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Therapeutic Potential of Targeting Sphingosine Kinases and Sphingosine 1-phosphate in Hematological Malignancies

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Running title: SPHK/S1P in malignant hematological disorders

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Abstract

Sphingolipids such as ceramide, sphingosine, and sphingosine 1-phosphate (S1P), are bioactive molecules that have important functions in a variety of cellular processes, which include proliferation, survival, differentiation and cellular responses to stress. Sphingolipids have a major impact on determination of the cell fate by contributing to either cell survival or death. While ceramide and sphingosine are usually considered to induce cell death, S1P promotes survival of cells. Sphingosine kinases (SPHKs) are the enzymes that catalyze the conversion of sphingosine to S1P. There are two isoforms, SPHK1 and SPHK2, which are encoded by different genes. SPHK1 has recently been implicated in contributing to cell transformation, tumor angiogenesis, and metastatic spread, as well as cancer cell multidrug-resistance. More recent findings suggest that SPHK2 also has a role in cancer progression. This review is an overview of our understanding of the role of SPHKs and S1P in hematopoietic malignancies and provides information on the current status of SPHK inhibitors with respect to their therapeutic potential in the treatment of hematological cancers.

Key words: apoptosis, ceramide, drug-resistance, leukemia, lymphoma, multiple myeloma, sphingosine, sphingosine 1-phosphate

INTRODUCTION

The most spectacular success so far in the field of targeted therapy of hematological malignancies has been the introduction of the BCR/ABL1 tyrosine kinase ATP-competitive inhibitor, imatinib, which produces a major cytogenetic response in the vast majority of newly-diagnosed chronic phase chronic myelogenous leukemia (CML) patients. However, imatinib resistance/intolerance have led to the development of additional tyrosine kinase inhibitors, which have demonstrated effectiveness as salvage therapies or alternative first-line treatments for CML [1].

CML is a unique disorder in that BCR/ABL1 is sufficient for disease initiation and progression. In contrast, most hematological malignant disorders are more complex and display multiple genetic and/or epigenetic aberrations which affect many signaling pathways, including those responsible for cell proliferation, survival, differentiation, metabolism and drug-resistance. It is highly unlikely that single targeted agent therapy will be sufficient for successful treatment of these more complex diseases. Therefore, the use of rational combinations of appropriately targeted drugs might provide viable treatment options and these could also be combined with traditional chemotherapy.

In 1996, the "sphingolipid rheostat" model was proposed, based on evidence showing that ceramide, sphingosine and sphingosine 1-phosphate (S1P) differentially regulate cellular signaling pathways involved in proliferation and survival. The suggestion was that growth factors, cellular stress and inflammatory mediators might alter the balance between ceramide and S1P in order to control cell fate [2, 3]. This was supported by the finding that ceramide induces cell growth arrest and apoptosis, whereas S1P induces cell growth. Over the following years, many efforts were made to elucidate the molecular signaling pathways by which ceramide and S1P cause their effects. These studies have also revealed important roles for ceramide and S1P in the pathology of several human disorders, including cancer [4]. Therefore, from a therapeutic perspective, these findings have provided the rationale for manipulating the ceramide/S1P balance with small molecule inhibitors, in order to, for example, induce apoptosis of cancer cells. One particular target regulating the sphingolipid rheostat is the enzyme, sphingosine kinase (SPHK), which catalyzes the formation of

S1P. There are two isoforms, SPHK1 and SPHK2 that are encoded by different genes and which are involved in hematological malignancies [5]. In this review, we focus on the emerging evidence that SPHKs may indeed represent a target for innovative treatment of patients suffering from hematological malignant disorders.

Sphingosine 1-phosphate

Ceramide, sphingosine and S1P are bioactive sphingolipids involved in a wide range of cellular processes, including cell proliferation, apoptosis, autophagy, motility, angiogenesis and inflammation. Ceramide can be deacylated by ceramidases to form sphingosine, which in turn is phosphorylated by SPHKs to produce S1P. S1P can then be dephosphorylated by S1P phosphatases or lipid phosphate phosphatases or irreversibly cleaved by S1P lyase (**Figure 1**). S1P is generally produced within the cell and binds to either intracellular proteins (see below) or, upon export, functions as a ligand for five heterotrimeric G protein-coupled receptors, referred to as $S1P_1$ to $S1P_5$. S1P binding to these receptors regulates angiogenesis, lymphocyte trafficking through blood and lymphoid organs, inflammation and cell transformation. This involves activation of signaling pathways that include Ras/MEK/extracellular signal-regulated kinase-1/2 (ERK-1/2), phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), Rac, Rho and phospholipase C (PLC) [6]. S1P is exported from cells through both ATP-dependent and -independent mechanisms. ATP-dependent export mechanisms, such as in erythrocytes, mast cells, and platelets, involve members of the ATP-binding cassette (ABC) super family of transporters, including ABCC1, ABCA1 and ABCG2. The spinster homolog 2 (SPNS2) is a transporter which plays an important role in exporting S1P from endothelial cells and B- and T-lymphocytes via an ATP-independent mechanism [7].

The regulation of intracellular targets by S1P binding also affects inflammation, immediate early gene expression and replicative immortality. For example, tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) is an essential component in the TNF-α/nuclear factor-κB (NF-κB)

signaling pathway. It has been reported that S1P confers E3 ligase activity on TRAF2. TRAF2 catalyzes the Lys63-polyubiquitination of the receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which serves as a scaffold platform for activation of the NF-κB pathway and regulates cell survival and inflammatory and immune responses. However, the role of SPHK1 in TRAF2-NFκB signaling is controversial. For instance, studies have shown that TNFα-mediated activation of NF-κB and cytokine production is unaffected in macrophages deficient in both SPHK1 and SPHK2. S1P has also been shown to bind to and inhibit the activity of histone deacetylases (HDACs) 1 and 2 leading to increases in histone acetylation, thereby inducing expression of c-Fos and the cell cycle negative regulator, p21. Furthermore, S1P binds to human telomerase reverse transcriptase (hTERT) to increase its stability, enhance telomere integrity and prevent senescence. The binding of S1P to hTERT prevents its interaction with makorin ring finger protein 1 (MKRN1), an E3 ubiquitin ligase that polyubiquitinates hTERT and promotes its proteasomal degradation (see [6] for details).

A role for S1P in cancer was initially suggested by the finding that the concentration of S1P in the plasma of cancer patients is elevated, suggesting that S1P might promote tumor growth via S1P receptors [8, 9]. Indeed, S1P binding to S1P receptors promotes carcinogenesis through crosstalk with different receptor tyrosine kinases that involves transactivation [10, 11], integrative S1P-receptor tyrosine kinase complex formation [12] and regulatory loop amplification [13]. Clinical relevance is evident from studies showing that high expression of SPHK1 and $S1P_1$ and $S1P_3$ receptors in estrogen positive breast tumors are associated with poor prognosis [14].

Sphingosine kinases

SPHK1 and SPHK2 belong to a family of proteins highly conserved throughout eukaryotes, ranging from yeasts to humans. SPHK1 and SPHK2 genes are located on human chromosome 17 (17q25.2) and 19 (19q13.2), respectively. Although the human isoforms vary considerably in size (384 and

618 amino acids for SPHK1 and SPHK2, respectively), they share 80% similarity and 45% overall sequence identity. SPHK2 contains additional regions at its N-terminus that are involved in regulating membrane localization and a proline-rich insert in the middle of its amino-acid primary sequence (**Figure 2**). SPHKs display differential expression during development, as well as different subcellular localization. Indeed, SPHK2 possesses a functional nuclear localization signal (NLS) and can shuttle in and out of the nucleus. In addition to being localized in the cytosol and the plasma membrane, SPHK2 can associate with mitochondria and under stress conditions with the endoplasmic reticulum (ER). In contrast, SPHK1 is distributed in the cytosol and the plasma membrane. These observations indicate that SPHKs have distinct biological roles. However, mice with genetic deletion of either *Sphk1* or *Sphk2* developed normally, suggesting there is considerable functional redundancy. In contrast, deletion of both genes is embryonic lethal due to severely disturbed neurogenesis and angiogenesis [15, 16].

