effect on cell proliferation [122]; induces proteasomal degradation of SK1 [124]
ABC294640	(3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide)	No inhibition at 100 µM	10 µM [91]	Efficacious in animal models of cancer [92], rheumatoid arthritis [128], ulcerative colitis [129] etc.	Off-target effects include antagonism of ERα [131] and proteasomal degradation of SK1 [93]
ROMe ((*R*)-FTY720 methylether)	(2*R*)-2-Amino-3-(O-methyl)-(2-(4′-n-octylphenyl)ethyl)propanol	No inhibition at 100 µM	16 µM [95]	N.D.	
SLR080811	[(S)-2-[3-(4-octylphenyl)-1,2,4-oxadiazol-5-yl] pyrrolidine-1-carboximidamide]	12 µM [133]	1.3 µM [133]	Increases blood S1P in wildtype mice [133]	
K145	3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione	No inhibition at 10 µM	6.4 µM [132]	Efficacious in animal model of cancer [132]	
SKi (SKI-II)	2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole	16 µM [29]	6.7 µM [29]		Also inhibits dihydroceramide desaturase (K_i 0.3 µM) [143]
Amgen 82	(2*R*,4*S*)-2-(hydroxymethyl)-1-(4-((4-(4-(trifluoromethyl)phenyl)thiazol-2-yl)amino)phenethyl)piperidin-4-ol	IC_{50} 20 nM (hSK1) IC_{50} nM (mSK1) [137]	IC_{50} 114 nM (hSK2) IC_{50} >5 µM (mSK2) [137]	Reduces plasma S1P but does not reduce tumor volume [137]	Cell viability affected only at high concentrations [137]
ATP competitive					
MP-A08	4-methyl-N-[2-[[2- [(4-methylphenyl) sulfonylamino]phenyl]iminomethyl] phenyl] benzenesulfonamide	27 µM [144]	6.9 µM [144]	Efficacious in animal model of cancer [144]	

