Sphingosine kinases as druggable targets

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Contents

Abstract

There is substantial evidence that the enzymes, sphingosine kinase 1 and 2, which catalyse the formation of the bioactive lipid, sphingosine 1-phosphate are involved in physiological and pathophysiological processes. In this chapter, we appraise the evidence that both enzymes are druggable and describe how isoform-specific inhibitors can be developed based on the plasticity of the sphingosine-binding site. This is contextualised with the effect of sphingosine kinase inhibitors in cancer, pulmonary hypertension, neurodegeneration, inflammation and sickling.

Keywords: sphingosine kinase, sphingosine 1-phosphate, cancer, pulmonary hypertension, inflammation, neurodegeneration, sickling

1. Sphingosine kinases

There are two isoforms of sphingosine kinase, termed SphK1 and SphK2, which catalyse the phosphorylation of sphingosine (Sph) to form the bioactive lipid, sphingosine 1-phosphate (S1P). SphK1 and SphK2 are encoded by different genes, are localised in distinct sub-cellular compartments and have different biochemical properties and inhibitor sensitivities (Pyne et al. 2016a). There are splice variants of both SphK1 (SphK1a-c) and SphK2 (SphK2a, b) that, for each isozyme, are distinguished by their N-terminal sequences. Although the relative expression, tissue distribution and function of the various splice variants is not well established, the differing N-terminal sequences are known to influence biochemical properties. Thus, the 36 amino acid N-terminally extended SphK2b isoform has higher activity compared with the SphK2a isoform, indicating that the N-terminus might regulate catalysis (Billich et al. 2003). Moreover, SphK1b, which contains an additional N-terminal 86

amino acids is more resistant to SphK inhibitor-induced ubiquitin-proteasomal degradation compared with SphK1a (Loveridge *et al*., 2010). SphK1 and SphK2 exhibit some redundancy in function, as evident from studies in *sphk1*$^{-/-}$ or *sphk2*$^{-/-}$ mice, which are phenotypically healthy while deletion of both genes is embryonic lethal (Mizugishi et al. 2005). In this regard, SphK1 and SphK2 appear to regulate discrete sub-cellular pools of S1P that govern both overlapping and/or non-overlapping biology (Pyne et al. 2016a). Levels of S1P are controlled both by SphK1/2, acting on pools of Sph that are coupled to ceramide biosynthesis (*vide infra*), and by the activity of S1P lyase and S1P phosphatases (Pyne et al. 2016a). S1P lyase catalyses the irreversible cleavage of S1P to form (*E*)-2-hexadecenal and phosphoethanolamine (Aguilar & Saba, 2012) and represents the only exit point from the sphingolipid metabolic pathway, providing precursors for phospholipid biosynthesis (Nakahara et al. 2012). In contrast, S1P phosphatases, of which there are two isoforms, catalyse dephosphorylation of S1P to form Sph (Mandala, 2001). Once formed, S1P can be released from cells by transporters (including Spns2 (Hisano et al. 2012), MFsd2b (Vu et al. 2017) and some ABC proteins (Kobayashi et al. 2009)) to subsequently bind to a family of S1P-specific G protein-coupled receptors (GPCR), $S1P_{1-5}$, and thereby induce cellular responses (Blaho & Hla, 2014). However, S1P also acts inside cells by binding to intracellular targets, such as histone deacetylase (HDAC1/2) (Hait et al. 2009) and telomerase reverse transcriptase (hTERT) (Panneer Selvam et al. 2015), to regulate epigenetic programmes and senescence. Therefore, S1P is a rather versatile bioactive lipid, being able to act both extracellularly and intracellularly to regulate biological processes.

2. Role of S1P and ceramide

S1P is involved in regulating cardiovascular function, neuronal development and immune cell trafficking as well as other physiological processes (Pyne et al. 2016a). Not surprisingly, then, S1P is implicated in various pathophysiological conditions, including cardiovascular disease, autoimmune/inflammatory disease, neurodegeneration and cancer (Pyne et al. 2016a; Pyne et al. 2017). Therefore, targeting the S1P signalling pathway is a potential therapeutic option for a number of

diseases. There are two objectives here. The first is to suppress S1P signalling *via* its receptors and intracellular targets, either by antagonism of the receptors or by inhibition of SphK1/2 activity. The second, which is particularly relevant to cancer, is to promote an increase in cellular ceramide and Sph levels by blocking conversion of the latter to S1P, again by inhibition of SphK1/2 activity. Indeed, the physiological actions of ceramide and Sph generally oppose the actions of S1P and have been shown to promote senescence and induce apoptotic cell death in mammalian cells (Hannun & Obeid, 2008; Hannun & Obeid, 2011). In contrast, S1P is involved in promoting cell growth (Spiegel & Milstien, 2011). Therefore, the aim in elevating ceramide levels is to induce cytotoxicity in cancer cells.

3. Sphingolipid biosynthesis

De novo synthesis of ceramide begins with the condensation of serine and palmitoyl CoA, catalysed by serine palmitoyltransferase, to form 3-ketosphinganine as the rate limiting step in ceramide biosynthesis (Hannun & Obeid, 2008). 3-Ketosphinganine reductase then converts 3-ketosphinganine into dihydrosphingosine (sphinganine), which is acylated to produce dihydroceramide by ceramide synthases (CerS). Finally dihydroceramide desaturase (Degs1) catalyses the formation of ceramide by introduction of a 4,5-*trans* double bond. Ceramide can also be formed by the hydrolysis of sphingomyelin, catalysed by sphingomyelinase, which is part of the 'salvage pathway'. Ceramide is deacylated to Sph by ceramidase, and finally Sph can be either phosphorylated by SphK1 or SphK2, to form S1P, or it can be converted back into ceramide, catalysed by CerS (Figure 1).

4. The sphingolipid rheostat

The dynamic interconversion of ceramide, Sph and S1P has been termed the 'sphingolipid rheostat' (Pyne et al. 1996, Cuvillier et al. 1996). In order for this pathway to be considered a rheostat the forward reactions (ceramide → Sph → S1P) and backward reactions (S1P → Sph → ceramide) should be regulated in a reciprocal manner. In order to achieve this it is probable that the enzymes involved in the interconversion of ceramide, Sph and S1P are allosterically regulated. Indeed, it has been

reported that S1P inhibits CerS2 by binding to two residues that are part of an S1P receptor-like motif found only in CerS2 (Laviad et al. 2008). This might therefore provide the essential molecular regulation that enables a rheostat to function. Inhibition of CerS2 by S1P would bias the rheostat toward the synthesis of S1P. In addition, the inhibition of CerS2 by S1P is expected to reduce *de novo* synthesis of long chain ceramides by redirecting dihydrosphingosine toward dihydrosphingosine 1-phosphate formation where SphK1 negatively regulates *de novo* ceramide synthesis (Siow et al., 2015). Regulation of SphK1 by ERK-1/2 catalysed phosphorylation (Pitson et al. 2003) stimulated by extracellular mediators is a mechanism by which the forward flux can be enhanced. In addition, regulation of the rheostat might be achieved by an unidentified sensor that can modulate forward and backward fluxes to determine the position of the rheostat, thereby programming cell death *versus* survival. Indeed, homeostatic sensors have been identified for regulating *de novo* ceramide synthesis (Siow et al., 2015). Thus, SphK inhibitors are expected to increase ceramide synthesis through modulation of both *de novo* and salvage pathways.

However, the sphingolipid rheostat model has additional complexity. For example, different molecular species of ceramide do not always promote cell death and, in some cases, can promote cell survival (Saddoughi & Ogretmen, 2013). In addition, ceramide can be converted by ceramide kinase into ceramide 1-phosphate (C1P) (Hoeferlin et al. 2013), which is a pro-survival signal. Moreover, the presence or absence of the key enzymes in various cellular compartments will determine whether the rheostat can operate in the manner expected, and distinct enzyme forms appear to regulate discrete lipid pools. Thus, at the plasma membrane, neutral sphingomyelinase catalyses the formation of ceramide (Hannun & Obeid, 2008), which can then be converted into Sph which is used by SphK1 to produce S1P. However, the Sph substrate for SphK2 at the endoplasmic reticulum (ER) derives, at least in part, from the action of acid sphingomyelinase in the lysosome to generate ceramide, which is then deacylated by acid ceramidase (Hannun & Obeid, 2008). Ceramide is also formed by the *de novo* pathway in the ER (Hannun & Obeid, 2008). A ceramide transporter protein (CERT) transfers

ceramide from the ER to the Golgi apparatus where it is converted into complex sphingolipids e.g. sphingomyelin and glycosphingolipids, which are then transported to various cellular compartments, including the plasma membrane (Yamaji & Hanada, 2015). Therefore, there is both spatial and temporal regulation in the interconversion of ceramide, Sph and S1P in mammalian cells, which can influence cellular fate and offer opportunities for therapeutic intervention.

S1P-dependent signalling might enable cells to undergo positive selection by conferring enhanced survival. Therefore, the sphingolipid rheostat might have evolved in order to endow organisms with the capacity to use sphingolipids in interconverted and branched pathways to induce pleiotropy, thereby providing a wider repertoire of biological functions that enhance cell renewal and survival. In this regard, the ability of cancer cells to up-regulate SphK1 expression as part of a 'non-oncogenic addiction' (Vadas et al. 2008) and to drive the forward reaction of the sphingolipid rheostat, might enable positive selection of a clonally expanded cancer cell population with replicative immortality. Therefore, pharmacological inhibition of SphK activity in cancer, for example, is geared to subverting the gain in cell survival achieved by simultaneously enhancing ceramide formation to shift the balance toward cell death. This requires the development of effective isoform selective inhibitors of SphK1 and SphK2 that exhibit minimal or no 'off-target' effects.

5. Concept of modulation of the sphingolipid rheostat

It is not immediately obvious why inhibition of SphK should necessarily increase ceramide levels with a pro-apoptotic outcome. One mechanistic explanation might involve dis-inhibition of the allosteric effects of S1P on CerS2. This enzyme has no activity using C16:0-CoA and very low activity using C18:0 CoA, but instead utilises longer acyl-chain CoAs (C20–C26) for ceramide synthesis (Laviad et al. 2008). In contrast to C16:0 ceramide, which has been shown to promote growth, longer chain species of ceramide are apoptotic (Saddoughi & Ogretmen, 2013). Therefore, enhanced activity of CerS2 in the sphingolipid rheostat, due to a blockade of S1P formation by SphK inhibitors, could

potentially bias synthesis toward apoptotic ceramides. A second possibility is that the conversion of ceramide to Sph, catalysed by ceramidase, might be inhibited by Sph that has accumulated as a consequence of inhibiting SphK activity, and there is evidence for product inhibition using epidermal ceramidase (Yada et al. 1995). Furthermore, rectifying sensors or relief of allosteric inhibition of enzymes involved in S1P and dihydrosphingosine 1-phosphate metabolism might induce a 'rippling effect' throughout the *de novo* ceramide, 'salvage' and complex sphingolipid biosynthetic pathways, adding complexity to therapeutic approaches that target sphingolipids. In addition, other conceptual considerations require evaluation, such as whether the sphingolipid rheostat involves graded or switch responses, involving 'threshold' levels of specific sphingolipids.