Sphingosine kinase 1

Three SPHK1 isoforms have been identified, which result from alternative splicing and differ only in their N-terminal regions [15]. SPHK1a is expressed in the central nervous system, the kidney, endothelial cells, megakaryocytes and platelets. SPHK1a appears to be the main contributor to plasma S1P levels. In contrast, SPHK1b has a 14 amino-acid N-terminal extension. SPHK1c (sometimes referred also to as SPHK1b based on antibody identification of 42 *versus* 51 kDa isoforms) has an 86 amino-acid extension at the N-terminus. The N-terminal 86 amino-acid extended SPHK1 isoform (termed here SPHK1b) is very much more stable in cells compared with SPHK1a and appears to be associated with chemo-resistance of cancer cells. For example, the sphingosine kinase inhibitor, SKI-II [SKi, 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole)] induces the proteasomal degradation of SPHK1a and SPHK1b in androgen-sensitive LNCaP prostate cancer cells and this is associated with apoptosis of these cells [17]. SKI-II also induces proteasomal degradation of SPHK1a in androgen-independent LNCaP-AI cells, but fails to reduce

SPHK1b levels [17] and these cells do not undergo apoptosis. It should be noted that most studies focus on SPHK1a and the relative importance of the splice variant forms remains unclear. SPHK1 exhibits intrinsic catalytic activity [18], which can be further up-regulated by a wide range of growth factors, cytokines, hormones and adhesion molecules which include epidermal growth factor (EGF), TNF-α, androgens and platelet endothelial cell adhesion molecule (PECAM) -1. Activation of SPHK1 is via phosphorylation, catalyzed by ERK-1/2. ERK-1/2 catalyzes phosphorylation of SPHK1 at Ser 225 [19], which induces its activation and translocation from the cytoplasm to the plasma membrane and which also involves its binding to calcium- and integrin-binding protein 1 (CIB1) [20]. SPHK1 phosphorylation is transient, being reversed by protein phosphatase 2A (PP2A) [21]. The S1P formed by SPHK1 can be released from cells to activate S1P receptor-mediated signaling, in a process termed "inside-out" signaling [22].

Over-expression of SPHK1 induced neoplastic transformation of NIH3T3 fibroblasts, although it is not yet established whether SPHK1 is an oncogene as there are no reported activating mutations in cancer. Vadas et al. [23] have defined a non-oncogenic addiction of cancer cells to SPHK1. Nevertheless, SPHK1 expression levels are up-regulated in several malignancies, including glioblastoma, non-Hodgkin lymphomas (NHL), prostate cancer, colon adenocarcinoma, non-small-cell lung cancer and chemoresistant acute leukemia [24, 25]. Moreover, high SPHK1 expression levels in tumors is associated with poor patient survival in glioblastoma, gastric cancer, breast cancer and cholongiocarcinoma [4]. It should be noted that in some of these studies SPHK1 mRNA levels were analyzed [24, 25], whereas in others, SPHK1 protein levels were investigated [13, 14, 24, 25].

A systematic review and meta-analysis of literature data on SPHK1 expression in human cancers as compared to healthy tissue has been recently published [26]. This analysis included 4,673 patients from 7 countries and 19 types of cancer. Overall, this study demonstrated that SPHK1 positivity/high expression in tumors was significantly associated with various types of cancers and reduced 5-year and overall survival. The important role played by SPHK1 in cancer cell biology is substantiated by the fact that chemical targeting of SPHK1 reduced tumor growth in xenograft

mouse models of established human tumor cell lines [27-30] and decreased therapeutic resistance in prostate cancer [31-33], pancreatic adenocarcinoma [34] and breast cancer cells [35].

Sphingosine kinase 2

Two SPHK2 isoforms (SPHK2-S/SPHK2a and SPHK2-L/SPHK2b) have been identified, encoded by alternative start codon usage [16]. When compared with SPHK2a, SPHK2b possesses an additional 36 amino acids and is more abundantly expressed in a range of human tissues and cultured cells. Like SPHK1, SPHK2 displays intrinsic catalytic activity that can be further increased by ERK-1/2-catalyzed phosphorylation on Ser 351 and/or Thr 578 (Ser 387 and Thr 614 on SPHK2b)[36]. SPHK2a localizes to either the nucleus or the cytoplasm and accumulates in the nucleus under conditions of stress [16].

In the nucleus, SPHK2 produces S1P which binds to and inhibits HDAC 1/2 activity, resulting in increased histone acetylation and the subsequent expression of the cyclin-dependent kinase inhibitor p21, an inhibitor of cell cycle progression and the transcriptional regulator, c-Fos [37]. SPHK2 contains a NLS and a putative nuclear export signal (NES). Phosphorylation in the NES (either Ser 419 or Ser 421 of SPHK2b) by protein kinase D results in the export of SPHK2 from the nucleus into the cytoplasm [38]. SPHK2 can play a pro-apoptotic role when associated with the endoplasmic reticulum, by generating S1P which is channeled into biosynthesis of pro-apoptotic ceramide [39]. Further evidence to suggest a pro-apoptotic role for SPHK2 is the finding that the BH3-binding domain of SPHK2 sequesters and inhibits the pro-survival Bcl-2 family member, Bcl-xL [40], while S1P formed by SPHK2 affects mitochondrial membrane permeability and cytochrome c release to induce apoptosis [41].

However, several other more recent studies have suggested a pro-survival role for SPHK2 as its knock-down enhanced apoptosis and increased the sensitivity of cancer cells to chemotherapy [42-45]. This is supported by the finding that shRNA knock-down of SPHK2 in MCF7 human breast cancer cells results in delayed growth of cancer cells in immunocompromised mice [46]

208 Furthermore, the SPHK2 inhibitor ABC294640 decreased cell proliferation in a number of solid
209 cancer types *in vitro* and induced autophagic cell death in kidney, prostate and breast tumor cell
210 lines [47-49] and inhibited *in vivo* growth of breast adenocarcinoma, hepatocellular and renal
211 carcinoma [47, 48]. However, ABC294640 has since been found to inhibit and induce the proteasomal
212 degradation of SPHK1 and dihydroceramide saturase [50, 51], which may contribute to its anti-tumor
213 effects *in vitro* and *in vivo*.
214
215 **Sphingosine kinase inhibitors**
216 The advance in our understanding of the role of SPHKs in disease has provided impetus for the
217 development of small molecule inhibitors of these enzymes. Selective inhibitors of SPHK1 with
218 nanomolar potency include PF-543 [52] and Genzyme compound 51 [53]. These inhibitors are effective in
219 a number of animal disease models. For instance, PF-543 decreases sickling of red blood cells *in*
220 *vitro* and *in vivo* [54] and reduces cardiac remodelling following post myocardial infarction where
221 SPHK1/S1P/$S1P_1$ participate in cardiac inflammation [55]. SPHK2-selective inhibitors include
222 ABC294640, K145, SLR080811 and ROMe [(*R*)-FTY720-methyl-ether)], which exhibit micromolar
223 potency. ABC294640 is in phase 1/2 clinical trials for refractory/relapsed diffuse large B cell
224 lymphoma (DLBCL) (NCT02229981) and multiple myeloma (MM) (NCT02757326). The use of
225 SPHK2 inhibitors suggests a conserved role of SPHK2 in regulating common signalling pathways in
226 different cancers. For instance, ROMe inhibits DNA synthesis in breast cancer cells [56] and induces
227 the autophagic death of leukemic T-ALL cell lines [57]. There are also inhibitors that target the ATP-
228 binding site of SPHK. For instance, MP-A08 inhibits SPHK1 and SPHK2 with low micromolar
229 potency [29] and reduces cellular S1P levels, while elevating cellular ceramides, sphingosine, and
230 dihydrosphingolipids. This appears to underlie the mechanisms by which MP-A08 induces apoptosis
231 and inhibits cell proliferation and colony formation *in vitro*.
232 To date, there are no high-potency SPHK2-specific inhibitors. However, with the solved crystal
233 structures of SPHK1 in the absence and presence of SPHK inhibitors (SKI-II [58], PF-543 [59] and

