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Sphingolipids in Disease

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Sphingolipids in Disease

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Preface

Until the late 1980s, sphingolipids were believed to represent structural components of the plasma membrane, whose function was to provide a protective barrier to the cell. This picture dramatically changed within the last years. Sphingolipids are now recognized signals for fundamental cellular processes such as proliferation, survival, cell death, adhesion, migration, angiogenesis, and embryogenesis. The explosion of knowledge regarding sphingolipids was facilitated by biochemical studies of their signaling properties, the cloning of enzymes of the sphingolipid metabolism, development of genetic models for determining their physiologic roles, and the establishment of biochemical, biophysical, and optical methods for their detection and quantitation. The next step in the evolution of sphingolipids will be the transfer of basic insights into the biochemistry and cell biology of human disease. The recent success of the sphingolipid drug, Fingolimod, a sphingosine 1-phosphate agonist, which rapidly became a therapy for multiple sclerosis, exemplifies the potential of targeting sphingolipids for the treatment of human disorders. The aim of our two volumes in this series—*Sphingolipids*: *Basic Science and Drug Development* and *Sphingolipids in Disease*—is to define the state of the art of sphingolipid biology and to present preclinical developments and early clinical applications of this fascinating class of lipids.

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Part I
Sphingolipids in Cancer

Sphingosine Kinase/Sphingosine 1-Phosphate Signaling in Cancer Therapeutics and Drug Resistance

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Contents

Abstract In this chapter, roles of bioactive sphingolipids, specifically sphingosine kinase 1 (SK1) and 2 (SK2) and their product—sphingosine 1-phosphate (S1P)—will be reviewed with respect to regulation of cancer growth, metastasis, chemotherapeutics, and drug resistance. Sphingolipids are known to be key bioeffector molecules that regulate cancer proliferation, angiogenesis, and cell death. Sphingolipid molecules such as ceramide and S1P have been shown to control cancer cell death and proliferation, respectively. Roles of S1P have been described with respect to their intracellular and extracellular pro-survival and drug resistance

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functions mostly through S1P receptor (S1PR1-5) engagement. Identification of novel intracellular SK/S1P targets has broadened the existing complex regulatory roles of bioactive sphingolipids in cancer pathogenesis and therapeutics. Thus, deciphering the biochemical and molecular regulation of SK/S1P/S1PR signaling could permit development of novel therapeutic interventions to improve cancer therapy and/or overcome drug resistance.

Keywords SK1 • SK2 • S1P • S1PR • Ceramide • Cancer and Drug resistance • Anti-Cancer therapeutics

1 Introduction

Sphingolipids are structural and functional components of biological membranes (Ogretmen and Hannun 2004; Ponnusamy et al. 2010), which contribute to maintenance of membrane fluidity and subdomain structure. They are also implicated in bioeffector roles in cancer pathogenesis (Hannun and Obeid 2008; Ogretmen and Hannun 2004). Bioactive sphingolipids such as ceramide, sphingosine, and S1P are important in cell death pathways (apoptosis, necrosis, autophagy, anoikis), cancer proliferation, migration, inflammation, and drug resistance (Hannun and Obeid 2008; Ogretmen and Hannun 2004; Ponnusamy et al. 2010; Saddoughi et al. 2008). This chapter will focus on the roles of SK/S1P/S1PR signaling in cancer cell growth, therapeutics, drug resistance, and metastasis.

2 Sphingolipid Metabolism

The de novo sphingolipid synthesis pathway (Fig. 1) begins with the condensation of serine and palmitoyl-coA catalyzed by serine palmitoyl transferase (SPT) (Dolgachev et al. 2004; Reynolds et al. 2004) leading to 3-ketosphinganine generation, which is rapidly reduced to dihydrosphingosine. Dihydrosphingosine is then *N*-acylated by a family of six dihydroceramide synthases (CerS1-6, also known as longevity associated gene, LAG, homologues, LASS1-6), which show preference for varying fatty acyl chain length specificity to synthesize dihydroceramide (Futerman and Hannun 2004). Then, a double bond is introduced between carbons 4 and 5 of the sphingosine backbone to generate ceramide (Kraveka et al. 2007; Michel et al. 1997). Ceramide is at the center of the sphingolipid metabolism, displaying mainly antiproliferative and pro-apoptotic roles (Ogretmen and Hannun 2004; Ponnusamy et al. 2010). Ceramide can be deacylated by ceramidases to generate sphingosine, which is rapidly phosphorylated by SK1 and SK2 to generate S1P, a pleiotropic lipid elucidating pro-survival, anti-apoptotic, metastatic, and/or chemoresistance functions in various cancers (Spiegel and Milstien 2003). S1P can be dephosphorylated by two S1P phosphatases (S1PP1 and S1PP2) in a reversible reaction to generate sphingosine (Mandala 2001; Mao et al. 1999; Spiegel and Milstien 2003), or S1P can be irreversibly cleaved by S1P lyase to form ethanolamine-1-phosphate and hexadecenal (Ikeda et al. 2004). Recently, S1P and