6. Sub-cellular localisation of SphK1 and SphK2

SphK1 is predominantly localised in the cytoplasm and is recruited to the plasma membrane in response to stimulation by extracellular ligands e.g. growth factors, TNFα and S1P (Pyne et al. 2016a). Once localised at the plasma membrane, SphK1 is thought to access its lipid substrate *via* a gating mechanism that involves opening of a flap-like lid to the substrate binding site (Adams et al. 2016). The surface of this lid, denoted lipid binding loop-1 (LBL-1), contains a hydrophobic patch that has been implicated in recruitment of SphK1 to membranes and may potentially insert into the plasma membrane (Adams et al. 2016). Recruitment of SphK1 to the plasma membrane is promoted by the action of extracellular signal-regulated kinases, ERK-1/2, which catalyse phosphorylation of a serine (Ser225) within an extended surface-exposed regulatory loop (the 'R-loop') (Pitson et al. 2003; Pitson et al., 2005). Translocation of SphK1 to the plasma membrane also requires the calcium and integrin-binding protein 1, CIB1, which functions as a molecular chaperone (Jarman et al. 2010). SphK1 is additionally recruited to the plasma membrane in an ERK-1/2-independent manner involving G_q-coupled GPCRs (ter Braak et al., 2009). Thus, a Ser225Ala SphK1 mutant retains the capacity to translocate to the plasma membrane in response to muscarinic receptor stimulation. Both wild type (WT) and constitutively activated forms of G_q (*e.g.* $G\alpha_q^{R183C}$, $G\alpha_q^{Q209L}$-EE) promote this alternative

mode of translocation (ter Braak et al., 2009), which might potentially involve association of G_q with the SphK1 C-terminal tail sequence (Adams et al. 2016). Indeed, this region of SphK1 is known to facilitate binding to other proteins, namely TNF receptor-associated factor 2 (TRAF2, in response to TNFα stimulation) and protein phosphatase 2 (PP2A, for dephosphorylation of Ser225) (Xia et al. 2002; Barr et al. 2008). Moreover, SphK1 adopts a dimeric quaternary structure that might allow coordinated coupling of plasma membrane association with phosphorylation of Ser225 in the R-loop, catalytic activity and protein-protein interaction (Lim et al. 2011b; Adams et al. 2016). Thus, the regulation of catalytic activity is likely to be more complex than a simple targeting of the protein to its substrate pool, potentially involving direct structural modulation of the enzyme's phosphoryl transfer capacity. In particular, the catalytic centre of the enzyme features a key loop, the 'T-loop', that presents the ATP co-substrate to the lipid. The T-loop is located at the N-terminal end of a short helix, α5, and exhibits structural plasticity that is likely to be important for kinase activity. The C-terminal end of helix-α5 lies proximal to and may be capped by the SphK1 C-terminal protein-binding sequence, deletion of which results in constitutive activation (Hengst et al. 2010a). It is therefore possible that protein binding to the C-terminal sequence modulates capping and thence T-loop structure to regulate activity (Adams et al., 2016).

In contrast, SphK2, is localised at the endoplasmic reticulum or is associated with mitochondria (Maceyka et al. 2005). In this regard, SphK2 contains both a nuclear localisation sequence (NLS) and a nuclear export signal sequence (NES) that enables the enzyme to shuttle in and out of the nucleus and to be exported from the nucleus after phosphorylation of the NLS by protein kinase D (Ding et al. 2007). S1P generated in the nucleus can regulate epigenetic programmes via the inhibition of HDAC1/2 activity (Hait et al. 2009). In addition, S1P binds to other intracellular targets that affect gene expression, such as the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) (Parham et al. 2015).

7. S1P receptors

S1P-specific GPCRs, $S1P_1$-$S1P_5$ (Chun et al. 2002; Kihara et al. 2014) are involved in numerous physiological processes in the cardiovascular, immune and nervous systems and can participate in regulating vascular barrier integrity, trafficking of lymphocytes and astrocyte function (Blaho & Hla, 2014). However, S1P receptors also participate in the pathophysiology of autoimmune/inflammatory diseases and cancer. Therefore, members of the S1P receptor family are of significant interest as therapeutic targets, with drug discovery most advanced for ligands acting *via* $S1P_1$, such as fingolimod (FTY720) and ozanimod (Gonzalez-Cabrera et al. 2014). The latter and phosphorylated FTY720 serve as agonists for $S1P_1$ but trigger internalisation of the receptor, resulting in a functional antagonism that inhibits T-cell trafficking (Hla & Brinkmann, 2011). FTY720 is currently licensed for treatment of multiple sclerosis (MS) while ozanimod is in Phase III and Phase II clinical trials for MS and ulcerative colitis, respectively. FTY720 phosphate, acting *via* S1P receptors, also regulates astrogliosis and nerve remyelination (Bigaud et al. 2014). Therefore, in terms of the development and utility of SphK inhibitors, a key question is whether these compounds can reduce the bioavailability of S1P at S1P receptors as a pharmacologically useful alternative to ligands targeting the receptors themselves. There is a substantial literature that relates to the release of S1P formed by SphK1 from cells and action on S1P receptors, both in terms of autocrine (termed 'inside-out' signalling) and paracrine mechanisms (Takabe et al. 2008). However, there is far less evidence that SphK2 can perform a similar function. Nevertheless, SphK2 catalyses the phosphorylation of FTY720, and phosphorylated FTY720 is released from cells to functionally antagonise $S1P_1$ on T-cells. This underlies, in part, the pharmacological action of fingolimod and, additionally, suggests that SphK2 can indirectly regulate S1P receptor function.

8. Intracellular actions of S1P

In addition to its action on S1P receptors, there is evidence that S1P binds to and regulates intracellular target proteins to induce cell responses (Figure 2). These represent additional targets for therapeutic intervention. However, at present there is a paucity of information concerning binding affinities for S1P and the stoichiometry at putative targets. Current understanding is further compromised by the lack of information concerning the concentration of S1P in various sub-cellular compartments. Nevertheless, intracellular targets of S1P have been directly related to fundamental biological processes that can potentially contribute to disease pathology. For instance, S1P formed by SphK1 has been reported to confer E3 ligase activity on TRAF2, where S1P is modelled to bind to its RING finger (Alvarez et al. 2010). TRAF2 has been shown to associate with SphK1 (Xia et al., 2002), thereby enabling the S1P formed to be in close proximity with its target. TRAF2 mediates the K63 polyubiquitination of RIP1, which participates in regulating pro-inflammatory TNFα/NFκB signalling. However, other reports have suggested that while TNFα-stimulated NF-kB activation is indeed reduced in *Traf2*$^{-/-}$ keratinocytes, it is not affected by the loss of SphK1 (Etemadi et al. 2015). An additional intracellular role for SphK1 is in endosomal processing/endocytic signalling and neurotransmission (Shen et al. 2014). Indeed, knockdown of SphK1 modulates endocytic recycling and results in dysfunctional neurotransmission, an outcome that is rescued by WT SphK1 but not by SphK1 that is mutated in the hydrophobic LBL-1 patch implicated in membrane targeting (V268Q-SphK1) (Shen et al. 2014).

S1P formed by SphK2 also has intracellular roles. For example, nuclear localised SphK2 forms a complex with histone H3 and HDAC1/2 in the promoters of *c-fos* and *p21* genes (Hait et al. 2009). The S1P formed by SphK2 inhibits HDAC1/2 to preserve histone acetylation at specific lysine residues, thereby enhancing gene transcription and implicating SphK2 in epigenetic regulation. S1P has also been shown to bind to the transcription factor PPARγ to enhance expression of genes in endothelial cells linked with neovascularisation (Parham et al. 2015). In addition, S1P formed by

SphK2 binds to prohibitin 2 (PHB2), which regulates mitochondrial assembly and function (Strub et al. 2011). SphK2-derived S1P also stabilises hTERT; this catalytic subunit of telomerase maintains telomeres and its activity is increased in cancer cells (Panneer Selvam et al. 2015). Finally, S1P activates the β-site amyloid precursor protein (APP) cleaving enzyme-1 (BACE1), suggesting a role for S1P formed by SphK2 in β amyloid accumulation and synaptic spread in neurodegenerative disease (Takasugi et al. 2011).

9. Role of SphK1 and SphK2 in disease and utility of inhibitors

The case for pharmacological development of SphK1 and SphK2 inhibitors is dependent largely on whether unequivocal target validation can be demonstrated. Therefore, the following sections describe a number of instances where SphK1 or SphK2 play key roles in diseases. Figure 3 summarises isoform-selective and dual inhibitors of SphK1 and SphK2, which have recently been reviewed (Pitman et al. 2016).

9.1 S1P levels in blood

Kharel et al. (2012) reported that the treatment of mice with the SphK2 inhibitor, SRL080811, increased blood S1P levels. This contrasts with the effect of SphK2 inhibitor, ABC294640, which lowers S1P levels (Beljanski et al. 2011). However, the latter might be attributed to indirect effects on SphK1 expression, as ABC294640 has been shown to induce the proteasomal degradation of SphK1 in cancer cells (see below). Significantly, SRL080811 recapitulated the elevation of blood S1P levels observed in *sphk2*$^{-/-}$ mice, suggesting that SphK2 might exhibit additional activities in regulating S1P uptake into cells (Kharel et al. 2012).

9.2 Role of SphK1 and SphK2 in cancer

In patients, SphK1 expression levels are higher in tumours compared with normal tissue and this is associated with poor prognosis in breast (Watson et al 2010; Ohotski et al. 2013), colon (Tan et al.

2014), head and neck carcinoma (Facchinetti et al. 2010), prostate (Malavaud et al. 2010) and other cancers (Pyne & Pyne, 2010). Indeed, there are numerous examples of a role for SphK1 in cancer. For instance, the over-expression of the K-RasG12V oncogene stimulates translocation and activation of SphK1 and this promotes cellular transformation (Gault et al. 2012). SphK1 also participates in stimulating neovascularisation of tumours involving paracrine signaling by S1P leading to angiogenesis and lymphangiogenesis (Anelli et al. 2010). In addition, cross-regulation between SphK1, $S1P_3$ and Notch promote cancer stem cell proliferation to increase tumorigenesis (Hirata et al. 2014). Significantly, *sphk1*$^{-/-}$ mice have reduced S1P levels and decreased tumour multiplicity and volume in 4-NQO-induced head and neck squamous cell carcinoma carcinogenesis (Shirai et al. 2011).

Furthermore, the stability of Bcr-Abl1 (a fusion gene involved in promoting haematological cancers) is reduced upon siRNA knockdown of SphK1 in imatinib-resistant K562/IMA-3 chronic myeloid leukemia (CML) cells or in *SphK1*$^{-/-}$ mouse embryonic fibroblasts (Salas et al. 2011). In addition, treatment of a murine model of breast cancer metastasis with the selective SphK1 inhibitor, SPHK1-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 metastasis to lymph nodes and lungs and decreases overall tumour burden (Nagahashi et al. 2012). Loss of systemic, but not tumour SphK1 prevents S1P elevation and inhibits TRAMP-induced prostate cancer growth in *TRAMP*$^{+/+}$ *sphk1*$^{-/-}$ mice or lung metastasis of multiple cancer cell types in *sphk1*$^{-/-}$ mice (Ponnusamy et al. 2012). SphK1 is also over-expressed and constitutively activate in primary acute myeloid leukemia (AML) patient blasts but not in normal mononuclear cells (Powell et al. 2017). Inhibition of SphK1 with the dual SphK1/SphK2 inhibitor, MP-A08, or shRNA knockdown reduced AML cancer cell survival (Powell et al. 2017). In addition, MP-A08 induces caspase-dependent cell death *via* down-regulation of MCl-1 in AML cell lines, primary AML patient blasts and isolated AML patient leukemic progenitor/stem cells. MP-A08 also reduced tumour burden and increased overall survival in orthotopic AML patient-derived xenografts (Powell et al. 2017).

There is also evidence for a role for SphK2 in cancer. Indeed, low-level SphK2 expression has been shown to enhance cell survival and proliferation and promote neoplastic transformation *in vivo* and this is associated with plasma membrane localised SphK2, while high expression promoted cell death (Neubauer et al. 2016). The reason why both localisation and function of SphK2 are altered is unknown, but these are clearly concentration-dependent. This might reflect competition for binding partners, such that high concentrations of SphK2 *via* its BH3 domain can sequester and inactivate the anti-apoptotic protein, Bcl2 (Liu et al. 2003). SphK2 inhibitors have utility in cancer. Thus, treatment of early stage and advanced prostate cancer cells with the SphK2 selective inhibitor, ABC294640, induces inhibition of growth, proliferation, and cell cycle progression. Furthermore, oral treatment of mice with ABC294640 reduced xenograft tumour growth (Schrecengost et al. 2015). In addition, the SphK2 selective inhibitor, (*R*)-FTY720 methylether (ROMe), induces the autophagic death of T cell acute lymphoblastic leukemia (T-ALL) cell lines and patient lymphoblasts (Evangelisti et al. 2014). SphK2 also confers an ability on tumour cells to evade the immune system. Thus, *SPHK2*$^{-/-}$ MCF-7 breast cancer cells display retarded growth *in vivo* and tumour associated macrophages exhibit an anti-tumour phenotype (Weigert et al. 2009).

Analysis of a large gene expression data set from CD138^{+} bone marrow plasma cells from newly diagnosed myeloma patients revealed increased *SphK2* expression compared to healthy controls (Wallington-Beddoe et al. 2017). In addition, the SphK2 inhibitors, K145 and ABC294640, induce a caspase-3-dependent apoptosis of multiple myeloma (MM) cells *via* an ER stress/sustained unfolded protein response (UPR), and non-lethal concentrations of K145 and bortezomib synergise to promote sustained UPR and CCAAT-enhancer-binding protein homologous protein (CHOP)-induced apoptosis of MM cells (Wallington-Beddoe et al. 2017, Pyne & Pyne, 2017).