234 Amgen compound 23 [60] and with ADP[58]), it has been possible to define the sphingosine substrate
235 binding site (named the 'J-channel' due to its shape), the nucleotide binding site and detail of the
236 interaction of sphingosine-competitive inhibitors [60] and an ATP-competitive inhibitor [29]. In the future
237 this will help inform on the design of isoform-selective inhibitors by identifying and exploiting key
238 differences between SPHK1 and SPHK2.
239 Dual SPHK1/SPHK2 inhibitors include SKI-II (also called SKi) [61] and Amgen compound 82 [62, 63].
240 SKI-II inhibits human SPHK1 and SPHK2 with micromolar potency [63] and induces the proteasomal
241 degradation of SPHK1 in cancer cells [17]. *In vivo* effects include the reduction of tumor volume [64],
242 reduced bronchial hyper-responsiveness, prevention of cerebral preconditioning and increased
243 atherosclerosis in low-density lipoprotein receptor deficient (LDL-$R^{-/-}$) mice.
244 The therapeutic potential afforded by targeting SPHKs continues to fuel a drive to generate small
245 molecule inhibitors for several disease indications.

246

247 **SPHINGOSINE METABOLISM AND SIGNALING IN MALIGNANT HEMATOPOIETIC**
248 **DISORDERS**
249 S1P displays well-known mitogenic and anti-apoptotic activities. Several factors [e.g. platelet-
250 derived growth factor (PDGF)] that promote proliferation/survival have been shown to activate
251 SPHK1 in hematological malignancies such as T-cell large granular lymphocytic leukemia (T-LGL
252 [65]). We will now review the evidence which links SPHKs and S1P with the pathobiology of
253 malignant blood disorders (summarized in Table 1).
254 It should not be forgotten however, that S1P acts as a major chemoattractant which directs the
255 egress of healthy hematopoietic stem cell from bone marrow [66] as well as their homing and
256 engraftment in the same compartment [67].
257
258

Chronic myelogenous leukemia

Some CML patients are either initially refractory to imatinib treatment or develop resistance and experience disease relapse. Second- and third-generation BCR/ABL1 inhibitors have been developed for treating imatinib-resistant patients and are being successfully used in the clinic [68]. However, even the introduction of these new drugs has not completely solved the problem of tyrosine kinase inhibitor resistance in CML patients as this leukemia can be driven independently of BCR/ABL1 [69].

Several lines of evidence support the possibility that SPHK1 and its regulation of the sphingolipid rheostat have an important role in CML. Baran et al. [70] demonstrated that imatinib increased the generation of C18-ceramide in sensitive, but not resistant K562 cells [70]. This was correlated with higher expression levels of SPHK1 in imatinib-resistant K562 cells. Indeed, the knock-down of SPHK1 expression by siRNA in these resistant cells decreased S1P levels and increased the sensitivity to imatinib, thereby providing evidence that SPHK1 was responsible for the acquisition of resistance to imatinib. This was supported by the finding that the overexpression of SPHK1 in K562 cells increased the total S1P/C18-ceramide level ratio approximately 6-fold and prevented apoptosis in response to imatinib. Interestingly, this is associated with a ~2-fold increase in BCR/ABL1 protein expression. A link between SPHK1 and BCR/ABL1 was evinced by the finding that the siRNA knock-down of SPHK1 resulted in a decrease in BCR/ABL1 protein levels [70]. This is important in terms of linking SPHK1 with clinical prognosis in CML as BCR/ABL1 levels are directly proportional to the extent of imatinib resistance in CML cell lines [71-73] and in patients [74].

Additional studies demonstrated that increased expression of SPHK1 in imatinib-resistant cells was due to over-activation of the PI3K/Akt/mTOR signaling pathway [75]. This was a significant finding as activation of this pathway has been linked with tyrosine kinase inhibitor resistance in CML cells[76-80]. In addition, ERK-1/2 and Janus kinase (JAK) 2 are implicated in regulating the expression of SPHK1 in BCR/ABL1-transformed cells [81].

SPHK1/S1P signaling also enhances BCR/ABL1 protein stability via a mechanism that involves $S1P_2$-dependent inhibition of the proteasomal degradation of BCR/ABL1 in imatinib-resistant K562 and LAMA-4 human CML cells [82]. S1P binding to $S1P_2$ prevented BCR/ABL1 dephosphorylation and degradation via inhibition of PP2A activity. Moreover, molecular or pharmacologic interference of SPHK1/$S1P_2$ signaling restored PP2A-dependent BCR/ABL1 dephosphorylation and enhanced imatinib- or nilotinib-induced growth inhibition in primary $CD34^+$ mononuclear cells (obtained from either chronic phase or blast crisis CML patients), imatinib-resistant K562 or LAMA4 cells and 32Dcl3 murine progenitor cells, expressing the wild-type or mutant (Y253H or T315I) BCR/ABL1. This model was supported by evidence demonstrating that the abrogation of SPHK1/$S1P_2$ signaling enhanced the growth-inhibitory effects of nilotinib in 32D/T315I-BCR-ABL1-derived mouse allografts [82].

These findings support the notion that inhibiting SPHK1/$S1P_2$ signaling might represent a novel approach for targeting either wild-type or mutant BCR/ABL1, thereby overcoming resistance to tyrosine kinase inhibitors in CML cells. In this regard, treatment with the SPHK1 inhibitor, SKI-II, impaired cell cycle progression and induced apoptosis in K562 cells. Moreover, SKI-II acted synergistically with imatinib to inhibit cell growth and survival and affected the clonogenic potential and viability of primary cells from CML patients, including one patient harboring the imatinib-insensitive T315I mutation [83]. The anti-apoptotic activity of SPHK1 in BCR/ABL1-harboring CML cells is dependent on the expression of Bcl-2 family members. Thus, imatinib treatment failed to down-regulate anti-apoptotic Bcl-xL and myeloid cell leukemia-1 (Mcl-1) levels in LAMA84 cells overexpressing SPHK1, as well as increasing the expression of pro-apoptotic Bim in LAMA84/Neo cells [84]. Mcl-1 appears to have a critical role in mediating the anti-apoptotic function of SPHK1. This was evidenced by studies showing that combined treatment of K562 and primary cells from CML patients with SKI-II and the proteosomal inhibitor bortezomib caused apoptosis accompanied by down-regulation of Mcl-1 [85]. A schematic on the relationship between SPHK1/S1P, BCR/ABL1 and imatinib resistance is presented in **Figure 3**.