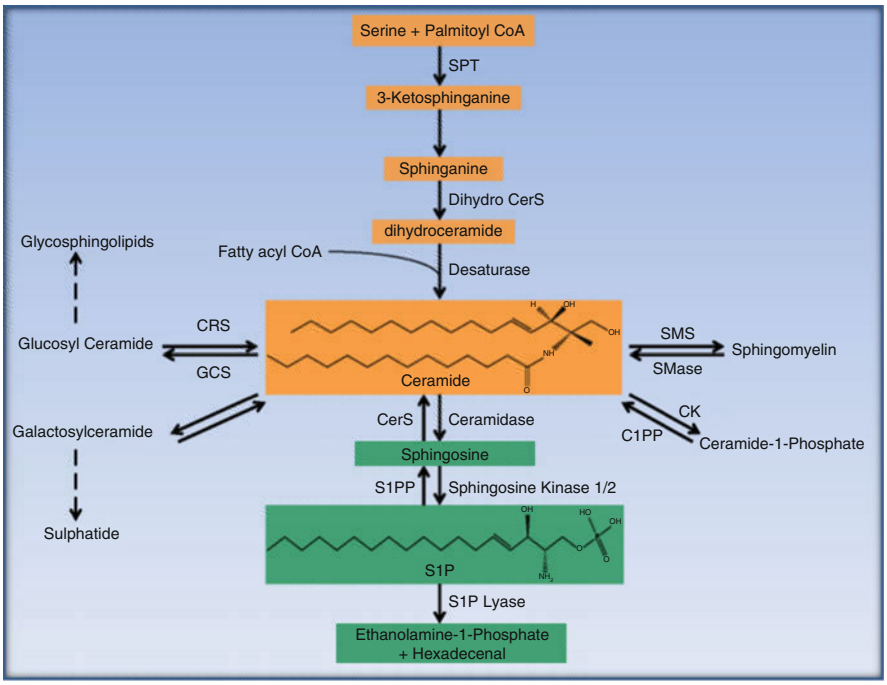

Fig. 1 *De novo sphingolipid synthetic pathway*. The initial step is the condensation of serine and palmitoyl-coA by serine palmitoyl transferase (SPT) followed by the action of ceramide synthases (CerS) and desaturase (DES) to generate ceramide. Ceramide is also generated by the degradation of sphingomyelin (SM) by sphingomyelinase (SMase) or by the action of cerebrosidase (CRS) on glycosphingolipids also by the action of ceramide 1-phosphate phosphatase (C1PP). Ceramide is further metabolized by ceramidase (CDase) to yield sphingosine, which is used as a substrate by SK1 and SK2 to generate S1P. S1P can be dephosphorylated by S1P phosphatases (S1PP) to generate sphingosine, or it can be irreversibly cleaved by S1P lyase into ethanolamine 1-phosphate and C_{18} fatty aldehyde (hexadecenal). Ceramide is further metabolized to generate complex glucosyl and galactosyl-ceramide or glycolipids. Ceramide can be acted upon by sphingomyelin synthase to generate sphingomyelin or by ceramide kinase to generate ceramide 1-phosphate (C1P). *C1P* ceramide 1-phosphate, *C1PP* ceramide 1-phosphate phosphatase, *CDase* ceramidase, *CerS* ceramide synthase, *CRS* cerebrosidase, *DES* desaturase, *GCS* glucosyl ceramide synthase, *S1P* sphingosine 1-phosphate, *S1PP* S1P phosphatase, *SK1* sphingosine kinase 1, *SK2* sphingosine kinase 2, *SM* sphingomyelin, *SMase* sphingomyelinase, *SPT* serine palmitoyl transferase

hexadecenal have been shown to promote MOMP (mitochondrial outer membrane permeabilization). Interestingly, S1P and hexadecenal have been shown to cooperate with BAK and BAX, respectively, to regulate MOMP and cellular responses to apoptosis (Chipuk et al. 2012).

3 Ceramide/S1P Rheostat in Cancer

The fate of a cell is determined by the balance between ceramide and S1P signaling (not necessarily a quantitative ratio for the amount of lipids, but a biological/metabolic balance between these two signaling arms of sphingolipids with