9.3 Role of SphK1 in pulmonary hypertension

There is also evidence that clearly supports a significant role for SphK1 in pulmonary hypertension. For instance, SphK1 expression is increased in the lungs and pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension (Chen et al. 2014). Increased SphK1 expression is also evident in remodelled pulmonary arteries of patients with idiopathic pulmonary arterial hypertension and in the Sugen5416/hypoxia/normoxia (Su/Hx/Nx PAH) rat model of occlusive pulmonary hypertension (Gairhe et al. 2016). The potential of SphK1 as a therapeutic target in pulmonary hypertension is further supported by studies with *sphk1*$^{-/-}$ mice, which exhibit lower right ventricular systolic pressure, decreased right ventricular hypertrophy (RVH) and less severe pulmonary vascular remodelling in response to chronic hypoxia (Chen et al. 2014). Additionally, the SphK1/2 inhibitor, SKi (SKI-II), prevented the development of hypoxia-dependent pulmonary hypertension (Chen et al. 2014). The utility of SphK1 inhibitors is also evident in other disease models. Thus, the SphK1 inhibitor, SLP7111228, reduced plasma S1P levels and the percentage of occluded pulmonary arteries, while increasing the percentage of non-occluded pulmonary arteries in the Su/Hx/Nx PAH rat model (Gairhe et al. 2016). Finally, administration of the highly selective SphK1 inhibitor, PF-543, in a mouse hypoxic model of pulmonary hypertension reduced right ventricular hypertrophy (MacRitchie et al. 2016), thereby suggesting that the SphK1 inhibitor might be protective in heart failure. In this regard, PF-543 also reduced chronic cardiac inflammation and ameliorated cardiac remodelling and dysfunction *in vivo* following myocardial infarction in mice (Zhang et al. 2016).

9.4 Role of SphK1 and SphK2 in the central nervous system

SphK2-derived S1P promotes the survival of dopaminergic neurons that are involved in the development of Parkinson's disease. In this regard, SphK2 expression is decreased in the *substantia nigra* region in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease mouse model (Sivasubramanian et al. 2015). The localisation of SphK2 to the mitochondria might be important here, as mitochondrial dysfunction is a feature of Parkinson's disease. Indeed, the inhibition

of SphK2 activity reduces the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, the co-activator of PPARγ), nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM) (Sivasubramanian et al. 2015). There is also evidence for a role for SphK in Alzheimer's disease (AD). In this regard, β-amyloid peptide fragment 25-35 (Aβ25-35) toxicity is associated with a marked down-regulation of SphK1 expression (Yang et al. 2014). Moreover, siRNA knockdown of SphK1 expression increased Aβ load and worsened learning and memory ability in *APP/PS1* transgeneic mice, a model for AD (Zhang et al. 2013). SphK2 activity is also up-regulated in brains from patients with AD and S1P formed by SphK2 activates BACE1 (Takasugi et al. 2011).

9.5 Role of SphK1 and SphK2 in inflammation

SphK1 has a pro-inflammatory role in cancer (Liang et al. 2013; Pyne & Pyne, 2013) and rheumatoid arthritis (Baker et al. 2010) but is protective in neuro-inflammation (Grinkina et al. 2012). For instance, colitis-associated cancer is linked to an amplification loop involving SphK1, $S1P_1$, NFκB, STAT3 and IL-6 (Liang et al. 2013), and *sphk1*$^{-/-}$ mice are protected from dextran sulphate sodium-induced ulcerative colitis (Snider et al. 2009). Similarly, *sphk1*$^{-/-}$ mice are protected against development of TNFα-induced arthritis (Baker et al. 2010). Furthermore, enforced over-expression of SphK1 increases IL-12 formation from dendritic cells and siRNA knockdown of SphK1 in these cells prevents IFN-γ formation from Th1 cells (Jung et al. 2007). However, administration of the SphK1 inhibitor, PF-543, to mice increased disease progression in the experimental auto-immune encephalomyelitis (EAE) model of MS with enhanced infiltration of $CD4^+$ T-cells, $CD11b^+$ monocytes and $F4/80^+$ macrophages in the spinal cord (Pyne et al. 2016b).

In contrast with deletion of *sphk1*, *sphk2* deficiency does not affect the severity of inflammatory arthritis, even though the SphK2-inhibitory compound, ABC294640, worsens hTNFα-induced arthritis (Baker et al. 2013). The explanation for this apparent paradox may lie in the fact that ABC294640 does

not have a pharmacologically clean profile, but also induces proteasomal degradation of SphK1 and inhibits Degs1 in cancer cells (McNaughton et al. 2016). A pro-inflammatory role for SphK2 is evident from studies demonstrating that the number of infiltrating $CD3^+$ T cells, $CD11b^+$ neutrophils and macrophages are reduced in the fibrotic kidney from *sphk2*$^{-/-}$ mice compared with WT mice (Bajwa et al. 2016). In addition, increased mRNA expression levels of CXCL1 and CXCL2 were evident in WT and *sphk1*$^{-/-}$ mice but not *sphk2*$^{-/-}$ mice (Bajwa et al. 2016). Furthermore, *sphk2*$^{-/-}$ mice are protected against EAE (Imeri et al. 2016), and the SphK2 inhibitor, (*R*)-FTY720 methyl ether (ROMe), prevents inflammatory cell infiltration into the spinal cord and reduces disease progression in this model (Barbour et al. 2017). Further evidence that SphK2 has a pro-inflammatory function was obtained in studies demonstrating that the SphK2 inhibitor, ABC294640, can produce anti-inflammatory outcomes in some scenarios, namely in rodent models of inflammatory bowel disease (IBD) (French et al. 2010; Maines et al. 2010; Maines et al. 2008) and collagen-induced arthritis (Fitzpatrick et al. 2011).

Therefore, there is sufficient evidence to suggest that both SphK1 and SphK2 inhibitors could have utility for treatment of inflammatory disease. However, isoform-specific inhibitors may be important for any future clinical deployment in order to maintain homeostatic control of S1P through compensatory effects exerted by the SphK isoform that is not targeted. Dual SphK1/SphK2 inhibitors, whilst potentially exhibiting pharmacological activity, may carry a risk of undesirable effects arising from blanket, unmitigated reduction in cellular S1P.

9.6 S1P and sickling

S1P levels are substantially increased in the blood of humans with sickle cell disease (SCD) and in SCD transgenic mice. SphK1, the expression of which is increased in this condition, is responsible for the elevation of S1P in the blood in a murine model of SCD, but was found to promote sickling in an S1P receptor-independent manner in isolated erythrocytes (Zhang et al. 2014). A direct role for

elevated S1P levels as a driving factor in SCD was shown by the fact that the SphK1 inhibitor, PF-543, reduced sickling, haemolysis, and inflammation in SCD mice (Zhang et al. 2014). Circulating IL-2, IL-6, and IL-17A were also significantly reduced in SCD mice. Moreover, shRNA knockdown of SphK1 in hematopoietic stem cells decreased erythrocyte sickling and reticulocytes in SCD chimeras (Zhnag et al. 2014). Therefore, in terms of targeting SphK1 it is necessary to consider the specific disease context. For instance, PF-543 lowers IL-6/IL-17A in SCD mice but worsens disease progression in an EAE mouse model (Zhang et al. 2014).

10. Significance and questions concerning the sphingolipid rheostat

As discussed above, pharmacologically active SphK inhibitors can increase the ceramide/S1P ratio in cells. Conversely, elevated levels and/or activity of SphK is expected to decrease the ceramide/S1P ratio. Consistent with this, enforced expression of SphK1 reduces the C18-ceramide/S1P ratio in K-562 human erythroleukemia cells and prevents imatinib-induced apoptosis (Baran et al. 2007). In addition, the ceramide/S1P ratio is increased in response to imatinib in imatinib-sensitive LAMA84 cells, while the ratio is unaltered in imatinib-resistant cells. Similarly, daunorubicin-sensitive but not -insensitive leukaemia cells (CML, AML and ALL) exhibit an elevated ceramide/S1P ratio, and sensitivity to daunorubicin is restored by inhibiting SphK1 activity (Sobue et al. 2008). There are other examples (e.g. MP-A08 in multiple myeloma) where SphK inhibitors increase ceramide and reduce S1P levels, with subsequent induction of apoptosis (Wallington-Beddoe et al. 2017). However, controversy has arisen over whether the sphingolipid rheostat is modulated by SphK1 inhibitors and whether these compounds have utility in the treatment of cancer. These questions arise because the nanomolar-potent selective SphK1 inhibitor, PF-543 (K_i 3.6 nM), fails to induce cytotoxicity in head and neck cancer cells (Schnute et al. 2012). However, in this study PF-543 failed to increase ceramide levels (but did reduce S1P levels), unless cells were incubated with Sph (Schnute et al. 2012). An assessment of whether PF-543 induces apoptosis of cancer cells in the presence of Sph was not undertaken. In principle, the lack of effect of PF-543 on ceramide levels might also be due to its

potential modulation of other targets involved in sphingolipid metabolism so as to rebalance the changes in ceramide levels caused by inhibition of SphK1. These issues require further investigation. In addition, certain Sphk1 inhibitors (e.g. SKi, ABC294640) also indirectly inhibit Degs1 and we have proposed that combined inhibition of Degs1 and SphK1 activity might be necessary to growth arrest and/or kill certain types of cancer cells (McNaughton et al. 2016). Thus, SphK1 inhibition alone might not be sufficient, and in order to kill cancer cells it might be necessary to modulate both *de novo* ceramide and sphingolipid rheostat pathways. In addition, the N-terminal SphK1b splice variant (with an additional 86 amino acids, Mr = 51 kDa) can exhibit resistance to SphK1 inhibitors in terms of proteasomal degradation (Loveridge et al. 2010). Proteasomal degradation of SphK1 might be required in order to disrupt the sphingolipid rheostat to increase ceramide levels and to promote growth arrest and/or kill cancer cells.

11. SphK inhibitor-induced proteasomal degradation of SphK1

In addition to reversible inhibition of catalytic activity, SphK inhibitors induce the ubiquitin-proteasomal degradation of SphK1, which occurs through two distinct mechanisms. First, inhibitors, such as PF-543, appear to bind to SphK1 to induce a conformational change that promotes proteasomal degradation of the enzyme (Byun et al. 2013). In this regard, there is a strong correlation between the concentrations of inhibitor required to induce proteasomal degradation in cells and the K_i for inhibition of purified SphK1 (Byun et al. 2013). Second, relatively weak inhibitors of SphK1, such as SKi (K_i 17 μM, Lim et al. 2011b), activate the proteasome to remove SphK1 (along with c-Myc and cyclin D1) in human prostate cancer cells (Loveridge et al., 2010; Watson et al 2013). In this regard, SKi induces the proteasomal degradation of both SphK1a (42 kDa) and SphK1b (51kDa) in androgen-sensitive LNCaP prostate cancer cells, and this is associated with increased C22:0 and C24:0 ceramide and Sph levels, reduced S1P levels and the activation of apoptosis (Loveridge et al., 2010). In contrast, androgen-independent LNCaP-AI cells fail to undergo apoptosis in response to SKi, and this is associated with the proteasomal degradation of SphK1a, but not SphK1b, with no change in ceramide levels

(Loveridge et al., 2010). The resistance of SphK1b might be a consequence of a compensatory enhanced expression of SphK1b and/or post-translational-dependent stabilisation of SphK1b in androgen-independent prostate cancer cells. Thus, treatment of LNCaP-AI cells with siRNA (to reduce SphK1 expression) in combination with SKi (to promote proteasomal degradation) is able remove SphK1b and thereby induce apoptosis of these cells (Loveridge et al., 2010). Activation of the proteasome involves additional inhibition of Degs1 by SKi (McNaughton et al. 2016). Therefore, we have proposed that Degs1 might function to limit protein degradation flux through the proteasome in cancer cells. Indeed, SKi increases the levels of multiple molecular species of dihydroceramide in prostate cancer cells (Loveridge et al. 2010). Interestingly, the SphK2 selective inhibitor, ABC294640 also inhibits Degs1 (Venant et al. 2015; McNaughton et al. 2016) and induces proteasomal degradation of SphK1a in LNCaP-AI cells (McNaughton et al. 2016). Both ABC294640 and SKi induce an increase in p53 and p21 expression levels in these cells, indicative of an enhanced senescent programme. The mechanism of action of these inhibitors is validated by siRNA knockdown of Degs1, which increases p53 expression, while siRNA knockdown of SphK1 and Degs1 increases p21 expression in these cancer cells (McNaughton et al. 2016).