Fig. 1

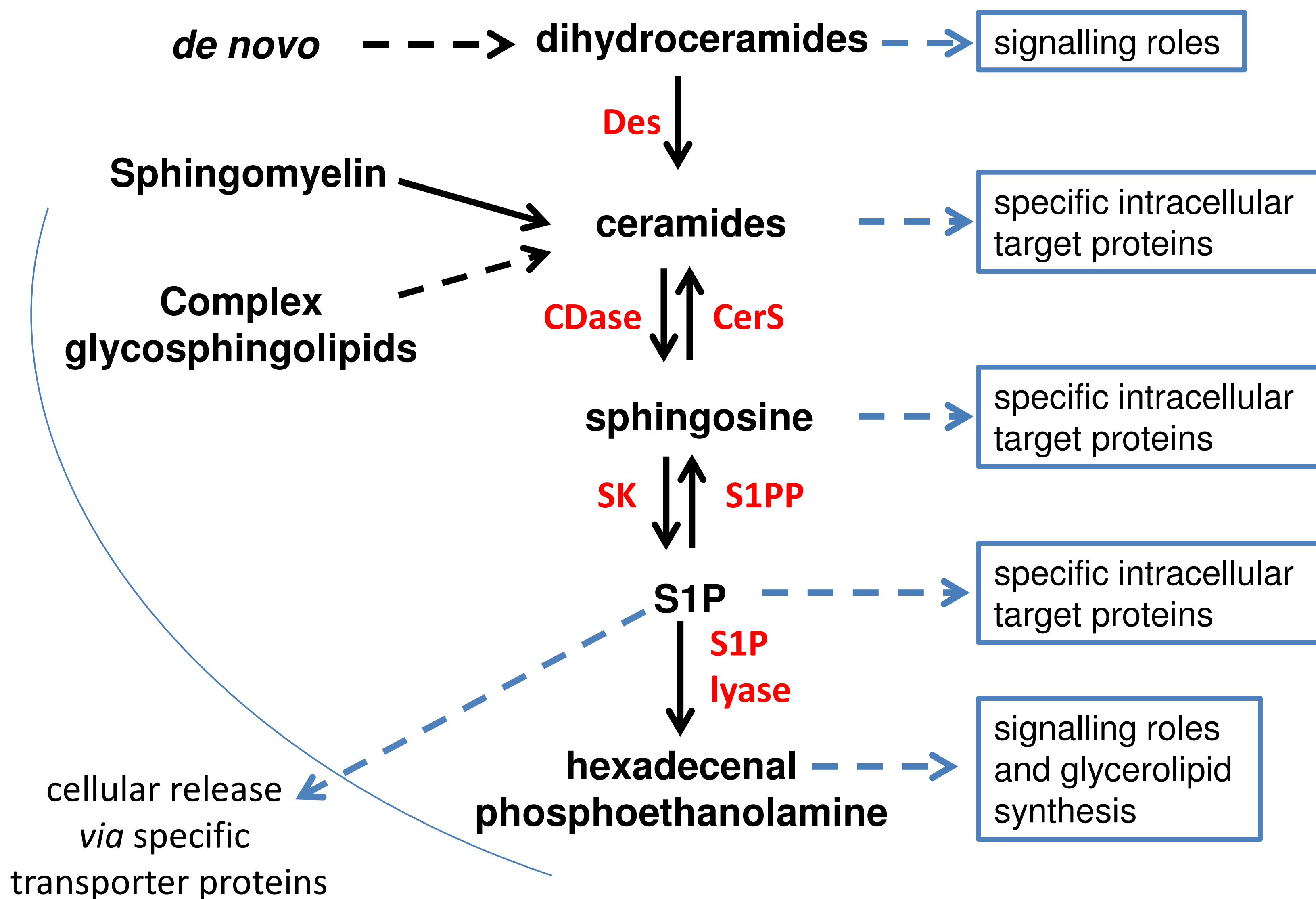

Fig. 2

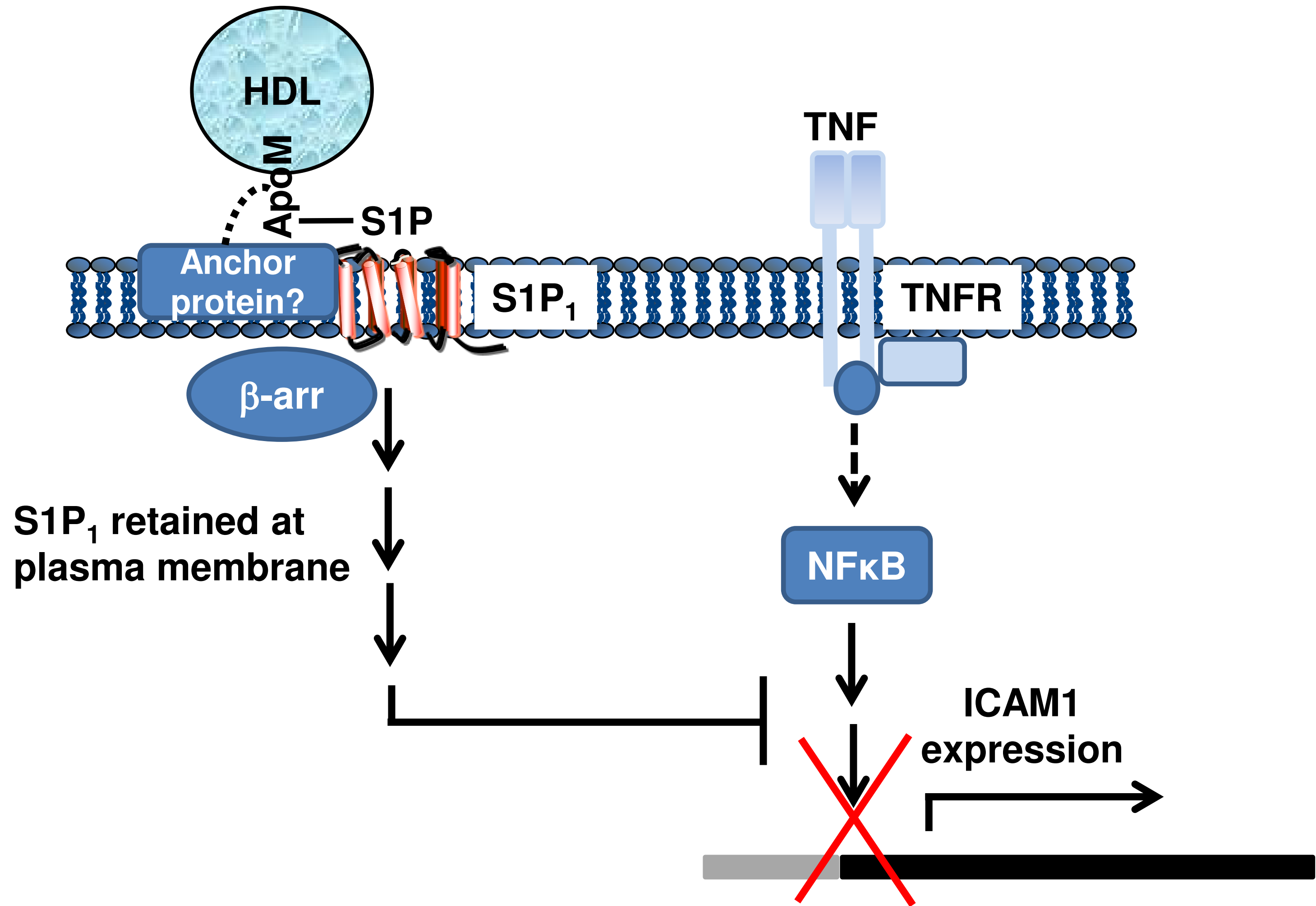

Fig. 3