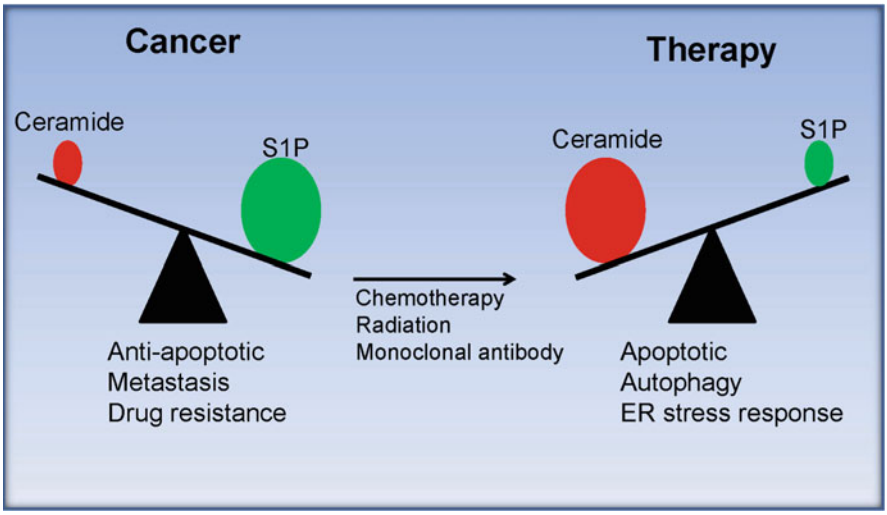

Fig. 2 *Ceramide-S1P rheostat in cancer and therapy*. There exists a balance or rheostat in ceramide to S1P signaling in cancer. A shift towards ceramide accumulation leads to pro-apoptotic, autophagic, ER stress response, and anti-survival effects, whereas a dynamic shift towards S1P accumulation leads to pro-survival, anti-apoptotic, metastatic, and drug-resistant phenotypes. Potential therapeutic approaches will be to increase ceramide and decrease S1P in cancer cells by chemotherapy, radiation, monoclonal antibody, and other molecular approaches. *ER* endoplasmic reticulum, *S1P* sphingosine 1-phosphate

opposing functions), which is often referred to as the ceramide/S1P rheostat (Fig. 2). There exists a dynamic balance between ceramide and S1P signaling, and when a shift towards ceramide is achieved by stress signaling such as radiation, heat, and chemotherapy treatment, this drives cells to undergo cell death and antiproliferation (Hannun and Obeid 2008). On the other hand, when the balance shifts towards S1P accumulation, cells exert pro-survival, anti-apoptosis, and/or chemoresistance (Ponnusamy et al. 2010). Increases in endogenous ceramide by chemotherapeutic agents, TNF-α, CD95, hypoxia, DNA damage, and heat stress can activate cell death pathways. Also, increases in ceramide via inhibiting ceramide metabolism or overexpressing CerS lead to cell death, in general. Moreover, overexpression of bacterial SMase, which generates ceramide by degradation of sphingomyelin, was shown to induce apoptosis (Meyers-Needham et al. 2012a). In contrast, inhibition of de novo ceramide generation by fumonisin B1 blocks ceramide-mediated cell death by chemotherapeutic drugs.

Ceramide has various established intracellular targets such as PP1, PP2A, I2PP2A, cathepsin D, and protein kinase Cζ, which mediate its apoptotic/cell death functions (Fox et al. 2007; Heinrich et al. 2000; Ogretmen and Hannun 2004; Wang et al. 2005). Our laboratory identified telomerase to be a nuclear target of ceramide (exogenous C_6-ceramide or C_{18}-ceramide generated by CerS1) which decreased c-Myc-mediated activation of hTERT promoter in lung cancer cells (Ogretmen et al. 2001). In fact, ceramide deacetylates Sp3 transcription factor

and deacetylated Sp3 recruits HDAC1 to the hTERT promoter, thereby decreasing hTERT expression/activity (Wooten-Blanks et al. 2007; Wooten and Ogretmen 2005). We also showed that ceramide binds I2PP2A and relieves PP2A-mediated tumor suppression functions in A549 lung cancer cells. Ceramide mediates the degradation of c-Myc by PP2A activation via prevention of I2PP2A-dependent inhibition of PP2A (Mukhopadhyay et al. 2009).

Recently, ceramides with different fatty acid chain lengths were suggested to have distinct functions. For example, in head and neck cancer cells, CerS1-generated C_{18}-ceramide suppressed tumor growth, whereas CerS6-generated C_{16}-ceramide increased tumor growth/proliferation (Senkal et al. 2011). These distinct and unexpected functions of endogenous ceramides might be due to their downstream targets regulated via ceramide/protein interactions and/or their subcellular localization at distinct biological membranes or membrane microdomains. Although mechanisms that regulate CerS expression and function are largely unknown for de novo ceramide generation, new insights about the modulation of CerS expression suggested that epigenetic and posttranscriptional control by concerted functions of HDAC1 and microRNA-574-5-mediated targeting of CerS1 mRNA is involved in its repression in HNSCC (Meyers-Needham et al. 2012b). In addition, dimerization of CerS proteins has been shown to regulate their function for the generation of ceramide (Laviad et al. 2012).