12. Progress towards isoform-selective SphK1 and SphK2 inhibitors

Although ATP-competitive inhibitors have been discovered recently (MP-A08, Figure 3) (Powell et al. 2017), work to develop SphK inhibitors to date has been heavily focused on compounds that are competitive for the lipid substrate binding site. A number SphK1-selective inhibitor chemotypes have been developed with inhibitory potency in the nanomolar range. Notable representatives of these that have proved useful as tool compounds include SKI-5c (Li et al. 2016), SKI-178 (Hengst et al. 2010b), VPC96091 (Lynch et al. 2106), RB-005 (Baek et al. 2013), Genzyme-51 (Xiang et al. 2010), Amgen-23/82 (Gustin et al. 2013), SLP7111228 (Patwardhan et al. 2015) and PF-543 (Schnute et al. 2012) (Figure 3) (reviewed in Pitman et al. 2016). Since 2013, the development of SphK1-selective inhibitors has been assisted by the emergence of co-crystal structures for this isozyme, firstly with the

non-selective inhibitor, SKi (also referred to as SKI-II) (Wang et al. 2013), and a representative (Amgen-23) of a derivative series (Gustin et al. 2013), and more recently with PF-543 (Wang et al. 2014). This latter compound has risen to prominence in biochemical and pharmacological studies by virtue of its low nanomolar potency for SphK1 inhibition (K_i 3.6 nM) coupled with greater than 100-fold selectivity over SphK2 (Schnute et al. 2012). The development of correspondingly potent and selective inhibitors for SphK2 has lagged behind that of SphK1 inhibitors, and, until very recently, the most selective compounds—ABC294640 (French et al. 2010), K145 (Liu et al. 2013), ROMe (Lim et al. 2011a), SG-12/14 (Kim et al. 2005), SLP120701 (Patwardhan et al. 2015)), SLR080811 (Congdon et al. 2015) (Figure 3)—have been limited to activity in the micromolar range and with only modest levels of selectivity in several cases. Examples of nanomolar potent SphK2-selective inhibitors are now just beginning to emerge however, as seen with VT-20dd (Childress et al. 2017) and Pfizer-27c (Schnute et al. 2017) suggesting that efforts to develop SphK2 inhibitors are gaining traction at last. The most advanced SphK2 inhibitor to date in terms of clinical development remains ABC294640 (Yeliva®), although (as noted above) its pharmacological profile is undoubtedly complicated by actions on other proteins, limiting its utility as a tool to fully explore the potential of SphK2 as a target. Thus, the development of clean, potent SphK2-selective inhibitors remains a key objective in the field, with ongoing efforts in our own laboratory and elsewhere to unlock the structural determinants for rational design of such compounds. As yet there are no SphK2 crystal structures available to assist this effort, although there is strong sequence homology between the two isozymes, thereby permitting the development of reasonable structural models based on the available SphK1 crystal structures.

We have recently reviewed the structure of SphK1 and discussed current structure-function hypotheses in detail elsewhere (Adams et al. 2016; Pyne et al. 2017). In brief, the enzyme comprises two domains, a C-terminal domain (CTD) that hosts the lipid substrate binding site and an ATP-binding N-terminal domain (Figure 4A). The CTD folds as a two-layer β-sandwich, with three 'lipid binding loops' (LBL-1 to LBL-3) occupying one face; a fourth loop, the regulatory R-loop, contains the ERK phosphorylation site for SphK1 and packs on the opposite face of the CTD β-sandwich (occluded from

view in Figure 4A). The cognate loop in SphK2 is greatly extended and contains the phosphorylation-dependent NES sequence. The different R-loops provide one of the main features of distinction between SphK1 and SphK2. In contrast, the three lipid binding loops are identical in length and exhibit 43-62% sequence identity across the isozymes (see supplementary Figure S1 in Adams et al. (2016) for sequence alignment). These loops pack so as to encapsulate a curved cavity, termed the 'J-channel', as binding site both for Sph substrate and the majority of inhibitors.

Ligand access to the J-channel is postulated (Adams et al. 2016) to involve hinged opening and closure of LBL-1, which comprises two reverse-paired helices (α7/α8). Residues in this loop together with LBL-2 provide the contact surface for the polar head group of the lipid and its central region. The majority of SphK inhibitors are substrate-mimetic and also comprise a polar, frequently basic, head group that is thought to engage the same region of the protein. Like LBL-1, LBL-3 is flap-like in structure; it contains an additional helix (α9), that packs antiparallel against α8, and this folding of LBL-3 provides much of the enclosure for the lipid tail (yellow surface in Figure 4B). Some 20 residues contribute to the direct ligand contact surface of the J-channel, and, of these, SphK2 differs from SphK1 in only three—Val304, Leu517 and Cys533, corresponding respectively to Ile174, Met272 and Phe288 in SphK1 (Figure 4C). In the SphK1 co-crystal structure with PF-543, Ile174 and Met272 flank the inhibitor's *p*-xylylene subunit; in principle, their replacement by Val and Leu in SphK2 might lead to isozyme distinguishing differences in the J-channel surface in this region. The third direct contact residue difference lies in the toe of the J-channel, which is stoppered by Phe288 in SphK1. Clearly, the replacement of this residue by a smaller one, Cys533 in SphK2, might generate a longer J-channel, a notion that has influenced the direction of a number of recent chemistry programmes that have usefully examined probing inhibitor tail subunits directed to the J-channel toe (Schnute et al. 2017; Childress et al. 2017; Xi et al. 2016; Congdon et al. 2016; Congdon et al. 2015).

The structural basis for discrimination of the Sphk isozymes by ligands remains under-addressed to date however, and, we suggest, is likely to be more complex and subtle than simply a rigidly extended J-channel as a consequence of a single residue interchange in the toe. In particular, we hypothesise that differences in neighbouring non-contact residues are likely to exert a significant impact in modulating the J-channel surface in both the toe and heel regions. For that reason, work is currently underway in our laboratory with ligand probes designed to explore the structural differences between the isozymes in these regions, and as part of this programme we have explored ligand docking to SphK2 homology models based on the structure of SphK1. These models do indeed suggest that toe expansion in SphK2 is likely to be a significant determinant of distinguishing ligand-binding preferences and that increased steric demand by ligands in the toe may discriminate against SphK1. This is the case with ABC294640 (Figure 5A), where the chlorine atom on the terminal phenyl group is predicted to be packed against Cys533 in SphK2. However, our models also suggest that the toe in SphK2 may not necessarily be rigidly expanded, rather that it may exhibit greater surface plasticity than in the case of SphK1. Thus, the toe surface in SphK2 is generated both by Cys533 and an adjacent side chain, Leu506. This latter residue is conserved in SphK1 (as Leu261) but is packed against two other non-conserved residues—Val304 (SphK1 Ile174) and Ile508 (SphK1 Leu263). Our models suggest that loosened packing of Leu506 may conspire with Phe/Cys exchange in the isozymes so as to allow SphK2 to accommodate more demanding ligand substituents in this region than SphK1, whilst still providing flexibility for good surface contact with tail subunits of similar size to those that fit SphK1 well.

In contrast to the toe region, however, our models suggest that the J-channel heel surface may be more contracted in SphK2 than in SphK1. Thus, the prediction here is that increased steric demand by ligands will discriminate against SphK2, whilst a combination of enhanced demand in the toe and reduced demand in the heel will favour binding to SphK2. This may be a factor in the remarkable selectivity switching seen between the two structurally related ligands, PF-543 and Pfizer-27c (Figure 3), where a reported 9-fold loss in potency against SphK1 coupled with a 148-fold gain in potency

against SphK2 (IC_{50} for SK2 is 2.4 nM and for SK1, it is 25 nM) results in a swing to net 10-fold SphK2-selective inhibition for Pfizer-27c (Schnute et al. 2017). The co-crystal structure of PF-543 reveals that the inhibitor's sulfonyl group is tightly fitted to the heel region in SphK1 (Figure 4B), a binding locus that we propose will experience significant steric compression in SphK2. Our models (Figure 5B) suggest that replacement of the phenylsulfonylmethyl tail of PF-543 by the terminal 5-methyl-1,2,4-oxadiazoly-3-yl group will alleviate compression in the SphK2 heel, whilst the methyl substituent, which is likely to be a little tight against toe residue Phe288 in SphK1, will be more comfortably accommodated by the cysteine (Cys533) in SphK2.

Another notable example of isozyme selectivity switching has been reported recently by Childress *et al*, where tail replacement in the potent SphK1-selective inhibitor, SLP7111228 (Ki 48 nM, >200-fold selectivity over SphK2) (Patwardhan et al. 2015), strongly enhanced activity against SphK2 but weakened activity against SphK1 to give pronounced overall SphK2-selective inhibition in VT-20dd (IC_{50} 90 nM, 100-fold selectivity over SphK1) (Figure 3) (Childress et al. 2017). The remarkable feature with VT-20dd is the compound's very long biphenyl tail subunit, which our models suggest cannot fit along the J-channel toe expansion trajectory postulated for accommodation of ABC29440, notwithstanding the proposed toe plasticity in that region. The very strong implication of this is that there is a second direction of expansion in the J-channel of SphK2 that is not readily accessible in SphK1. Our models suggest that this may involve rotation of phenylalanine Phe557 on LBL-3 to allow tunneling of the inhibitor's biphenyl group between LBL-2 and LBL-3 (Figure 5C). Toe plasticity in SphK2 is predicted to assist such tunneling, with the Cys588 side chain being displaced into the expansion region postulated to host the ABC29440 chlorine atom. SphK1 differs from SphK2 in a number of LBL-3 residues proximal to the proposed tunneling interface, and these differences, combined with the presence of the large conformationally restricted Phe288 toe residue, may require a higher degree of structural perturbation in SphK1 for adoption of a similar binding mode. The

associated energy penalty for such structural perturbation may account for the strong SphK2 selectivity with VT-20dd.

13. Conclusion

In this review, we have not attempted to comprehensively detail the field of SphK inhibitor development; others have recently reviewed the area (Lynch et al. 2016, Pitman et al. 2016). Rather, the intention has been to focus on a possible structural rationale for achieving isozyme-selective inhibition by exploitation of hypothesized structural differences between SphK1 and SphK2 in the toe and heel regions of the lipid substrate binding J-channel. Potent and selective SphK1 inhibitors are now relatively well established, at least at the level of tool compounds. However, the corresponding availability of SphK2-selective inhibitors has been problematic, with examples of nanomolar potent inhibitors with good selectivity over SphK1 only just beginning to be reported. Whilst the development of SphK1 inhibitors has been structurally enabled by protein crystallography in recent years, no SphK2 crystal structures have emerged to date. Nevertheless, homology models of SphK2 suggest plausible approaches for achieving SphK2-over-SphK1 selectivity with compounds that exhibit increased steric demand in the J-channel toe and reduced demand in the heel. The outlook for developing nanomolar potent SphK2 inhibitors with at least two orders of magnitude selectivity over SphK1 is encouraging, therefore, and may provide the tools for a more thorough exploration of the pharmacological potential of SphK2 as a target to complement ongoing investigations with SphK1 and the biochemical interplay between these two isozymes.

Figure legends

Figure 1: Schematic of the sphingolipid metabolic pathway.

Figure 2: Schematic illustrating various known targets of S1P as possible points of therapeutic intervention by blockade of S1P signalling through receptors or intracellular targets such as HDAC-1/2, PPARγ, TERT and prohibitin.

Figure 3: Structure of sphingosine kinase inhibitors. For references, see text. [a]SphK1 co-crystal structure available.

Figure 4: Structure of SphK1 and the lipid binding J-channel. **(A)** The co-crystal structure of SphK1 with bound PF-543 (PDB: 4V24) is shown, with the nucleotide-binding site marked by superimposition of ADP (pink surface) from the SphK1 co-crystal structure (3VZD) with Mg-ADP. Three lipid binding loops (LBL-1 to LBL-3, colour coded as indicated in the key) fold across a β-sandwich core to generate the Sph-binding J-channel (coloured mesh). **(B)** Detail of the J-channel surface (mesh) extracted from (A) and highlighting three isozyme differences in surface-contributory residues (SphK1: Ile174, Met272, Phe288); a fourth residue (Asp178) is conserved in SphK2 and plays a key role in anchoring the polar head groups of many SphK inhibitors. **(C)** As (B), but with surface removal to highlight the organization of residues relative to PF-543 (green stick); key regions of likely J-channel surface expansion and compression in SphK2 are marked.