These findings provide a powerful rationale for targeting SPHK1 in CML, not least because the overwhelming evidence suggests that SPHK1 has a critical and definitive role in regulating the oncogenic signaling gain of BCR/ABL1 through an S1P/$S1P_2$ receptor-dependent stabilization of BCR/ABL1. In addition, SPHK1 also confers resistance of CML cells to imatinib by maintaining Mcl-1 expression. Taken together these findings demonstrate functional association of SPHK1 with important oncogenes that underlie the hallmarks of cancer.

Large granular lymphocytic leukemia

LGL leukemia is a rare and incurable chronic disease, characterized by clonal expansion of either cytotoxic T-cells (T-LGL) or natural killer (NK) cells (NK-LGL) in blood and bone marrow. Somatic activating Signal Transducer and Activator of Transcription (STAT) 3 mutations have been shown to be specific for T-LGL leukemia and with a prevalence of up to 70% [86]. It has been shown that SPHK1 is activated by PDGF in T-LGL and its inhibition by SKI-I and SKI-II can significantly induce apoptosis of leukemic cells [65]. The importance of this finding is exemplified by the fact that SPHK1 is overexpressed in NK-LGL cells. Moreover, the mechanism by which SKI-II or SKI-178 induce apoptosis in NK-LGL cells is associated with increased ceramide and decreased S1P levels, consistent with inhibition of SPHK1 and in line with the predicted outcome of modulating the sphingolipid rheostat. Significantly,the apoptotic effect of SPHK1 inhibitors in NK-LGL cells was linked with decreased oncogenic JAK/STAT signaling [87].

Acute lymphoblastic leukemia

The prognosis for pediatric B-ALL and T-ALL patients has dramatically improved over the last two decades with survival rates of approximately 75-80% at 5-years. In contrast, the outcome of adult patients is much more severe [88]. Therefore, novel targeted therapies for treatment of B- and T-ALL are required, especially for adult cases.

It was recently shown that SPHK2 has a significant role in B-ALL by regulating the expression of c-MYC. This was based on the finding that genetic ablation of SPHK2 impaired leukemogenesis in a mouse model of B-ALL and pharmacologic inhibition with ABC294640 prolonged survival in mouse xenograft models of human disease [89]. Indeed, inhibition of SPHK2 reduced c-MYC expression in these leukemic cells and was associated with decreased acetylated K9 histone H3 levels within the c-MYC gene promoter and reduced c-MYC-regulated gene expression. These findings have provided preclinical proof-of-concept for targeting SPHK2/c-MYC as a broad-based therapeutic approach in B-ALL.

S1P prevents T-ALL Jurkat cell apoptosis induced by anti-Fas, TNF-α, serum deprivation and cell-permeable ceramides. This is associated with reduced caspase-3 activation, a consequence of inhibiting the release of cytochrome c and Smac/DIABLO from mitochondria [90]. Recently, we have shown that the SPHK1/2 inhibitor SKI-II [61] induced apoptosis of T-ALL cells, while the SPHK2-selective inhibitor ROMe [56] induced autophagic death of these cells [57]. SKI-II treatment induced an increase in SPHK1 protein levels in MOLT-4 cells, whereas it activated the ER stress/unfolded protein response (UPR) pathway in Jurkat and CEM-R cells as protective mechanisms in a sub-population of T-ALL cells [57]. Interestingly, we observed a synergistic effect of SKI-II with the classical chemotherapeutic drug, vincristine. In addition, we reported that SKI-II affected signaling pathways implicated in survival, proliferation, and stress response of T-ALL cells. These findings indicate that SPHK1 and/or SPHK2 are potential therapeutic targets for treating T-ALL [57].

The overwhelming evidence suggests that SPHK1 and SPHK2 have a critical role in regulating the expression and function of the oncogene c-MYC, which is the master transcriptional regulator of glycolytic gene products essential for the Warburg effect and to which cancer cells are addicted for production of ATP and biosynthetic intermediates [91]. The functional link between SPHKs and c-MYC provides a strong rationale for targeting these enzymes in both T-ALL and B-ALL.

Acute myelogenous leukemia

AML is a clonal disorder characterized by pronounced clinical and biological heterogeneity. Despite considerable advances in our understanding of pathogenesis, genomic alterations and prognostic factors, AML treatment has changed little in the last 40 years and the prognostic outcome remains poor for the majority of patients [92]. Over the last 10 years, there have been an increasing number of signaling pathways identified for targeting with new drugs in AML.

Studies on S1P and AML have shown that this bioactive lipid mobilizes intracellular Ca^{2+} in U937 cells and activates NF-κB [93] and is capable of inhibiting apoptosis in both U937 and HL-60 cells [90]. Importantly, several studies have identified SPHK1 as a potential drug target for AML treatment. For instance, BML-258 (SKI-I) is a micromolar potency, water-soluble, SPHK1 inhibitor and has been shown to decrease growth and survival of human AML U937 cells. This is associated with an increased ceramide : S1P ratio, cleavage of Bcl-2 and apoptosis [27]. Indeed, the pro-apoptotic effect of BML-258 was reversed by caspase inhibitors and by overexpression of Bcl-2. BML-258 also abrogates survival signaling pathways, including ERK-1/2 and Akt. The importance of these pathways in the apoptotic activity of BML-258 was demonstrated by the finding that overexpression of constitutively active Akt protected against BML-258-induced apoptosis. Importantly, BML-258 potently induced apoptosis in leukemic blasts isolated from patients with AML but was relatively sparing of peripheral blood mononuclear leukocytes from healthy donors. Moreover, BML-258 markedly reduced growth of AML xenograft tumors. These results suggested that SPHK1 inhibitors warrant attention as potential additions to the therapeutic arsenal in AML [27]. Subsequent studies have confirmed this conclusion, using inhibitors such as SKI-178 [94] and SKI-II [95].

SPHK1 activity has also been linked to multidrug-resistant (MDR) phenotype in AML HL-60 cells [96]. In this regard, treatment of chemosensitive HL-60 cells with either doxorubicin and etoposide produced a marked decrease in SPHK1 activity and an acute generation (around 50% increase) of the pro-apoptotic ceramide. However, doxorubicin and etoposide failed to induce apotosis of

chemoresistant HL-60/doxorubicin and HL-60/etoposide cells which overexpress MRP1 (ABCC1) and MDR1 (ABCB1), respectively. This difference in chemosensitivity can be explained by the finding that chemoresistant HL-60/doxorubicin and HL-60/etoposide cells express higher levels of SPHK1 activity and therefore are resistant to changes in ceramide levels upon treatment with these apoptotic agents. The mechanism by which SPHK1 is protective against apoptosis is linked with a reduction in ceramide levels and inhibition of mitochondrial cytochrome c efflux. Indeed, treatment of chemoresistant cells with cell-permeable ceramide led to SPHK1 inhibition and the induction of apoptosis, both of which were prevented by over-expression of SPHK1. More effective SPHK1 inhibitors might overcome the chemoresistance in AML. Indeed, the SPHK inhibitor, F-12509a increased the ceramide:S1P ratio and promoted apoptosis of both chemosensitive and chemoresistant AML cell lines with equal sensitivity [96].

Taken together, the evidence suggests that the regulation of the intrinsic apoptotic pathway in AML by SPHK1 and in particular the inhibition of the proteolytic processing of the oncogene, Bcl-2 is a critical component in promoting AML cell survival and chemoresistance. This therefore serves as another example of the important role that SPHK1 plays in augmenting oncogene function in hematological cancers.