In contrast to ceramide, S1P plays a pro-survival function. Sphingosine kinases and S1P-phosphatases/lyase are important players in the regulation of S1P generation/metabolism. SiRNA-mediated knockdown of SK1 inhibits cell proliferation and increases ceramide/S1P rheostat in pancreatic, prostate, and leukemia cancer cells (Akao et al. 2006; Baran et al. 2007; Guillermet-Guibert et al. 2009; Pchejetski et al. 2005). In contrast, overexpression of SK1 leads to increased cell proliferation by inducing G1/S phase transition and DNA synthesis (Olivera et al. 1999). Exogenous S1P addition was found to significantly inhibit apoptosis via increased Bcl-2 (Sauer et al. 2005) and Mcl-1 expression (Li et al. 2008) or decreased BAD and BAX expression (Avery et al. 2008). In particular, exogenous S1P prevents BAD/BAX translocation to mitochondria, thereby inhibiting the intrinsic cell death (mitochondrial) pathway (Betito and Cuvillier 2006). Also, SK1 overexpression has been shown to inhibit cytochrome c release and caspase-3 activation by the regulation of BCL-X_L, MCL1, and BIM in chronic myelogenous leukemia (CML) cells (Bonhoure et al. 2008).

Moreover, important in vivo findings indicate the involvement of S1P in cancer progression. For example, breast and prostate cancer cells that overexpressed SK1 formed tumors in mice, and ceramide/S1P was altered in these tumors (Nava et al. 2002; Pchejetski et al. 2005), which were resistant to doxorubicin and had increased neovascularization and decreased ceramide/S1P (Nava et al. 2002; Pchejetski et al. 2005). Interestingly, CML and leukemia cells, which are sensitive to imatinib and daunorubicin, respectively, had higher ceramide/S1P compared to resistant cells. Furthermore, increased SK1/S1P in response to camptothecin treatment in prostate cancer cells suggests a role for S1P signaling in chemoresistance (Fig. 3).

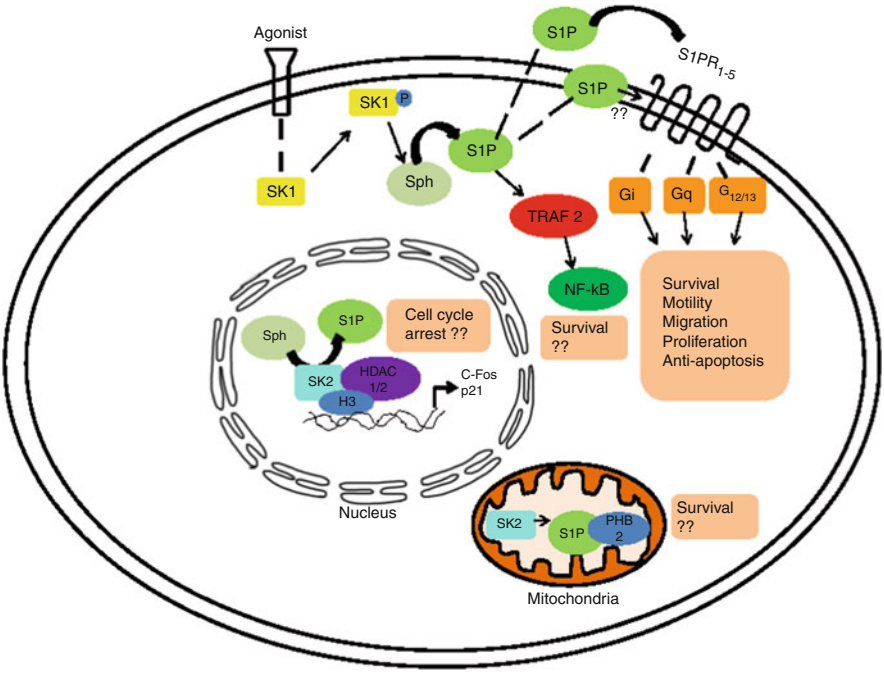

Fig. 3 *SK1/S1P signaling and intracellular targets of S1P*. SK/S1P signaling pathway involves the activation of SK1 by agonist-mediated receptor action. The activated SK1 translocates to the inner leaflet of the plasma membrane to utilize the substrate sphingosine to generate S1P. The S1P generated inside the cells are transported outside the cell by S1P transporters and furthermore engage in an autocrine or paracrine fashion to five G protein-coupled receptors specific for S1P ($S1PR_{1–5}$) to induce an array of downstream mechanisms involved in cell motility, survival, migration, and/or proliferation. S1P generated by SK1 has been reported to play intracellular function by binding with TRAF2 protein to regulate NF-κB function. S1P generated by SK2 has been shown to interact with HDAC1/2 in the nucleus to regulate transcription of p21 and c-Fos genes. Moreover, SK2-generated S1P also binds to prohibitin 2 (PHB2), mitochondrial protein which in turn regulates cytochrome c oxidase (Cox-2) in respiration complex assembly and function. *HDAC* histone deacetylase, *NF-κB* nuclear factor kappa light chain enhance of activated B cells, *PHB2* prohibitin 2; *TRAF2* TNF receptor associated factor 2