Figure 5: SphK2 binding models suggest probable determinants of isozyme selectivity for inhibitors. **(A)** A model of ABC294640 (yellow stick) is shown docked into the J-channel of a SphK2 homology model (green mesh / green stick) with the SphK1 J-channel (pink mesh / pink stick) superimposed from its co-crystal structure (3VZD) with inhibitor, SKi. Residues (1)–(8) are identified in the key (bottom right). Plasticity in the J-channel toe for SphK2 is postulated to be a key feature of distinction from SphK1, where residue differences in (2) and (4) may allow greater mobility in conserved Leu (3). This, coupled with the Phe-to-Cys exchange at (1) is proposed to allow accommodation of the Cl atom of SphK2-selective inhibitor ABC294640; in SphK1 the Cl

atom would be compressed against residues (1) and (3). **(B)** As (A), but showing a model of Pfizer-27c (yellow stick) docked to the SphK2 homology model (green mesh / stick). SphK2 is postulated to have a contracted heel surface proximal to Leu (6) as a second key feature of distinction from SphK1. The sulfonyl oxygens of structurally related SphK1-selective inhibitor, PF-543, known to occupy the expanded heel of SphK1, may compromise binding to SphK2, thereby conferring SphK1 selectivity. The modified tail of Pfizer-27c may circumvent the heel compression, consistent with a substantial gain in binding affinity and inhibitory potency against SphK2. Compression of the terminal methyl group of Pfizer-27c against Phe (1) in SphK1 is additionally predicted to contribute to the isozyme selectivity switch between PF-543 and Pfizer-27c. **(C)** As (A), but showing a model of VT-20dd (yellow stick) docked to the SphK2 homology model (green mesh / stick). Conformational mobility in SphK2 Cys (1) coupled with rotation of Phe (8) may allow 'tunneling' and accommodation of the long biphenyl tail of VT-20dd as an additional aspect of SphK2 toe plasticity. Limited conformational freedom for Phe (1) in SphK1 is likely to preclude such tunneling, requiring more substantial structural perturbation for binding to SphK1.

References

Adams DR, Pyne S, Pyne NJ (2016) Sphingosine Kinases: Emerging Structure-Function Insights. Trends Biochem Sci 41(5):395-409

Aguilar A, Saba JD (2012) Truth and consequences of sphingosine-1-phosphate lyase. Adv Biol Regul 52:17-30

Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T, Milstien S, Spiegel S (2010) Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. Nature 465:1084-8

Anelli V, Gault CR, Snider AJ, Obeid LM (2010) Role of sphingosine kinase-1 in paracrine/transcellular angiogenesis and lymphangiogenesis in vitro. FASEB J 24:2727-2738

Baek DJ, MacRitchie N, Pyne NJ, Pyne S, Bittman R (2013) Synthesis of selective inhibitors of sphingosine kinase 1. Chem Commun (Camb) 49(21):2136-2138

Bajwa A, Huang L, Kurmaeva E, Ye H, Dondeti KR, Chroscicki P, Foley LS, Balogun ZA, Alexander KJ, Park H, Lynch KR, Rosin DL, Okusa MD (2016) Sphingosine Kinase 2 Deficiency Attenuates Kidney Fibrosis via IFN-γ. J Am Soc Nephrol. 28(4):1145-1161

Baker DA, Barth J, Chang R, Obeid LM, Gilkeson GS (2010) Genetic sphingosine kinase 1 deficiency significantly decreases synovial inflammation and joint erosions in murine TNF-alpha-induced arthritis. J Immunol 185:2570-2579

Baker DA, Eudaly J, Smith CD, Obeid LM, Gilkeson GS (2013) Impact of sphingosine kinase 2 deficiency on the development of TNF-alpha-induced inflammatory arthritis. Rheumatol Int 33(10):2677-2681

Baran Y, Salas A, Senkal CE, Gunduz U, Bielawski J, Obeid LM, Ogretmen B (2007) Alterations of ceramide/sphingosine 1-phosphate rheostat involved in the regulation of resistance to imatinib-induced apoptosis in K562 human chronic myeloid leukemia cells. J Biol Chem 282(15):10922-10934

Barbour M, McNaughton M, Boomkamp SD, MacRitchie N, Jiang HR, Pyne NJ, Pyne S (2017) Effect of sphingosine kinase modulators on interleukin-1β release, sphingosine 1-phosphate receptor 1 expression and experimental autoimmune encephalomyelitis. Br J Pharmacol 174(2):210-222

Barr, RK, Lynn, HE, Moretti, PA, Khew-Goodall, Y, Pitson, SM (2008) Deactivation of sphingosine kinase 1 by protein phosphatase 2A. J Biol Chem 283:4994-35002

Beljanski V, Lewis CS, Smith CD (2011) Antitumor activity of sphingosine kinase 2 inhibitor ABC294640 and sorafenib in hepatocellular carcinoma xenografts. Cancer Biol Ther 11, 524-534

Bigaud M, Guerini D, Billich A, Bassilana F, Brinkmann V (2014) Second generation S1P pathway modulators: research strategies and clinical developments. Biochim Biophys Acta 1841:745-758

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

Blaho VA, Hla T (2014) An update on the biology of sphingosine 1-phosphate receptors. J Lipid Res 55:1596-1608

Byun HS, Pyne S, Macritchie N, Pyne NJ, Bittman R (2013) Novel sphingosine-containing analogues selectively inhibit sphingosine kinase (SK) isozymes, induce SK1 proteasomal degradation and reduce DNA synthesis in human pulmonary arterial smooth muscle cells. Medchemcomm 4(10)

Chen J, Tang H, Sysol JR, Moreno-Vinasco L, Shioura KM, Chen T, Gorshkova I, Wang L, Huang LS, Usatyuk PV, Sammani S, Zhou G, Raj JU, Garcia JG, Berdyshev E, Yuan JX, Natarajan V, Machado RF (2014) The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension. Am J Respir Crit Care Med 190:1032-1043

Childress ES, Kharel Y, Brown AM, Bevan DR, Lynch KR, Santos WL (2017) Transforming Sphingosine Kinase 1 Inhibitors into Dual and Sphingosine Kinase 2 Selective Inhibitors: Design, Synthesis, and in Vivo Activity. J Med Chem. 60(9):3933-3957

Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S, Tigyi G (2002) International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. Pharmacol Rev 54:265-269

Congdon MD, Childress ES, Patwardhan NN, Gumkowski J, Morris EA, Kharel Y, Lynch KR, Santos WL (2015) Structure-activity relationship studies of the lipophilic tail region of sphingosine kinase 2 inhibitors. Bioorg Med Chem Lett 25(21):4956-4960

Congdon MD, Kharel Y, Brown AM, Lewis SN, Bevan DR, Lynch KR, Santos WL (2016) Structure-Activity Relationship Studies and Molecular Modeling of Naphthalene-Based Sphingosine Kinase 2 Inhibitors. ACS Med Chem Lett. 7(3):229-234

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

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

Etemadi N, Chopin M, Anderton H, Tanzer MC, Rickard JA, Abeysekera W, Hall C, Spall SK, Wang B, Xiong Y, Hla T, Pitson SM, Bonder CS, Wong WW, Ernst M, Smyth GK, Vaux DL, Nutt SL, Nachbur U, Silke J (2015) TRAF2 regulates TNF and NF-κB signalling to suppress apoptosis and skin inflammation independently of Sphingosine kinase 1. Elife 4. pii: e10592

Evangelisti C, Evangelisti C, Teti G, Chiarini F, Falconi M, Melchionda F, Pession A, Bertaina A, Locatelli F, McCubrey JA, Beak DJ, Bittman R, Pyne S, Pyne NJ, Martelli AM (2014) Assessment of the effect of sphingosine kinase inhibitors on apoptosis,unfolded protein response and autophagy of T-cell acute lymphoblastic leukemia cells; indications for novel therapeutics. Oncotarget 5:7886-7901

Facchinetti MM, Gandini NA, Fermento ME, Sterin-Speziale NB, Ji Y, Patel V, Gutkind JS, Rivadulla MG, Curino AC (2010) The expression of sphingosine kinase-1 in head and neck carcinoma. Cells Tissues Organs 192(5):314-324

French KJ, Zhuang Y, Maines LW, Gao P, Wang W, Beljanski V, Upson JJ, Green CL, Keller SN, Smith CD (2010) Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. J Pharmacol Exp Ther 333(1):129-139

Fitzpatrick LR, Green C, Frauenhoffer EE, French KJ, Zhuang Y, Maines LW, Upson JJ, Paul E, Donahue H, Mosher TJ, Smith CD (2011) Attenuation of arthritis in rodents by a novel orally-available inhibitor of sphingosine kinase. Inflammopharmacology 19(2):75-87

Gairhe S, Joshi SR, Bastola MM, McLendon JM, Oka M, Fagan KA, McMurtry IF (2016) Sphingosine-1-phosphate is involved in the occlusive arteriopathy of pulmonary arterial hypertension. Pulm Circ 6:369-380

Gault CR, Eblen ST, Neumann CA, Hannun YA, Obeid LM (2012) Oncogenic K-Ras regulates bioactive sphingolipids in a sphingosine kinase 1-dependent manner. J Biol Chem 287:31794-31803

Gonzalez-Cabrera P, Brown S, Studer SM, Rosen H (2014) S1P signaling: New therapies and opportunities. F1000Prime Rep. 6:109

Grinkina NM, Karnabi EE, Damania D, Wadgaonkar S, Muslimov IA, Wadgaonkar R (2012) Sphingosine kinase 1 deficiency exacerbates LPS-induced neuroinflammation. PLoSOne 7:e36475

Gustin DJ, Li Y, Brown ML, Min X, Schmitt MJ, Wanska M, Wang X, Connors R, Johnstone S, Cardozo M, Cheng AC, Jeffries S, Franks B, Li S, Shen S, Wong M, Wesche H, Xu G, Carlson TJ, Plant M, Morgenstern K, Rex K, Schmitt J, Coxon A, Walker N, Kayser F, Wang Z (2013) Structure guided design of a series of sphingosine kinase (SphK) inhibitors. Bioorg Med Chem Lett 23(16):4608-4616

Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S, Spiegel S (2009) Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science 325:1254-1257

Hannun YA, Obeid LM (2008) Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol 9:139-150

Hannun YA, Obeid LM (2011) Many ceramides. J Biol Chem 286:27855-27862

Hengst JA, Guilford JM, Conroy EJ, Wang X, Yun JK (2010a) Enhancement of sphingosine kinase 1 catalytic activity by deletion of 21 amino acids from the COOH-terminus. Arch. Biochem. Biophys. 494:23-31

Hengst JA, Wang X, Sk UH, Sharma AK, Amin S, Yun JK (2010b) Development of a sphingosine kinase 1 specific small-molecule inhibitor. Bioorg Med Chem Lett 20(24):7498-7502

Hirata N, Yamada S, Shoda T, Kurihara M, Sekino Y, Kanda Y (2014) Sphingosine-1-phosphate promotes expansion of cancer stem cells via S1PR3 by a ligand-independent Notch activation. Nat Commun 5:4806

Hisano Y, Kobayashi N, Yamaguchi A, Nishi T (2012) Mouse SPNS2 functions as a sphingosine-1-phosphate transporter in vascular endothelial cells. PLoS One 7:e38941

Hla T, Brinkmann V (2011) Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation. Neurology 76(8 Suppl 3):S3-8

Hoeferlin LA, Wijesinghe DS, Chalfant CE (2013) The role of ceramide-1-phosphate in biological functions. Handb Exp Pharmacol 215:153-166

Imeri F, Schwalm S, Lyck R, Zivkovic A, Stark H, Engelhardt B, Pfeilschifter J, Huwiler A (2016) Sphingosine kinase 2 deficient mice exhibit reduced experimental autoimmune encephalomyelitis: Resistance to FTY720 but not ST-968 treatments. Neuropharmacology 105:341-350

Jarman KE, Moretti PA, Zebol JR, Pitson SM (2010) Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium- and integrin-binding protein 1. J Biol Chem 285:483-492

Jung ID, Lee JS, Kim YJ, Jeong YI, Lee CM, Baumruker T, Billlich A, Banno Y, Lee MG, Ahn SC, Park WS, Han J, Park YM (2007) Sphingosine kinase inhibitor suppresses a Th1 polarization via the inhibition of immunostimulatory activity in murine bone marrow-derived dendritic cells. Int. Immunol 19:411-426

Kharel Y, Raje M, Gao M, Gellett AM, Tomsig JL, Lynch KR, Santos WL (2012) Sphingosine kinase type 2 inhibition elevates circulating sphingosine 1-phosphate. Biochem J 447(1):149-157

Kihara Y, Maceyka M, Spiegel S, Chun J (2014) Lysophospholipid receptor nomenclature review: IUPHAR Review 8. Br J Pharmacol 171:3575-3594