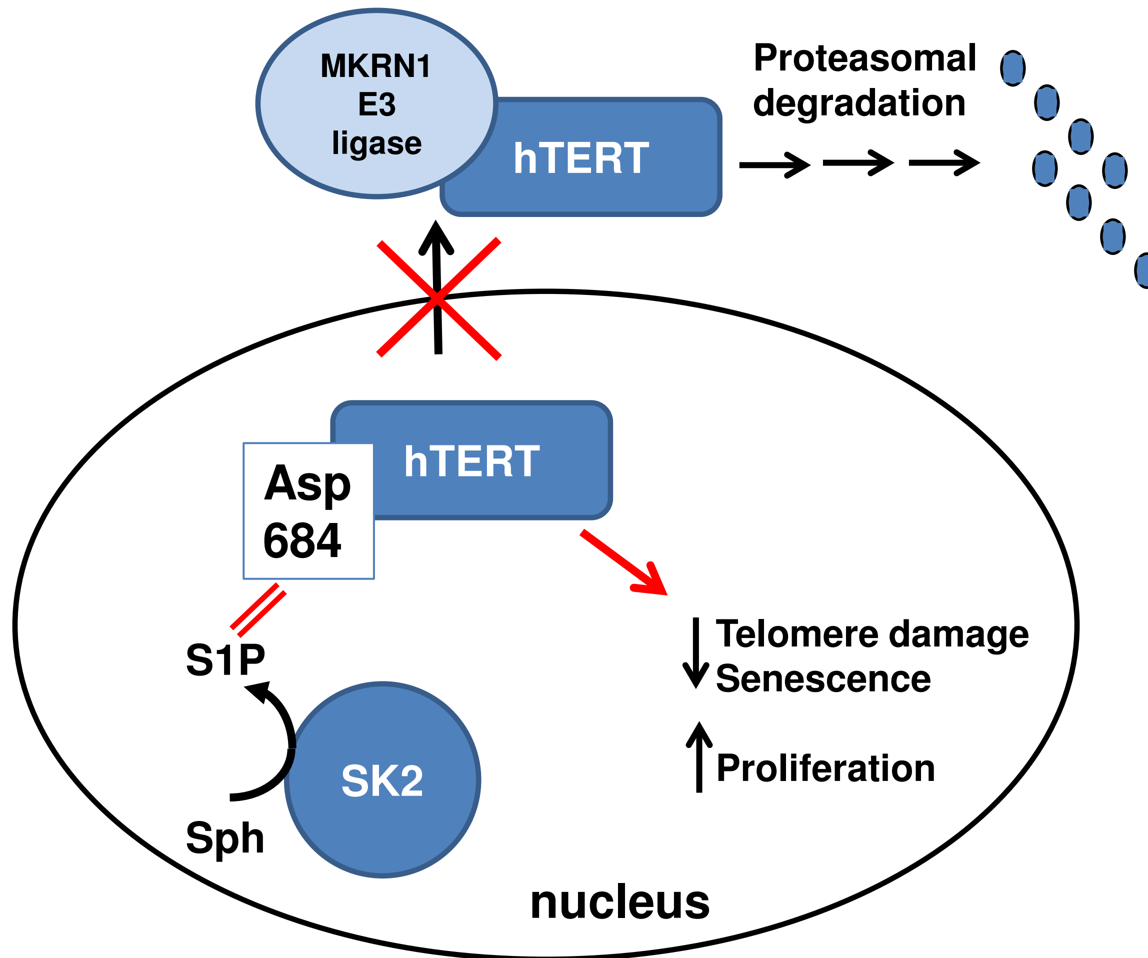

Fig. 4

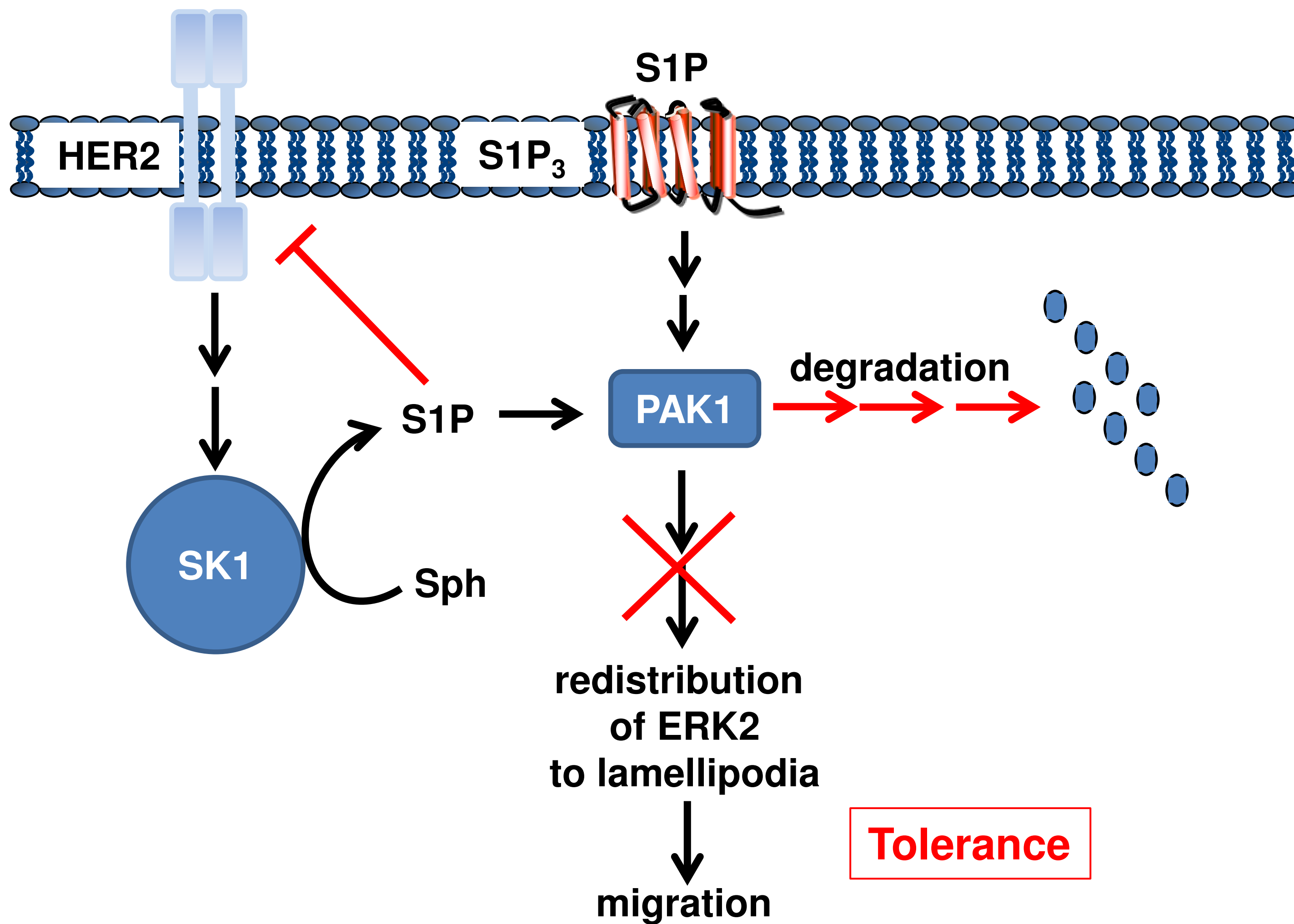

Fig. 5

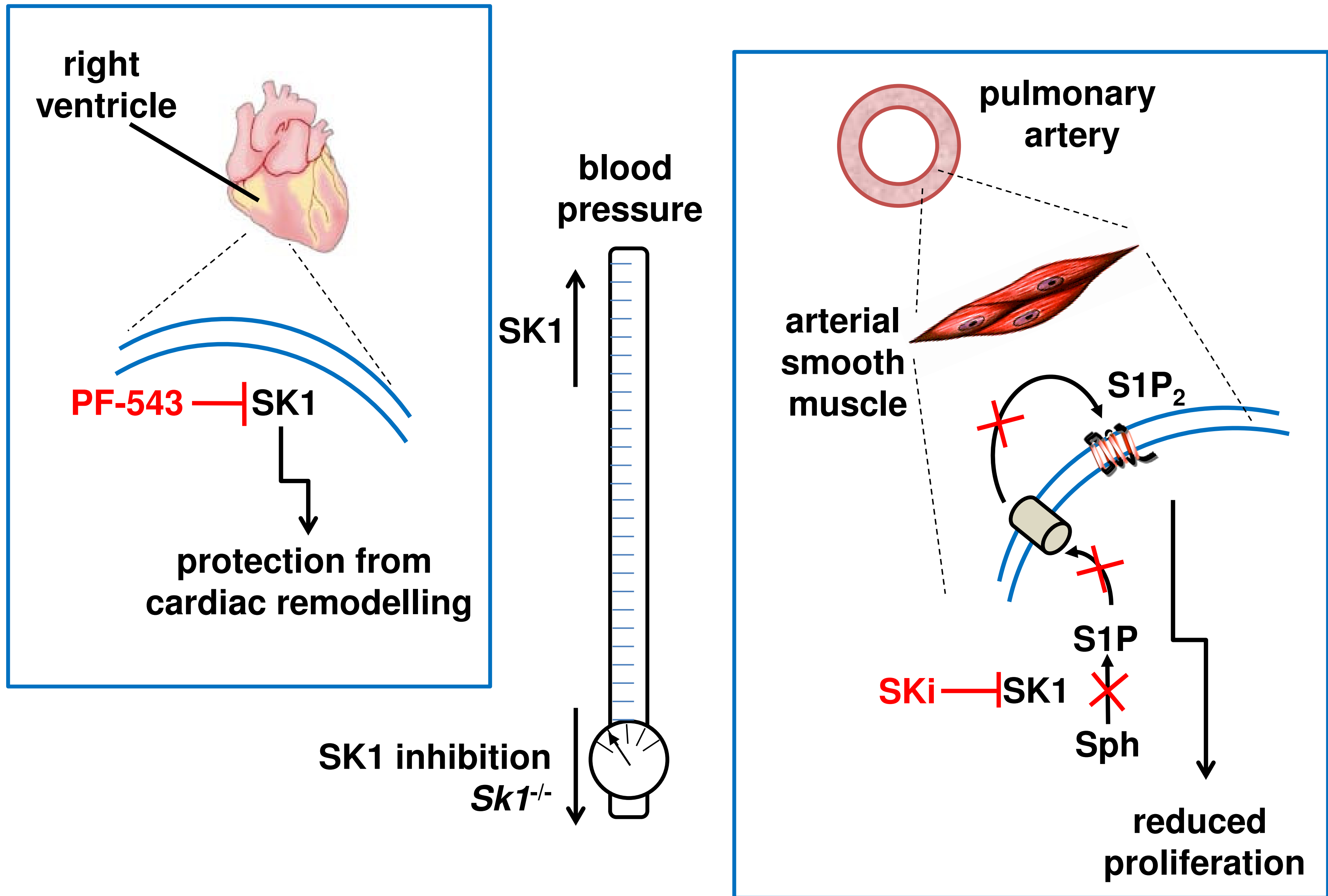

Fig. 6

(A)

C-terminal domain
N-terminal domain
β-sandwich core
ADP
Mg
α7
α8
PF-543

(B)

Phe173
(Ile174)
Ala175
Asp178
Ala339
ADP
W1
Met312
(Met272)
(Phe288)

Gly342 (NH)
Ala339 (CO)
Asp178
Ser168 (OH)
W1
Ile174
Ala175
Me
Phe173
Met272
J-channel
(Met312)
Phe288