4 S1P Signaling

S1P generated by SK1 has been shown to be secreted and engaged with S1P receptors ($S1PR_{1–5}$) to elicit various downstream responses involved in inflammation, cell migration, angiogenesis, and/or lymphocyte trafficking (Spiegel and Milstien 2003; Strub et al. 2010). S1PR1 is important in vascular maturation, immune cell trafficking, endothelial barrier function, and angiogenesis as displayed in S1PR1-null mice. Also the S1P–S1PR1 interaction reveals receptor tyrosine kinase (RTK) activation such as EGF- and PDGF-mediated pathways that dictate cellular growth and migration. Importantly, ATP-dependent ABC transport

proteins such as ABCC1, ABCA1, and ABCG2 are involved in S1P transport (Mitra et al. 2006; Sato et al. 2007; Takabe et al. 2010). Also, ABCC1 is involved in S1P export from mast cells independent of degranulation (Mitra et al. 2006). Recently, TOH/SPNS2 (two of hearts protein) has been identified as an S1P transporter in zebra fish, and interestingly TOH/SPNS2 acts upstream of MIL, an orthologue of S1PR2 involved in heart development (Kawahara et al. 2009; Osborne et al. 2008). SPNS2 was shown to be important for the transport of phosphorylated form of FTY-720, an immunomodulatory drug used for lymphocyte egress in multiple sclerosis. Importantly SPNS2-null mice had increased accumulation of mature T cells and a decreased T cell population in blood and secondary lymphoid organs (Fukuhara et al. 2012; Hisano et al. 2011). Thus, S1P-mediated signaling (Fig. 3) is important in various cellular processes, and how this mechanism is dysregulated in cancer needs further investigation. Recently, the structure of ligand-bound S1PR1 was solved, and data suggest that S1P might engage with the receptor within the plasma membrane (Hanson et al. 2012), indicating that lateral movements of S1P within the membrane or ligand swapping between S1PR1 and other receptors might be involved in this process.

5 Roles of SK in Cancer Pathogenesis

Sphingosine kinases (SK1 and SK2) are novel lipid kinases, which are evolutionarily conserved as diverse as in humans, mice, yeast, and plants. SK1 and SK2 are encoded by *SPHK1* and *SPHK2* genes in humans (Pitson 2011; Strub et al. 2010). SphK1 and SphK2 belong to the diacylglycerol kinase family and have five conserved domains C1–C5 (Spiegel and Milstien 2003). The unique catalytic domain is present between domains C1 and C3. SphK1 and SphK2 differ by the presence of a TM (transmembrane domain) present only in SphK2 and not in SphK1. Both SK1 and SK2 enzymes show substrate specificity towards D-e-sphingosine and D-e-dihydrosphingosine. Interestingly, SK1 and SK2 show different tissue distribution with SK1 expression higher in lung and spleen, whereas SK2 levels were found to be higher in the liver and the heart. SK1 is predominantly cytosolic in nature, whereas SK2 has been shown to localize both in the cytoplasm and nucleus (Kohama et al. 1998; Liu et al. 2000, 2002; Pitson 2011). Moreover, SK1 expression was detected at embryonic day 7 (E7) of mice and SK2 at E11 indicating the distinct functions of both of these isoenzymes. The functions of SK1 and SK2 seem redundant during development; SK1 and SK2 null mice survive, whereas the SK double knockout is embryonically lethal (Mizugishi et al. 2005; Pitson 2011; Spiegel and Milstien 2003).

5.1 SK1 and Cancer

SK1 has well-established pro-survival functions in various cancers. Indeed, by virtue of its transformation potential, SK1 is considered to be a bona fide oncogene

(Vadas et al. 2008). Upon transfection with SK1, untransformed NIH3T3 cells undergo transformation with the ability to form colonies in soft agar and tumors in nude mice (Xia et al. 2000). Activation of SK1 by agonists of GPCRs, protein kinases, proinflammatory cytokines, and small GTPases leads to its translocation to plasma membrane where it catalyzes the conversion of sphingosine to S1P. Importantly, phosphorylation of SK1 at Ser 225 by ERK1 and ERK2 is important for its activation and translocation to the plasma membrane (Pitson et al. 2003). This finding was further confirmed by S225A mutant of SK1 that lacks the phosphorylation site (Pitson et al. 2005). When SK1 S225A mutant was targeted to the plasma membrane by tagging with a myristoyl or palmitoyl moiety, the cells became transformed, via targeting to plasma membrane (Pitson et al. 2005). The membrane affinity and plasma membrane selectivity are determined by Thr54 and Asn89 residues of human SK1, wherein these residues interact specifically with phosphatidyl serine in the plasma membrane, thus making sphingosine available to generate S1P, which can be secreted outside the cell and engage with S1PRs to induce pro-survival functions (Stahelin et al. 2005). Interestingly, SK1 expression was found also to be important for Ras-mediated transformation. In cancers, such as breast, lung, ovary, stomach, and kidney, SK1 mRNA increased approximately twofold compared to paired normal tissues (French et al. 2003; Johnson et al. 2005; Kawamori et al. 2006). Also immunohistochemical studies with lung, colon, and breast cancer tissues were positive for SK1 expression in tumor tissues and/or carcinoma cells (Johnson et al. 2005; Kawamori et al. 2006; Kohno et al. 2006; Ruckhaberle et al. 2008). Moreover, microarray data show elevated SK1 in squamous cell carcinoma (Nindl et al. 2006), melanoma cancer (Talantov et al. 2005), *N*-methyl-*N*-nitrosourea-induced rat breast cancer (Chan et al. 2005), recurrent breast cancer after tamoxifen treatment (Ma et al. 2004), cervical cancer (Wong et al. 2003), head and neck cancer, and leukemia (Andersson et al. 2007; Ginos et al. 2004; Pyeon et al. 2007). Interestingly, siRNA-mediated downregulation of SK1 showed decreased migration of EGF, prolactin, and E2-induced migration of MCF-7 breast cancer cells (Doll et al. 2007; Sarkar et al. 2005), whereas downregulation of both SK1 and SK2 inhibited migration of MDA-MB-453 cells (Hait et al. 2005), indicating a possible redundancy/overlap in functions between these isoenzymes in cancer cell migration.