Kim JW, Kim YW, Inagaki Y, Hwang YA, Mitsutake S, Ryu YW, Lee WK, Ha HJ, Park CS, Igarashi Y (2005) Synthesis and evaluation of sphingoid analogs as inhibitors of sphingosine kinases. Bioorg Med Chem 13(10):3475-3485

Kobayashi N, Kobayashi N, Yamaguchi A, Nishi T (2009) Characterization of the ATP dependent sphingosine 1-phosphate transporter in rat erythrocytes, J Biol Chem 284:21192–21200

Laviad EL, Albee L, Pankova-Kholmyansky I, Epstein S, Park H, Merrill AH Jr, Futerman AH (2008) Characterization of ceramide synthase 2: tissue distribution, substrate specificity, and inhibition by sphingosine 1-phosphate. J Biol Chem 283(9):5677-5684

Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D, Takabe K, Kordula T, Milstien S, Spiegel S (2013) Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. Cancer Cell 23:107-120

Li ZH, Tao YF, Xu LX, Zhao H, Li XL, Fang F, Wu Y, Lu J, Li YH, Du WW, Ren JL, Li YP, Xu YY, Feng X, Wang J, He WQ, Pan J (2016) A novel sphingosine kinase 1 inhibitor (SKI-5C) induces cell death of Wilms' tumor cells in vitro and in vivo. Am J Transl Res 8(11):4548-4563

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

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

Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H, Payne SG, Bektas M, Ishii I, Chun J, Milstien S, Spiegel S (2003) Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. J Biol Chem 278(41):40330-40336

Liu K, Guo TL, Hait NC, Allegood J, Parikh HI, Xu W, Kellogg GE, Grant S, Spiegel S, Zhang S (2013) Biological characterization of 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) as a selective sphingosine kinase-2 inhibitor and anticancer agent. PLoS One 8(2):e56471

Loveridge C, Tonelli F, Leclercq T, Lim KG, Long JS, Berdyshev E, Tate RJ, Natarajan V, Pitson SM, Pyne NJ, Pyne S (2010) The sphingosine kinase 1 inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole induces proteasomal degradation of sphingosine kinase 1 in mammalian cells. J Biol Chem 285(50):38841-38852

Lynch KR, Thorpe SB, Santos WL (2016) Sphingosine kinase inhibitors: a review of patent literature (2006-2015). Expert Opin Ther Pat 26(12):1409-1416

MacRitchie N, Volpert G, Al Washih M, Watson DG, Futerman AH, Kennedy S, Pyne S, Pyne NJ (2016) Effect of the sphingosine kinase 1 selective inhibitor, PF-543 on arterial and cardiac remodeling in a hypoxic model of pulmonary arterial hypertension. Cell Signal 28:946-955

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

McNaughton M, Pitman M, Pitson SM, Pyne NJ, 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

Maines LW, Fitzpatrick LR, French KJ, Zhuang Y, Xia Z, Keller SN, Upson JJ, Smith CD (2008) Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. Dig Dis Sci 53(4):997-1012

Maines LW, Fitzpatrick LR, Green CL, Zhuang Y, Smith CD (2010) Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease. Inflammopharmacology 18(2):73-85

Malavaud B, Pchejetski D, Mazerolles C, de Paiva GR, Calvet C, Doumerc N, Pitson S, Rischmann P, Cuvillier O (2010) Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. Eur J Cancer 46(18):3417-3424

Mandala SM (2001) Sphingosine-1-phosphate phosphatases. Prostaglandins Other Lipid Mediat 64(1-4):143-156

Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL (2005) Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol 25:11113-11121

Nagahashi M, Ramachandran S, Kim EY, Allegood JC, Rashid OM, Yamada A, Zhao R, Milstien S, Zhou H, Spiegel S, Takabe K (2012) Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. Cancer Res 72:726-735

Nakahara K, Ohkuni A, Kitamura T, Abe K, Naganuma T, Ohno Y, Zoeller RA, Kihara A (2012) The Sjögren-Larsson syndrome gene encodes a hexadecenal dehydrogenase of the sphingosine 1-phosphate degradation pathway. Mol Cell 46(4):461-471

Neubauer HA, Pham DH, Zebol JR, Moretti PA, Peterson AL, Leclercq TM, Chan H, Powell JA, Pitman MR, Samuel MS, Bonder CS, Creek DJ, Gliddon BL, Pitson SM (2016) An oncogenic role for sphingosine kinase 2. Oncotarget 7(40):64886-64899

Ohotski J, Edwards J, Elsberger B, Watson C, Orange C, Mallon E, Pyne S, Pyne NJ (2013) Identification of novel functional and spatial associations between sphingosine kinase 1, sphingosine 1-phosphate receptors and other signalling proteins that affect prognostic outcome in estrogen receptor-positive breast cancer. Int J Cancer 132(3):605-616

Panneer Selvam S, De Palma RM, Oaks JJ, Oleinik N, Peterson YK, Stahelin RV, Skordalakes E, Ponnusamy S, Garrett-Mayer E, Smith CD, Ogretmen B (2015) Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation. Sci Signal 8:ra58

Parham KA, Zebol JR, Tooley KL, Sun WY, Moldenhauer LM, Cockshell MP, Gliddon BL, Moretti PA, Tigyi G, Pitson SM, Bonder CS (2015) Sphingosine 1-phosphate is a ligand for peroxisome proliferator-activated receptor-γ that regulates neoangiogenesis. FASEB J 29:3638-3653

Patwardhan NN, Morris EA, Kharel Y, Raje MR, Gao M, Tomsig JL, Lynch KR, Santos WL (2015) Structure-activity relationship studies and in vivo activity of guanidine-based sphingosine kinase inhibitors: discovery of SphK1- and SphK2-selective inhibitors. J Med Chem 58(4):1879-1899

Pitman MR, Costabile M, Pitson SM (2016) Recent advances in the development of sphingosine kinase inhibitors. Cell Signal 28(9):1349-1363

Pitson SM, Moretti PA, Zebol JR, Lynn HE, Xia P, Vadas MA, Wattenberg BW (2003) Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J 22:5491-5500

Pitson SM, Xia P, Leclercq TM, Moretti PA, Zebol JR, Lynn HE, Wattenberg BW, Vadas MA. (2005) Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. J Exp Med 201:49-54

Ponnusamy S, Selvam SP, Mehrotra S, Kawamori T, Snider AJ, Obeid LM, Shao Y,Sabbadini R, Ogretmen B (2012) Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signalling to regulate tumour metastasis. EMBO Mol Med 4:761-775

Powell JA, Lewis AC, Zhu W, Toubia J, Pitman MR, Wallington-Beddoe CT, Moretti PA, Iarossi D, Samaraweera SE, Cummings N, Ramshaw HS, Thomas D, Wei AH, Lopez AF, D'Andrea RJ, Lewis ID, Pitson SM (2017) Targeting sphingosine kinase 1 induces MCL1-dependent cell death in acute myeloid leukemia. Blood 129(6):771-782

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

Pyne NJ, Pyne S (2010) Sphingosine 1-phosphate and cancer. Nat Rev Cancer. 10(7):489-503

Pyne NJ, Pyne S (2013) Sphingosine 1-phosphate is a missing link between chronic inflammation and colon cancer. Cancer Cell 23:5-7

Pyne NJ, McNaughton M, Boomkamp S, MacRitchie N, Evangelisti C, Martelli AM, Jiang HR, Ubhi S, Pyne S. (2016b) Role of sphingosine 1-phosphate receptors, sphingosine kinases and sphingosine in cancer and inflammation. Adv Biol Regul 60:151-159

Pyne S, Adams DR, Pyne NJ (2016a) Sphingosine 1-phosphate and sphingosine kinases in health and disease: Recent advances. Prog Lipid Res 62:93-106

Pyne NJ, Adams DR, Pyne S (2017) Sphingosine Kinase 2 in Autoimmune/Inflammatory Disease and the Development of Sphingosine Kinase 2 Inhibitors. Trends Pharmacol Sci 38(7):581-591

Pyne NJ, Pyne S (2017) Sphingosine kinase 2 and multiple myeloma. Oncotarget. 8(27):43596-43597

Saddoughi SA, Ogretmen B (2013) Diverse functions of ceramide in cancer cell death and proliferation. Adv Cancer Res 117:37-58

Salas A, Ponnusamy S, Senkal CE, Meyers-Needham M, Selvam SP, Saddoughi SA, Apohan E, Sentelle RD, Smith C, Gault CR, Obeid LM, El-Shewy HM, Oaks J, Santhanam R, Marcucci G, Baran Y, Mahajan S, Fernandes D, Stuart R, Perrotti D, Ogretmen B (2011) Sphingosine kinase-1 and sphingosine 1-phosphate receptor 2 mediate Bcr-Abl1 stability and drug resistance by modulation of protein phosphatase 2A. Blood 117:5941-5952

Schnute ME, McReynolds MD, Kasten T, Yates M, Jerome G, Rains JW, Hall T, Chrencik J, Kraus M, Cronin CN, Saabye M, Highkin MK, Broadus R, Ogawa S, Cukyne K, Zawadzke LE, Peterkin V, Iyanar K, Scholten JA, Wendling J, Fujiwara H, Nemirovskiy O, Wittwer AJ, Nagiec MM (2012) Modulation of cellular S1P levels with a novel, potent and specific inhibitor of sphingosine kinase-1. Biochem J 444(1):79-88

Schnute ME, McReynolds MD, Carroll J, Chrencik J, Highkin MK, Iyanar K, Jerome G, Rains JW, Saabye M, Scholten JA, Yates M, Nagiec MM (2017) Discovery of a Potent and Selective Sphingosine Kinase 1 Inhibitor through the Molecular Combination of Chemotype-Distinct Screening Hits. J Med Chem 60(6):2562-2572

Schrecengost RS, Keller SN, Schiewer MJ, Knudsen KE, Smith CD (2015) Downregulation of Critical Oncogenes by the Selective SK2 Inhibitor ABC294640 Hinders Prostate Cancer Progression. Mol Cancer Res 13:1591-1601

Shen H, Giordano F, Wu Y, Chan J, Zhu C, Milosevic I, Wu X, Yao K, Chen B, Baumgart T, Sieburth D, De Camilli P (2014) Coupling between endocytosis and sphingosine kinase 1 recruitment. Nat Cell Biol 16:652-662

Shirai K, Kaneshiro T, Wada M, Furuya H, Bielawski J, Hannun YA, Obeid LM, Ogretmen B, Kawamori T (2011) A role of sphingosine kinase 1 in head and neck carcinogenesis. Cancer Prev Res (Phila) 4:454-462

Siow D, Sunkara M, Morris A, Wattenberg B (2015) Regulation of *de novo* sphingolipid biosynthesis by the ORMDL proteins and sphingosine kinase-1. Adv Biol Regul 57:42-54

Sivasubramanian M, Kanagaraj N, Dheen ST, Tay SS (2015) Sphingosine kinase 2 and sphingosine-1-phosphate promotes mitochondrial function in dopaminergic neurons of mouse model of Parkinson's disease and in MPP+ -treated MN9D cells in vitro. Neuroscience 290:636-648

Snider AJ, Kawamori T, Bradshaw SG, Orr KA, Gilkeson GS, Hannun YA, Obeid LM (2009) A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis. FASEB J. 23:143-152

Sobue S, Nemoto S, Murakami M, Ito H, Kimura A, Gao S, Furuhata A, Takagi A, Kojima T, Nakamura M, Ito Y, Suzuki M, Banno Y, Nozawa Y, Murate T (2008) Implications of sphingosine kinase 1 expression level for the cellular sphingolipid rheostat: relevance as a marker for daunorubicin sensitivity of leukemia cells. Int J Hematol 87(3):266-275

Spiegel S, Milstien S (2011) The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol 11:403-415

Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, Maceyka M, Price MM, Chen Q, Simpson DC, Kordula T, Milstien S, Lesnefsky EJ, Spiegel S (2011) Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. FASEB J 25:600-612

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

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

Tan SS, Khin LW, Wong L, Yan B, Ong CW, Datta A, Salto-Tellez M, Lam Y, Yap CT (2014) Sphingosine kinase 1 promotes malignant progression in colon cancer and independently predicts survival of patients with colon cancer by competing risk approach in South asian population. Clin Transl Gastroenterol 5:e51

ter Braak M, Danneberg K, Lichte K, Liphardt K, Ktistakis NT, Pitson SM, Hla T, Jakobs KH, Meyer zu Heringdorf D (2009) Galpha(q)-mediated plasma membrane translocation of sphingosine kinase-1 and cross-activation of S1P receptors. Biochim Biophys Acta 1791:357-370