Non-Hodgkin lymphomas

The outcome for patients with NHL has improved substantially over the past four decades. However, there remain NHL subtypes with a very poor prognosis. Interestingly, SPHK1 protein and mRNA levels were higher in 44 NHL patients than in controls (25 individuals with reactive lymphoid hyperplasia) and there was a clear trend toward increasing SPHK1 mRNA levels and clinical grade in this cancer [25].

Mantle cell lymphoma (MCL) is a distinct subset of B-cell non-Hodgkin lymphoma (NHL) characterized by the t(11;14) (q13;q32) chromosomal translocation and which results in overexpression of cyclin D1 and deregulation of the cell cycle [97]. Although intensive

413 polychemotherapy schemes and immunotherapy with anti-CD20 monoclonal antibody (Rituximab)
414 have improved the outcome of patients with MCL [98], no standard of care is currently available for
415 this cancer which remains incurable [99]. Several signal transduction pathways are aberrantly
416 activated in MCL, including NF-κB, PI3K/Akt/mTOR and JAK3/STAT3[100]. In addition, $S1P_1$
417 expression levels are elevated in MCL. In particular, this receptor was strongly expressed on the
418 surface of small lymphocytes forming primary lymphoid follicles and in the mantle zone of
419 secondary lymphoid follicles [101]. Interestingly, FTY720 (fingolimod, a pro-drug and functional
420 antagonist of $S1P_1$ used in the treatment of relapsing and remittent multiple sclerosis [102]) induced
421 the caspase-independent death of primary MCL tumor cells and MCL cell lines *in vitro*. Moreover,
422 FTY720 treatment resulted in the down-regulation of cyclin D1 and this was accompanied by an
423 accumulation of cells in G_0-G_1 and G_2-M phases of the cell cycle with a concomitant decrease in S-
424 phase entry. In addition, cytotoxicity was associated with a decrease in phosphorylated Akt levels.
425 Most importantly, the *in vivo* therapeutic efficacy of FTY720 was demonstrated in mice
426 xenografted with Jeko human MCL cell line [103]. These effects of FTY720 are likely mediated by its
427 inhibition of SPHK1 [104] or activation of PP2A [105].
428 Overexpression of $S1P_2$ was recently reported in several types of NHL, including follicular
429 lymphoma (FL), DLBCL, MCL and marginal-zone lymphoma (MZL) [106]. One of the most
430 aggressive subtypes of NHL is activated B cell–like DLBCL (ABC-DLBCL). This cancer remains a
431 challenge for effective therapy [107]. In this regard, STAT3 is known to be activated in ABC-DLBCL
432 cells and might be of significant clinical importance in terms of disease progression[108]. Intriguingly,
433 $S1P_1$ can activate STAT3 through JAK2 [109] and some ABC-DLBCL patients exhibit elevated $S1P_1$
434 and activated STAT3 levels. The importance of this finding was exemplified by the finding that
435 treatment with $S1P_1$ shRNA or FTY720 down-regulated STAT3 activity and caused tumor growth
436 inhibition in xenografts or syngeneic mouse models of lymphoma [110] (**Figure 4**). Moreover, the
437 overexpression of $S1P_1$ and high levels of phosphorylated STAT3 are associated with poor
438 prognosis in rituximab-treated DLBCL [111]. Very recently, it has been documented that $S1P_1$ is

overexpressed in 54.2% of 24 cases with primary testicular DLBCL and $S1P_1$ levels correlated with STAT3 phosphorylation [112].

Another rare and extremely aggressive variant of DLBCL is primary effusion lymphoma (PEL), a human herpes virus 8 (HHV8)-positive neoplasm that presents as an effusion within pleural or peritoneal cavities with no detectable tumor in individuals with human immunodeficiency virus infection or other immune deficiencies. In most cases, PEL cells also harbor the Epstein-Barr virus (EBV) genome [113]. PEL progresses rapidly despite chemotherapy, with a median survival of around 6 months [114]. It has been reported that ABC294640 induced dose-dependent caspase cleavage and apoptosis in $HHV8^+$ patient-derived PEL cells, thereby implicating a role for SPHK2 in this cancer. Also, ABC294640 down-regulated signaling pathways that are known for being activated in PEL and which are regulated by S1P, including MEK-ERK-1/2, PI3K/Akt/mTOR and NF-κB [115]. The role of SPHK2 was validated by induction of PEL cell apoptosis using SPHK2-specific siRNA. In addition, pharmacological inhibition of SPHK1 in PEL cells was associated with a dose-dependent accumulation of pro-apoptotic ceramide and a reduction of intracellular S1P. Finally, *in vivo* administration of ABC294640 induces tumor regression in an established human PEL xenograft model [115]. Sequential Phase 1 and 2a trials are on-going to identify the maximum tolerated dose and to evaluate safety, tolerability, toxicity, pharmacokinetics and pharmacodynamics of ABC294640 in patients with PEL (NCT02229981).

Taken together the data support a major role for both SPHK1 and SPHK2 and S1P receptor signaling systems in NHL, particularly with respect to the regulation of oncogenic JAK/STAT and Akt pro-survival functions. This might involve signaling loops involving SPHK1 and S1P that are subsequently released to act in an autocrine or paracrine manner to promote $S1P_1$ receptor-dependent JAK/STAT regulation of NHL growth.

Multiple myeloma

New classes of therapeutic agents have displayed remarkable efficacy in MM patients. Nevertheless, novel therapeutic approaches are still urgently needed to further improve patient outcome. The bone marrow microenvironment (e.g. stromal cells and immune cells) plays a central role in MM pathogenesis, by promoting tumor cell growth, survival and chemo-resistance [116]. A possible involvement of SPHKs in MM cell survival and chemo-resistance was first highlighted 10 years ago when it was shown that FTY720 was cytotoxic against both drug-sensitive and drug-resistant MM cell lines. This was also demonstrated in isolated tumor cells from MM patients who did not respond to conventional agents [117]. In this regard, FTY720 has been shown to induce caspase activation and poly(ADP-ribose) polymerase (PARP) cleavage. Importantly, FYT720 retained its cytotoxicity even in the presence of interleukin-6 (IL-6) or insulin-like growth factor-1 (IGF-1). It should be noted that IL-6 and IGF-1 are two of the most important cytokines, released by the bone marrow microenvironment, that support growth and survival of MM cells [118]. Importantly, growth of MM cells adherent to bone marrow stromal cells was significantly inhibited by FTY720 and this was associated with down-regulation of signaling pathways critical for MM pathobiology, including PI3K/Akt/mTOR, MEK/ERK-1/2, STAT3 and NK-κB [117].

Recent findings have highlighted that SPHK1 protein expression is elevated in MM cells and its inhibition resulted in apoptotic death of cancer cells due to the prevention of receptor tyrosine kinase phosphorylation and activation of death-associated protein kinase 1 (DAPK1) [119].

S1P might also play an important role in MM cell adhesion, which is dependent on α4β1 integrin and is crucial for the progression of the disease [120]. In this context, α4β1-dependent MM cell adhesion is up-regulated by the chemokine, CXCL12 [121]. S1P enhances α4β1-mediated MM cell adhesion and transendothelial migration stimulated by CXCL12 [122]. In particular, S1P promotes the generation of high-affinity α4β1 that efficiently binds α4β1 ligand and vascular cell adhesion molecule 1 (VCAM-1). Importantly, this is associated with an S1P-induced increase in talin-β1 integrin association. Furthermore, S1P cooperates with CXCL12 in enhancing α4β1-dependent

adhesion and spreading. The mechanism of this cooperation involves activation of the dedicator of cytokinesis 2 (DOCK2)-Rac1 pathway which is required for stimulation of MM cell adhesion via α4β1. The pathophysiological significance of these findings is evident from *in vivo* studies, which have demonstrated that S1P contributed to optimizing the interactions of MM cells with the bone marrow microvasculature and for their lodging inside the bone marrow [122].