Novel intracellular targets of SK1 have been recently established: SK1-generated S1P has been shown to bind to TRAF2 protein, an E3 ubiquitin ligase, modulating TNF-α-induced activation of NF-κB signaling (Alvarez et al. 2010). SK1 has been shown to bind TRAF2 to induce K63-mediated polyubiquitination of RIP1 leading to IκB degradation and subsequent stimulation of the NF-κB pathway (Fig. 3). These findings suggest SK1/S1P to be important driver of cancer progression.

Remarkably, a recent finding from our laboratory suggests that serum S1P generated by SK1, and not tumor S1P, was important for tumor metastasis to the lungs (Fig. 4). In this study, genetic ablation of systemic SK1 leads to increased breast cancer metastasis suppressor 1 (Brms1) expression via alterations of S1PR2 signaling in tumor cells, leading to suppression of metastasis. Furthermore, treatment with anti-S1P monoclonal antibody (Sphingomab) decreased lung metastasis

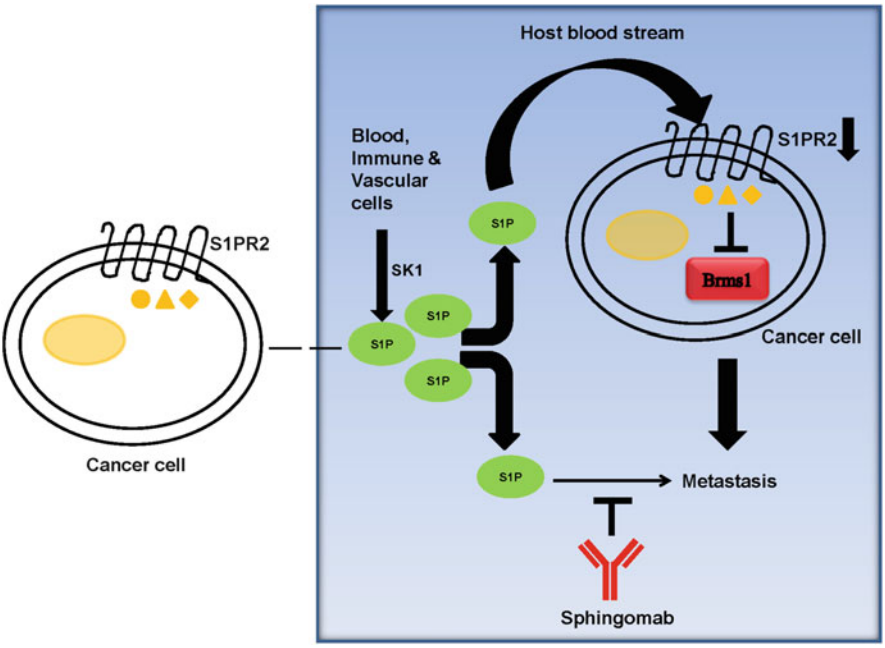

Fig. 4 *Role of systemic SK1/S1P in the modulation of S1PR2/Brms1 expression in lung tumor metastasis.* S1P generated in response to cancer cell exposure to bloodstream elevates systemic S1P levels thereby leading to the induction of S1PR2 expression and further leading to the suppression of Brms1, a master metastatic suppressor gene in cancer cells, inducing their metastatic potential. Use of pharmacologic inhibitors or antibody-based therapeutic tools (Sphingomab, Lpath Inc.) effectively modulates serum S1P via inhibition of cancer cell S1PR2 signaling to elevate tumor Brms1 levels and to suppress lung metastasis. *Brms1* breast cancer metastasis suppressor 1, *SK1* sphingosine kinase 1, *S1P* sphingosine 1 phosphate, *S1PR2* S1P receptor 2

in this model by neutralizing circulating/systemic S1P, inducing cancer Brms1 expression, and thus further proving the importance of SK1-generated systemic S1P in regulating tumor metastasis (Ponnusamy et al., 2012). Recently, the role of SK1 in the regulation of tumor metastasis has been also shown in a breast cancer model (Nagahashi et al. 2012). These data suggest that inhibition of systemic SK1/S1P and/or cancer S1PR2 signaling might inhibit tumor metastasis.