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

Venant H, Rahmaniyan M, Jones EE, Lu P, Lilly MB, Garrett-Mayer E, Drake RR, Kraveka JM, Smith CD, Voelkel-Johnson C (2015) The Sphingosine Kinase 2 Inhibitor ABC294640 Reduces the

Growth of Prostate Cancer Cells and Results in Accumulation of Dihydroceramides In Vitro and In Vivo. Mol Cancer Ther 14(12):2744-2752

Vu TM, Ishizu AN, Foo JC, Toh XR, Zhang F, Whee DM, Torta F, Cazenave-Gassiot A, Matsumura T, Kim S, Toh SES, Suda T, Silver DL, Wenk MR, Nguyen LN (2017) Mfsd2b is essential for the sphingosine-1-phosphate export in erythrocytes and platelets. Nature 550(7677):524-528

Wallington-Beddoe CT, Bennett MK, Vandyke K, Davies L, Zebol JR, Moretti PAB, Pitman MR, Hewett DR, Zannettino ACW, Pitson SM (2017) Sphingosine kinase 2 inhibition synergises with bortezomib to target myeloma by enhancing endoplasmic reticulum stress. Oncotarget 8(27):43602-43616

Wang Z, Min X, Xiao SH, Johnstone S, Romanow W, Meininger D, Xu H, Liu J, Dai J, An S, Thibault S, Walker N (2013) Molecular basis of sphingosine kinase 1 substrate recognition and catalysis. Structure 21(5):798-809

Wang J, Knapp S, Pyne NJ, Pyne S, Elkins JM (2014) Crystal Structure of Sphingosine Kinase 1 with PF-543. ACS Med Chem Lett 5(12):1329-1333

Watson C, Long JS, Orange C, Tannahill CL, Mallon E, McGlynn LM, Pyne S, Pyne NJ, Edwards J (2010) High expression of sphingosine 1-phosphate receptors, S1P1 and S1P3, sphingosine kinase 1, and extracellular signal-regulated kinase-1/2 is associated with development of tamoxifen resistance in estrogen receptor-positive breast cancer patients. Am J Pathol 177(5):2205-2215

Watson DG, Tonelli F, Alossaimi M, Williamson L, Chan E, Gorshkova I, Berdyshev E, Bittman R, Pyne NJ, Pyne S (2013) The roles of sphingosine kinases 1 and 2 in regulating the Warburg effect in prostate cancer cells. Cell Signal 25(4):1011-1017

Weigert A, Schiffmann S, Sekar D, Ley S, Menrad H, Werno C, Grosch S, Geisslinger G, Brüne B (2009) Sphingosine kinase 2 deficient tumor xenografts show impaired growth and fail to polarize macrophages towards an anti-inflammatory phenotype. Int J Cancer 125:2114-2121

Xi M, Ge J, Wang X, Sun C, Liu T, Fang L, Xiao Q, Yin D (2016) Development of hydroxy-based sphingosine kinase inhibitors and anti-inflammation in dextran sodium sulfate induced colitis in mice. Bioorg Med Chem 24(14):3218-3230

Xia P, Wang L, Moretti PA, Albanese N, Chai F, Pitson SM, D'Andrea RJ, Gamble JR, Vadas MA (2002) Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-alpha signalling. J Biol Chem 277:7996-8003

Xiang Y, Hirth B, Kane JL Jr, Liao J, Noson KD, Yee C, Asmussen G, Fitzgerald M, Klaus C, Booker M (2010) Discovery of novel sphingosine kinase-1 inhibitors. Part 2. Bioorg Med Chem Lett 20(15):4550-4554

Yada Y, Higuchi K, Imokawa G (1995) Purification and biochemical characterisation of membrane-bound epidermal ceramidase from guinea pig skin. J Biol Chem 270(20): 12677-12684

Yamaji T, Hanada K (2015) Sphingolipid metabolism and interorganellar transport: localization of sphingolipid enzymes and lipid transfer proteins. Traffic 16(2):101-212

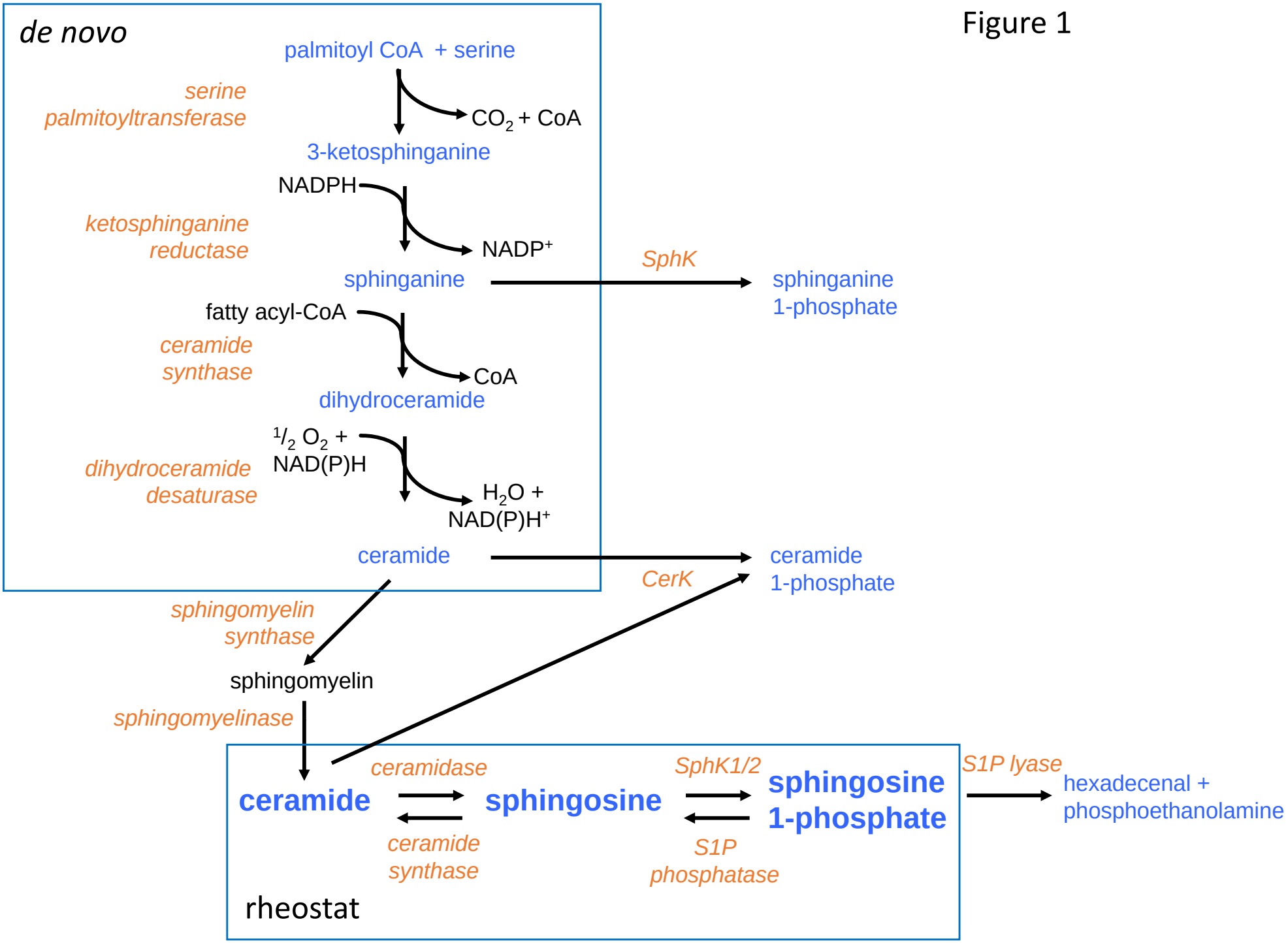
Yang Y, Wang M, Lv B, Ma R, Hu J, Dun Y, Sun S, Li G (2014) Sphingosine kinase-1 protects differentiated N2a cells against beta-amyloid25-35-induced neurotoxicity via the mitochondrial pathway. Neurochem Res 39:932-940

Zhang Y, Berka V, Song A, Sun K, Wang W, Zhang W, Ning C, Li C, Zhang Q, Bogdanov M, Alexander DC, Milburn MV, Ahmed MH, Lin H, Idowu M, Zhang J, Kato GJ, Abdulmalik OY, Zhang W, Dowhan W, Kellems RE, Zhang P, Jin J, Safo M, Tsai AL, Juneja HS, Xia Y (2014) Elevated sphingosine-1-phosphate promotes sickling and sickle cell disease progression. J Clin Invest 124(6):2750-2761

Zhang Y, Yu Q, Lai TB, Yang Y, Li G, Sun SG (2013) Effects of small interfering RNA targeting sphingosine kinase-1 gene on the animal model of Alzheimer's disease. J Huazhong Univ Sci Technolog Med Sci 33:427-432

Zhang F, Xia Y, Yan W, Zhang H, Zhou F, Zhao S, Wang W, Zhu D, Xin C, Lee Y, Zhang L, He Y, Gao E, Tao L (2016) Sphingosine 1-phosphate signaling contributes to cardiac inflammation, dysfunction, and remodeling following myocardial infarction. Am. J. Physiol. Heart Circ. Physiol 310:H250-261

Figure 1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fatty acyl-CoA
ceramide synthase
CoA
dihydroceramide
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dihydroceramide desaturase
H2O + NAD(P)H+
ceramide
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Figure 2

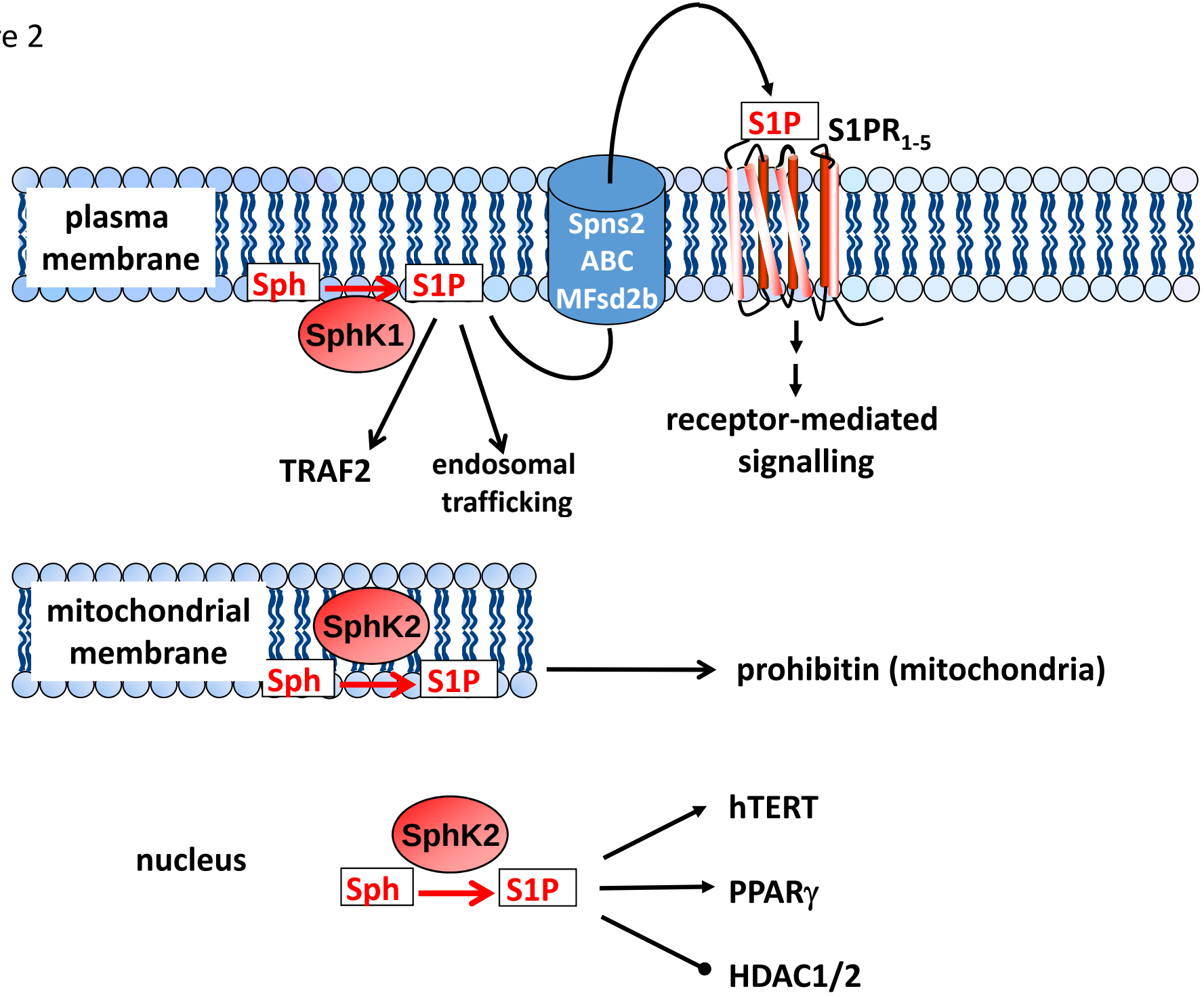
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Figure 3