More recently, SPHK2 has been found to be overexpressed in MM cell lines and in primary human bone marrow MM cells [123]. Down-regulation of SPHK2 by shRNA or treatment with ABC294640 inhibited proliferation and induced caspase 3-mediated apoptosis in both MM cell lines and primary cells and this can be achieved even in the presence of bone marrow stromal cells. Furthermore, ABC294640 directed c-MYC and Mcl-1 for proteasome degradation and increased pro-apoptotic Noxa gene transcription and protein expression and suppressed the growth of human MM.1S cells in a mouse xenograft cancer model [123].

Overall, these findings have provided the preclinical framework for clinical trials of SPHK inhibitors, used alone and/or combined with conventional and novel therapies to improve patient outcome in MM.

Conclusions and future perspectives

Over the last five years there have been major advances in understanding the role of S1P and SPHKs in hematological malignancies. Aberrant regulation of the sphingolipid metabolism is involved in the progression of malignancy and cancer cell drug-resistance. Therefore, a promising approach for targeted treatment of hematological malignancies is the development of SPHK inhibitors that increase pro-apoptotic sphingolipids such as ceramides while suppressing the synthesis of the anti-apoptotic S1P.

Suppression of SPHK1 by genetic ablation (siRNA or gene knockout murine models) or chemical inhibition have established the important role of this enzyme in cancer. Although our knowledge of SPHK2 is more limited, the burgeoning evidence also suggests a role in cancer. Therefore, highly

selective and potent SPHK2 inhibitors are eagerly awaited in order to provide a means for a more thorough interrogation of this enzyme in hematological cancers. SPHK1 and SPHK2 may not be the only relevant targets in cancer cells as the pathways regulating these enzymes and down-stream targets are also worthy of therapeutic targeting. The simultaneous inhibition of both SPHK1 and SPHK2 activity also warrants appraisal [5].

Finally, the potential for combining SPHK inhibitors (or other sphingolipid pathway components deregulated in cancer) with currently available therapeutic agents (either targeted or classic chemotherapeutic drugs) holds significant promise for improved disease-treatment outcome. However, the reported combinatory effects are scarce at present. One notable exception concerns CML. In this regard, there are impressive findings concerning the re-sensitization of imatinib-resistant CML cells by inhibiting SPHK1 activity [5]. It is in this disorder that the greatest translational advances will most likely be made. However, it is clear that better and more selective and potent SPHK inhibitors are required for the translation to the clinic. These compounds are already under development and it is hoped they will be tested in clinical trials in the near future.

Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Sphingolipid metabolism. Ceramide can be synthesized *de novo* or generated through the breakdown of sphingomyelin or complex glycosphingolipids (not shown) or by the acetylation of sphingosine. Phosphorylation of sphingosine by SPHK generates S1P. SMase: Sphingomyelinase. SMsynthase: Sphingomyelin synthase.

Figure 2. **Domain organization of SPHK isoforms and splice variants**. Blue represents untranslated exon regions while grey represents translated exon regions. All SPHK isoforms have five highly conserved regions: a catalytic domain (yellow), a lipid binding domain (black) and three ATP binding domains (red), one of which is split across an intron.

Figure 3. Role of SPHK/S1P signaling in CML. Imatinib resistance (red arrows) in CML is associated with elevated expression of BCR/ABL1 and SPHK1 (which may involve increased PI3K/Akt/mTOR, ERK-1/2 and JAK2 signaling). Over-expression of SPHK1 enhances BCR/ABL1 levels via $S1P_2$-mediated inhibition of PP2A, thereby preventing the dephosphorylation of BCR/ABL1 and reducing subsequent proteasomal degradation of BCR/ABL1. Over-expressed SPHK1 also maintains expression of the pro-survival protein, Mcl-1. Pharmacological inhibition of SPHK1/$S1P_2$ or activation of PP2A (black double arrows) counteracts these events and restores imatinib sensitivity.

Figure 4. Role of $S1P_1$ in NHL sub-type, activated ABC-DLBCL.

$S1P_1$ and STAT3 are up-regulated in ABC-DLBCL and associated with poor prognosis. Knockdown of $S1P_1$ using shRNA indirectly reduces STAT3 phosphorylation levels and inhibits tumor growth. The pro-drug FTY720 is phosphorylated by SPHK2 to FTY720-phosphate. This is released, binds to $S1P_1$ and induces proteasomal degradation of this receptor. The reduction of $S1P_1$ levels inhibits tumor growth *in vitro* and *in vivo*.

Table 1. The roles of SPHK and S1P in malignant hematological disorders.

Disorder	Component	Roles
CML	SPHK1	Enhanced expression by BCR/ABL1 and *vice versa*[79] Inhibition results in cytotoxicity and enhances imatinib sensitivity[68]
	S1P	Up-regulates anti-apoptotic Mcl-1[79] Binding to $S1P_2$ inhibits PP2A-dependent BCR/ABL1 dephosphorylation [80]
T-LGL	SPHK1	Activity stimulated by PDGF. Inhibition results in apoptosis [65]
NK-LGL	SPHK1	Enhanced expression. Inhibition results in apoptosis [85]
AML	SPHK1	Inhibition results in cytotoxicity of U937 xenografts in mice [27] Inhibition induces HL-60 cell cytotoxicity and sensitizes chemoresistant HL-60 cells [93]
	S1P	Elicits mitogenic signals through NF-κB activation in U937 cells [90] Inhibits apoptosis in U937 and HL-60 cells [88]
B-ALL	SPHK2	Oncogenic in mouse through c-MYC [87]
T-ALL	SPHK1	Inhibition results in apoptosis [57]
	SPHK2	Inhibition results in autophagic cell death [57]
	S1P	Induces apoptosis[88]
NHL	SPHK1	Enhanced expression [25]
PEL	SPHK2	Inhibition results in apoptosis [112]
	S1P	Regulates ERK 1/2, PI3K/Akt/mTOR, and NF-κB signaling [112]
MM	SPHK1	Inhibition prevents receptor tyrosine kinase phosphorylation and activation of DAPK1 [116]
	SPHK2	Over-expressed in MM cells. Inhibition results in down-regulation of cell proliferation and enhanced apoptosis [120]
	S1P	Plays a role in MM cell adhesion [117]