5.2 SK2 and Cancer

SK2 has been shown to predominantly localize to the nucleus, although cytoplasmic and ER localizations were also reported (Igarashi et al. 2003; Maceyka et al. 2005). SK2 localizes to the nucleoplasm and causes cell cycle arrest. Importantly, SK2 retains a nuclear export signal, and activation with phorbol myristate acetate (PMA) leads to protein kinase D-mediated phosphorylation and export from the nucleus. SK2, unlike SK1, possesses a BH-3 domain in its sequence that sequesters

Bcl-XL to diminish its anti-apoptotic functions (Liu et al. 2003). Interestingly, when SK2 is overexpressed, it induces apoptosis, cell cycle arrest, and/or caspase-3 activation (Liu et al. 2003). In contrast to the apoptotic roles, SK2 also displays anti-apoptotic functions. Knockdown of SK2 in HCT116 colon cancer cells and MCF7 breast cancer cells prevents doxorubicin-induced p21 expression and G2/M cell cycle arrest in a p53 independent manner (Sankala et al. 2007). SK2 knockdown decreased glioblastoma cell proliferation, whereas SK1 knockdown did not have any effects (Van Brocklyn et al. 2005). EGF activates SK2 at Ser351 and Thr578 (Hait et al. 2007) residues leading to EGF-mediated migration of MDA-MB-453 breast cancer cells. These studies elucidate SK2's anti-apoptotic and pro-proliferative effects.

Most importantly, the first identified intracellular function of S1P generated by SK2 was in the nucleus to inhibit HDAC1/2 enzymatic activity thereby preventing deacetylation of histone H3 (Hait et al. 2009). The SK2/HDAC repressor complex was found to be associated with promoter regions of P21 (CDKN1) and c-Fos genes thereby modulating their expression. Interestingly, another intracellular target of S1P has been identified as prohibitin 2 (PHB2), a protein regulating mitochondrial function (Strub et al. 2011). S1P generated by SK2 binds prohibitin 2 and not prohibitin 1 to regulate cytochrome c oxidase complex IV in mitochondrial respiration and function. Also, mitochondria isolated from SK2 knockout mice showed decreased association of PHB2 and cytochrome c oxidase (Strub et al. 2011) (Fig. 3).

Collectively, SK1- and SK2-generated S1P have distinct roles in the context of their subcellular localization and function. Also, SK1 and SK2 might also have overlapping functions in different cancer models for inducing pro-survival and anti-apoptosis, possibly via distinct mechanisms of action at different subcellular compartments.

6 Role of S1P in Autophagy

Autophagy is a cellular process to degrade long-lived proteins and organelles through lysosomes. The autophagic process can be protective or lethal; the protective autophagy pathway directs degradation of damaged organelles and recycling of amino acid to overcome nutrient deprivation, whereas lethal autophagy leads to caspase independent cell death involving Atg proteins (Kondo and Kondo 2006; Levine and Kroemer 2008). Use of SK2 inhibitor ABC294640 induces autophagic cell death in A498 cells (Beljanski et al. 2010). Mechanistically, Beljanski and colleagues reported that increased ceramide and sphingosine in response to ABC294640 treatment resulted in autophagy in vivo. Moreover, protective autophagy was activated in response to SK1 upregulation by lack of beclin 1 and mTOR inhibition (Lavieu et al. 2006). Recently, it was shown that S1P metabolism plays an important role in switching from protective autophagy to apoptosis through the involvement of SPP1 (S1P phosphohydrolase). It was found that doxorubicin-mediated autophagy was greatly decreased by SPP1 ablation with a concomitant increase in apoptosis by ceramide accumulation via de novo synthesis.

Ceramide then activates calpain to cleave Atg5 in SPP1-depleted cells to shift from protective autophagy to apoptosis (Lepine et al. 2011).