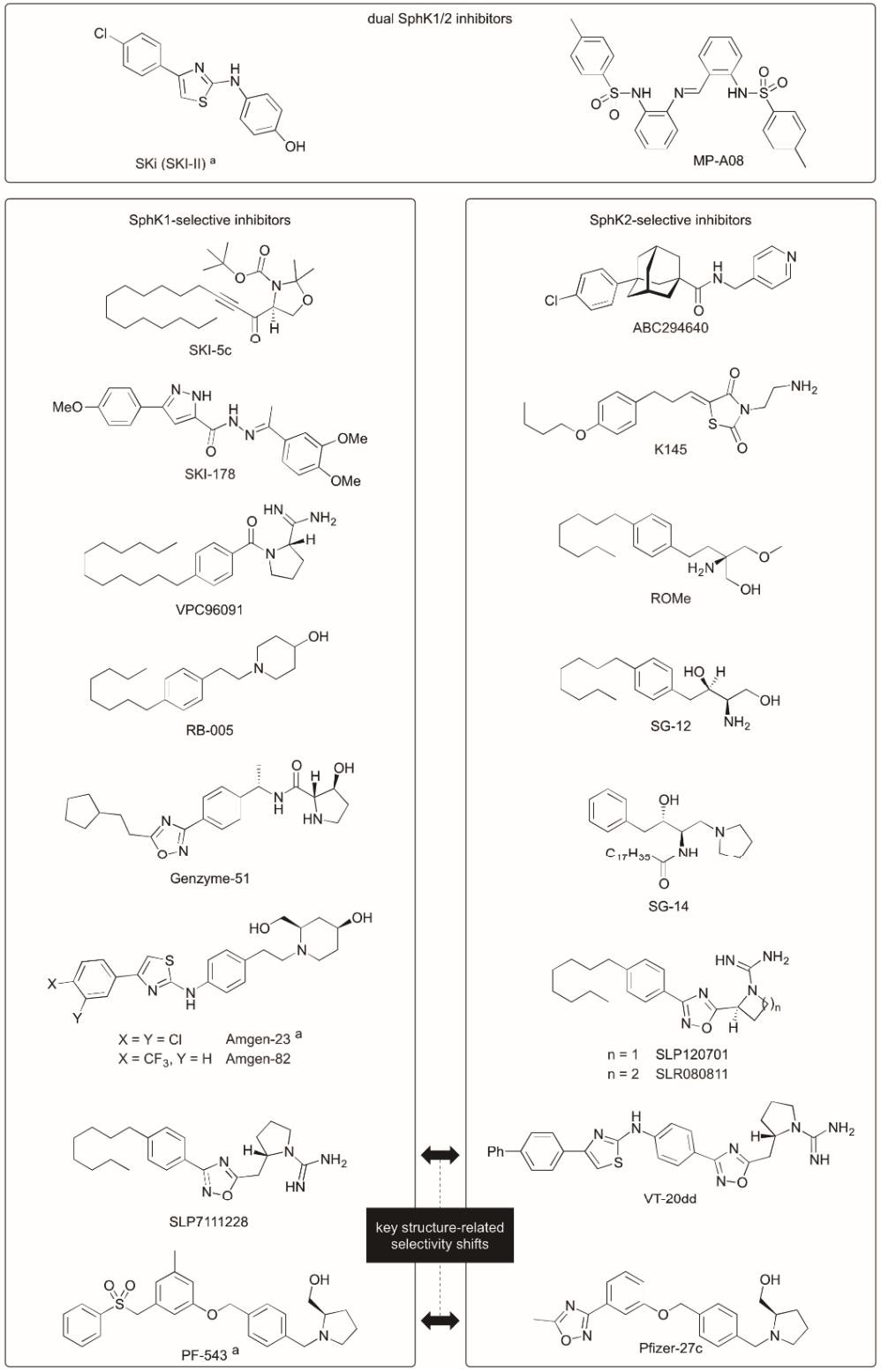
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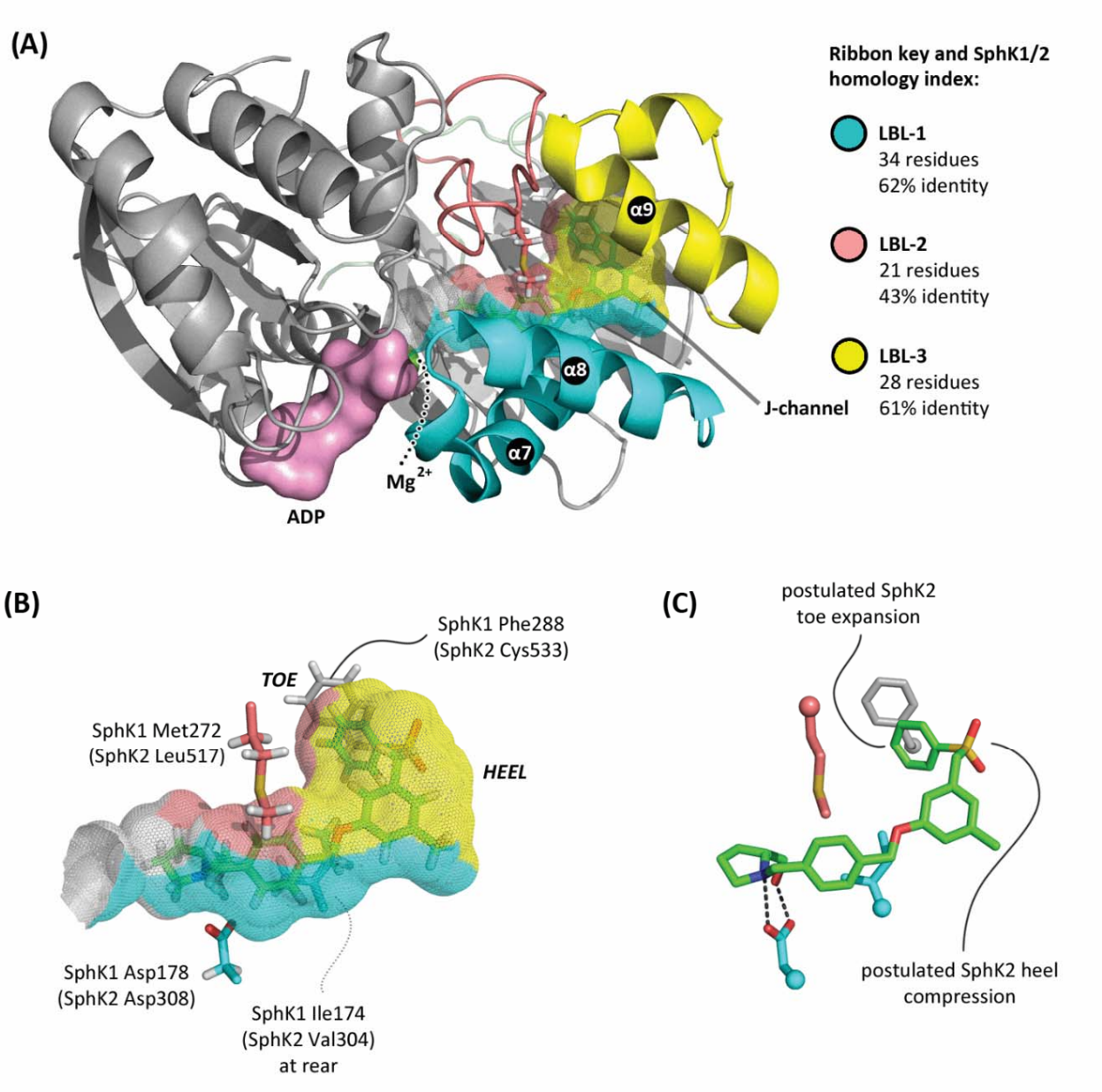
Figure 4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LBL-2
21 residues
43% identity
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Mg 2+
ADP
(B)
SphK1 Phe288
(SphK2 Cys533)
TOE
SphK1 Met272
(SphK2 Leu517)
HEEL
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(SphK2 Asp308)
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postulated SphK2
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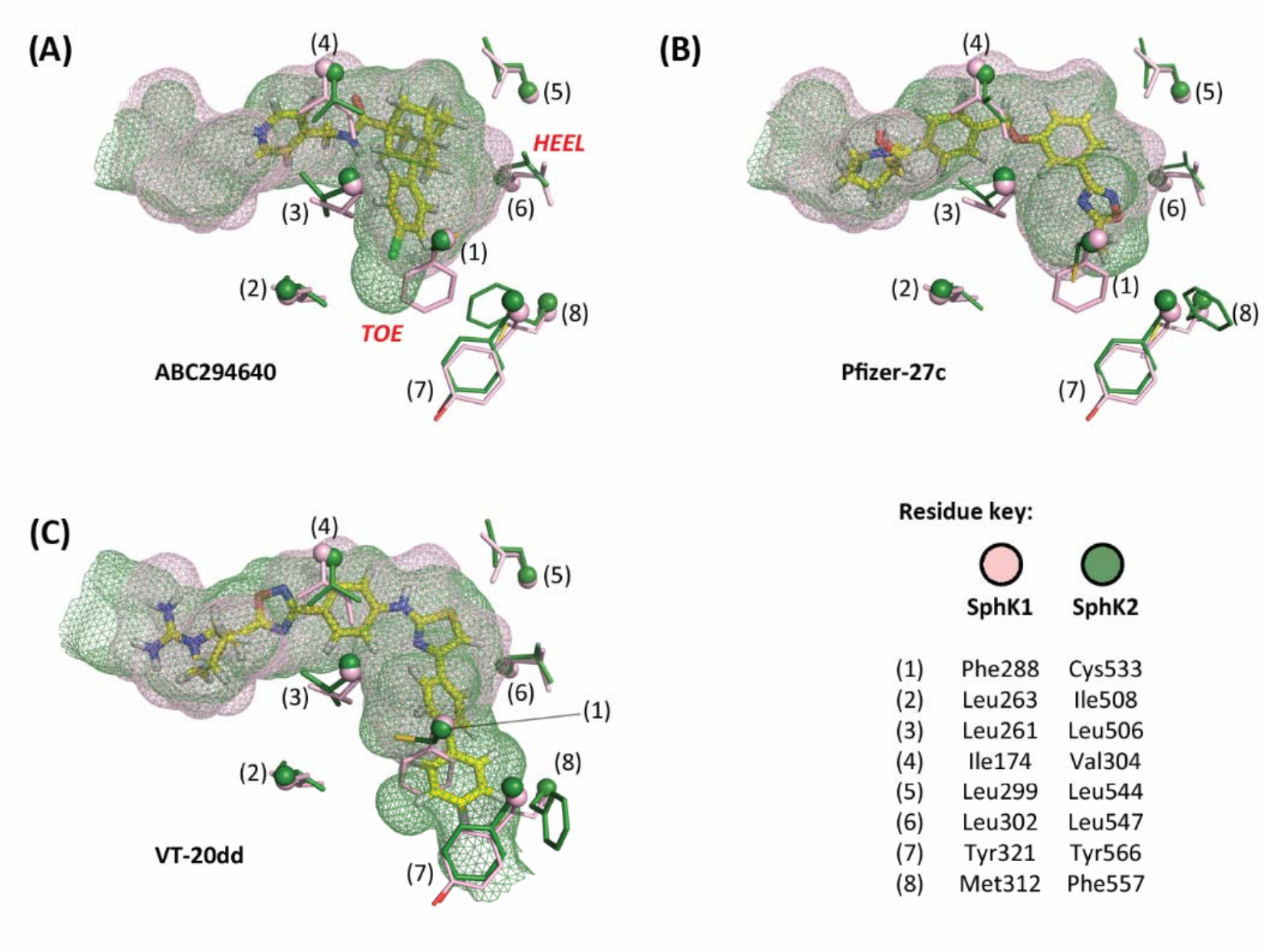
Figure 5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