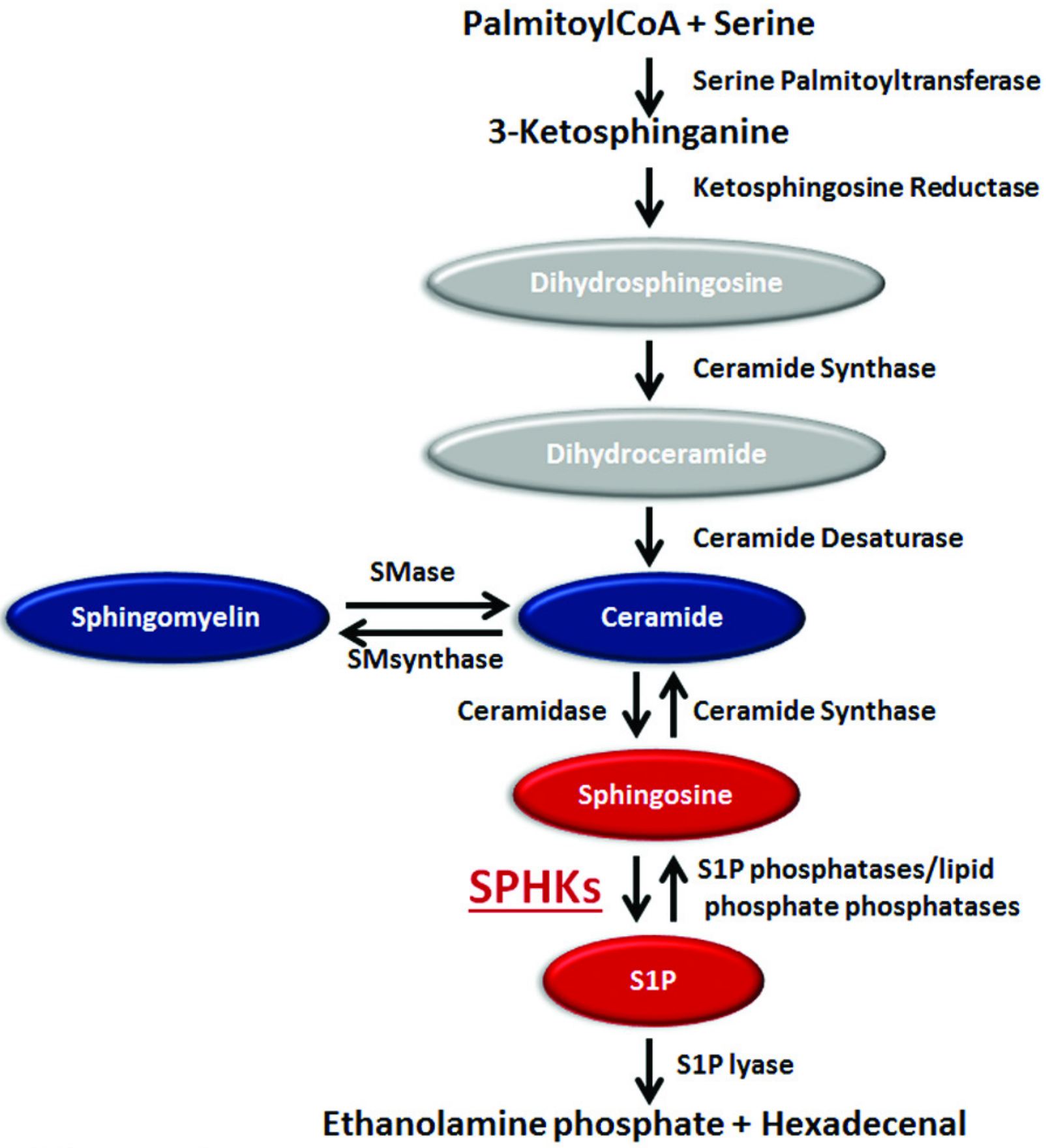
PalmitoylCoA + Serine
Serine Palmitoyltransferase
3-Ketosphinganine
Ketosphingosine Reductase
Dihydrosphingosine
Ceramide Synthase
Dihydroceramide
Ceramide Desaturase
SMase
Sphingomyelin
Ceramide
SMsynthase
Ceramidase
Ceramide Synthase
Sphingosine
SPHKs
S1P phosphatases/lipid phosphate phosphatases
S1P
S1P lyase
Ethanolamine phosphate + Hexadecenal

Figure 1

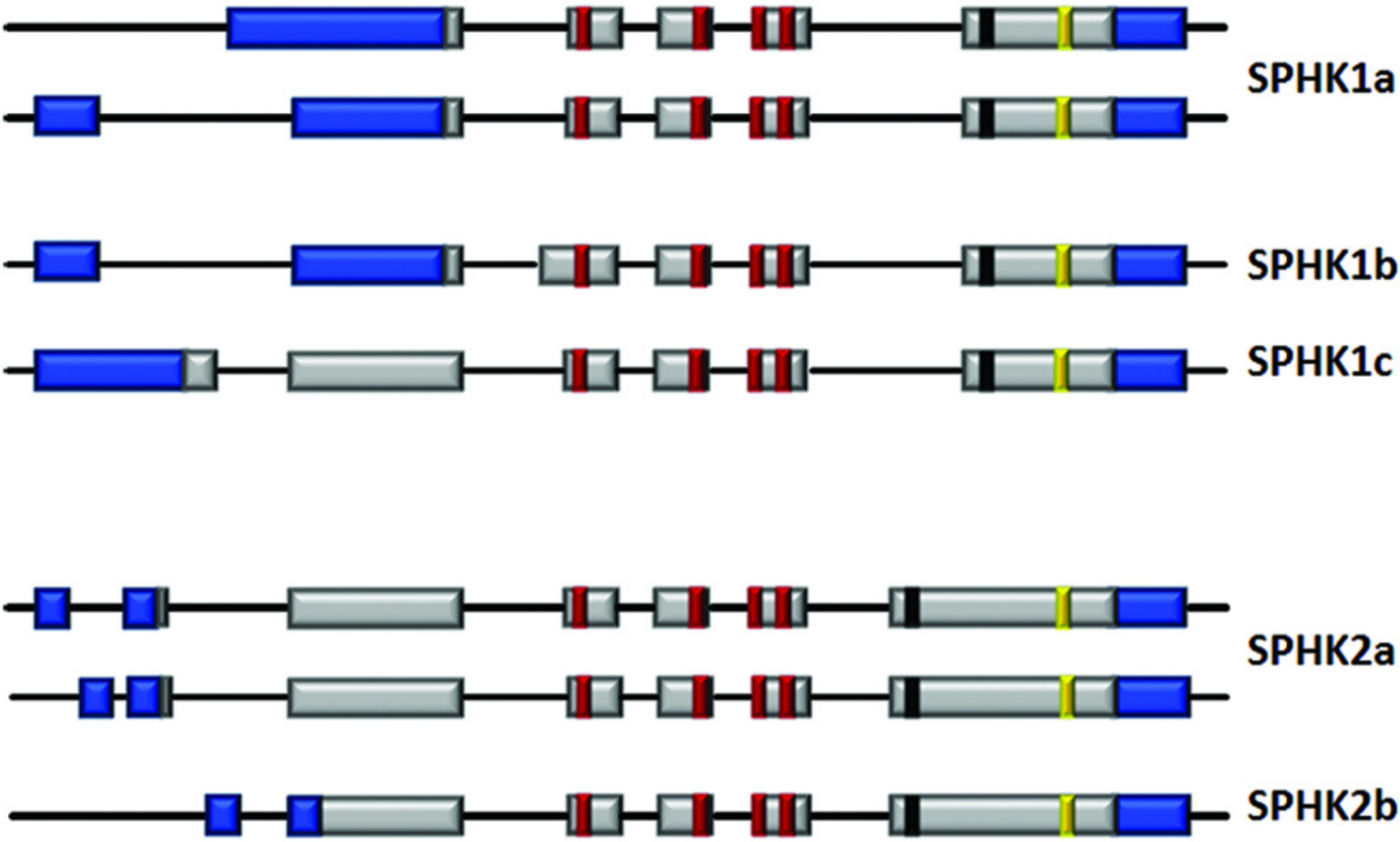

Figure 2

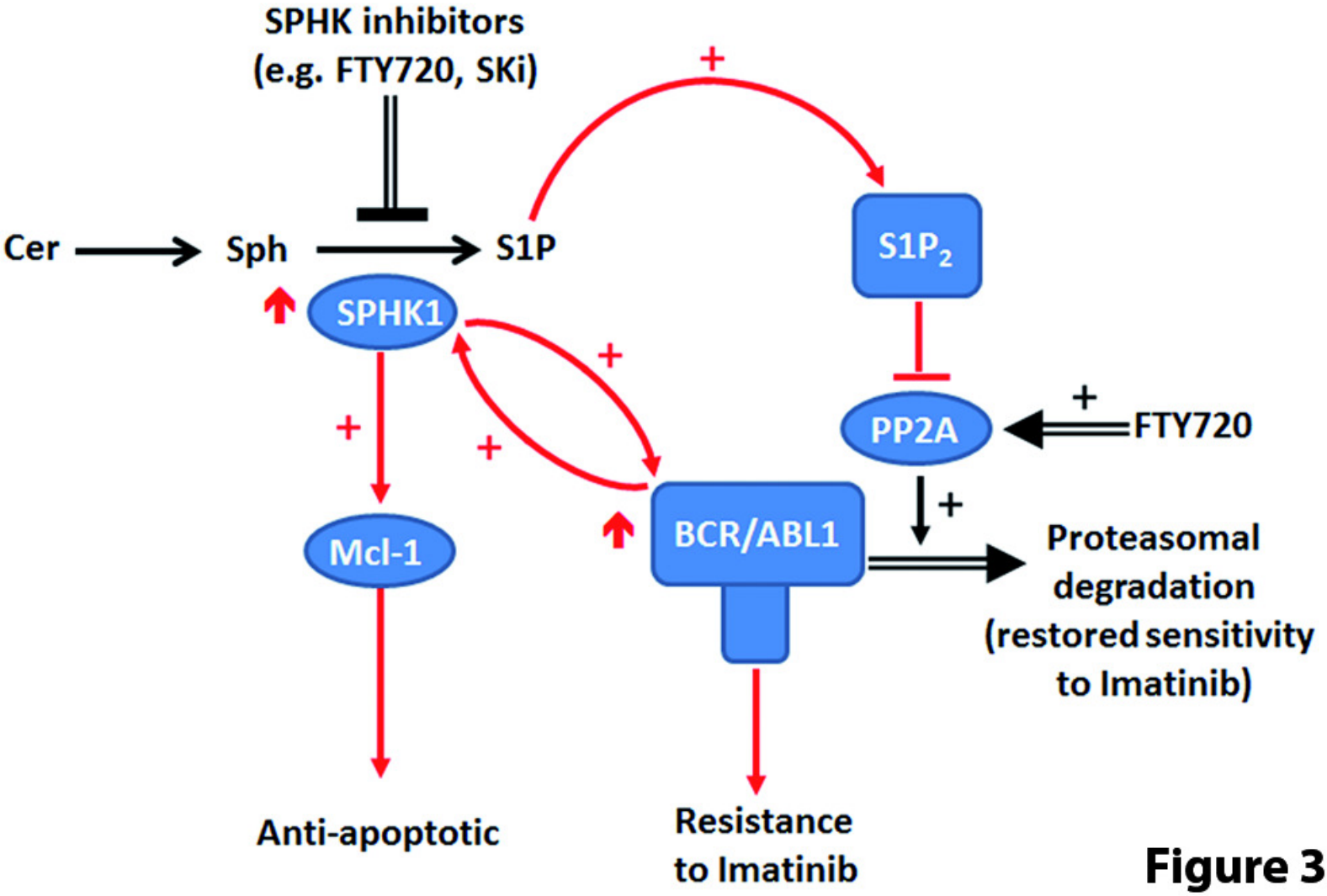
SPHK inhibitors
(e.g. FTY720, SKi)
Cer
Sph
S1P
SPHK1
S1P2
PP2A
FTY720
Mcl-1
BCR/ABL1
Proteasomal
degradation
(restored sensitivity
to Imatinib)
Anti-apoptotic
Resistance
to Imatinib

Figure 3

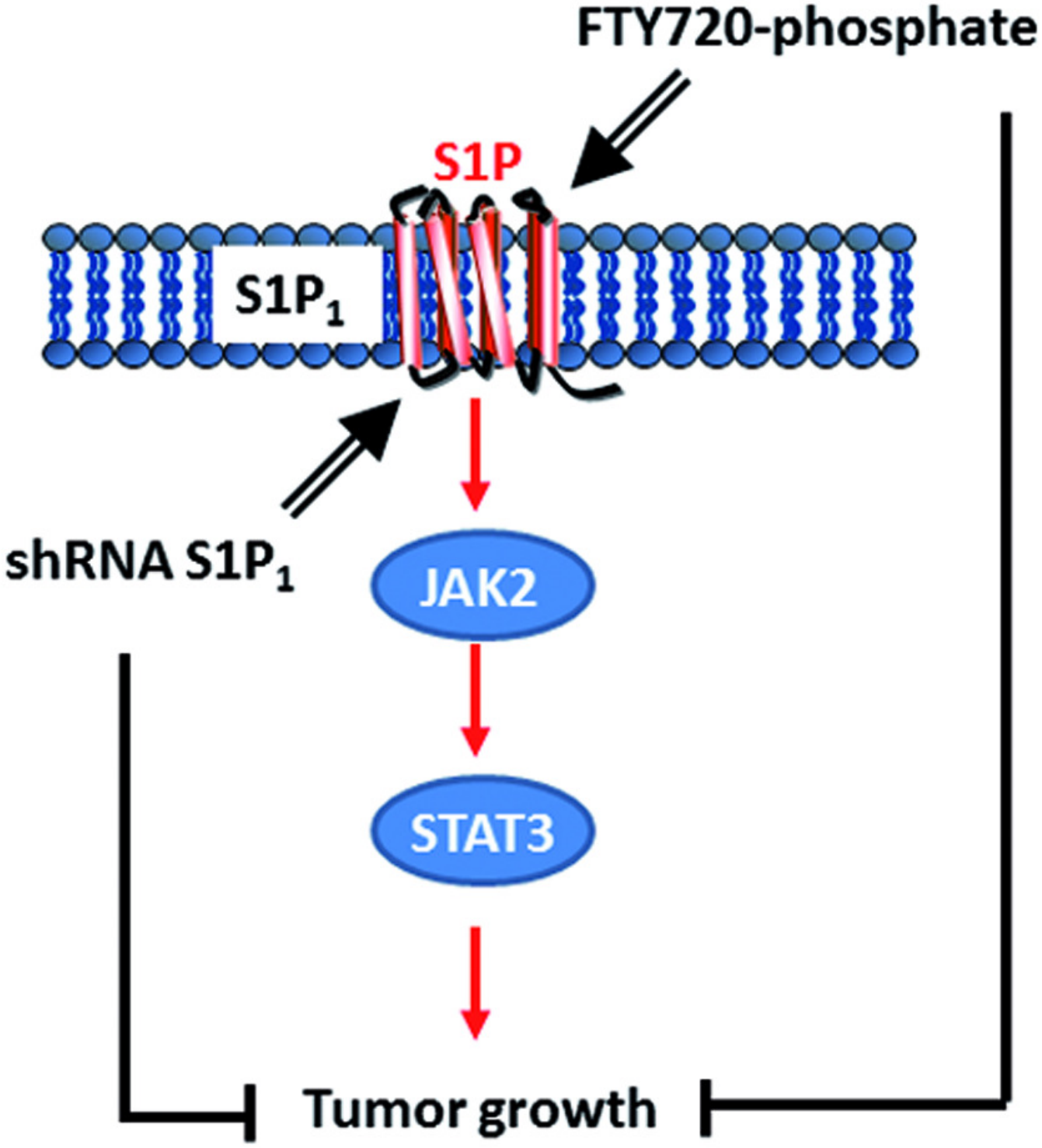
FTY720-phosphate
S1P
S1P1
shRNA S1P1
JAK2
STAT3
Tumor growth

Figure 4