7 SK1/S1P Signaling in Drug Resistance

SK/S1P protects cancer cells from drug-induced cell death; therefore, levels of bioactive sphingolipids have been shown to modulate drug resistance. For example, PC3 prostate cancer cells, which are androgen insensitive, are resistant to camptothecin treatment by upregulation of SK1 and S1P1/S1P3 signaling, and these cells became sensitive upon inhibition of SK and S1P formation (Bektas et al. 2005). Radiation-resistant prostate cancer cell line LNCaP showed sensitivity upon coadministration of TNF-α and γ-irradiation to increase the generation of sphingosine and decreased S1P formation (Nava et al. 2000). Moreover, treatment of LNCaP cells with SK inhibitor (*N*,*N*-dimethyl sphingosine) sensitized them to radiation-induced apoptosis. In addition, SK1 induced resistance of melanoma cells to ceramide and Fas-induced cell death. The role of SK1/S1P in breast cancer progression was also reported to be important. A microarray study conducted with 1,269 breast cancer tumor samples showed a significant increase in SK1 gene expression and showed a strong correlation between SK1 expression and poor prognosis (Ruckhaberle et al. 2008). Additionally SK1 expression was responsible for resistance to tamoxifen-induced cell death in MCF7 cells. Tamoxifen-resistant MCF7 cells showed elevated SK1 levels and inhibition of SK1 by DMS/SKI-II restored sensitivity of the cells to tamoxifen-induced apoptosis (Sukocheva et al. 2009). In erythroleukemia progression, overexpression of SK1 in non tumorigenic proerythroblasts showed increased tumorigenicity and resistance to cell death. Of note, a microarray-based transcriptome profile showed transcriptional upregulation of SK1 in tumorigenic proerythroblasts. These data suggest SK1 expression to be important in erythroleukemic progression (Le Scolan et al. 2005). Moreover, SK1 is overexpressed in glioblastoma multiforme (GBM) and targeted inhibition of SK1 with an isoform-specific drug; SK1-I significantly decreased tumor proliferation and markedly suppressed pro-survival AKT and its downstream targets (p70S6K and GSK3-α) (Kapitonov et al. 2009; Knapp et al. 2010). SK1-I not only inhibited S1P generation but also increased ceramide to induce apoptosis (Kapitonov et al. 2009). In endometrial cancer, compared with healthy endometrial tissues, the cancer tissues had increased SK1 activity (2.6-fold) and elevated S1P (1.6-fold) in serum, indicating a significant change in sphingolipid metabolism in driving cancer progression (Knapp et al. 2010).

In CML, ceramide/S1P rheostat plays a crucial role in conferring drug resistance. K562 CML cells generate endogenous C_{18}-ceramide in response to imatinib treatment, and interestingly K562 cells which show resistance to imatinib treatment have increased SK1 expression. Importantly, siRNA-mediated knockdown of SK1 sensitized K562-imatinib-resistant cells to apoptosis. These studies showed that overexpression of SK1 induces drug resistance in CML cells by altering the ceramide/S1P rheostat towards S1P accumulation (Baran et al. 2007). Recently,

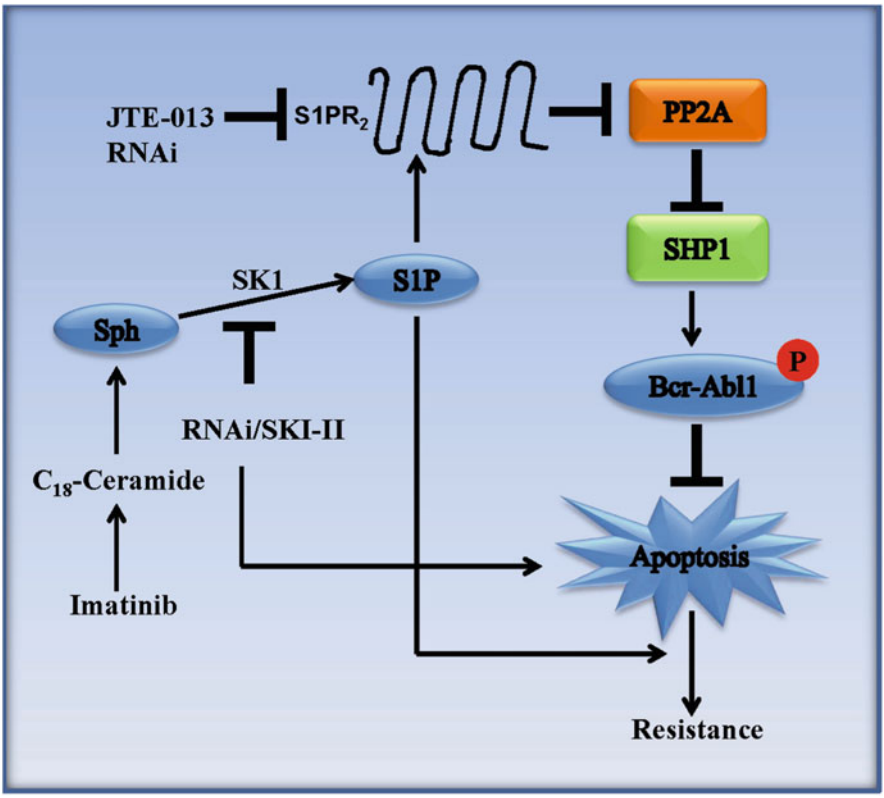

Fig. 5 *Role of SK1/S1P/S1PR-mediated drug resistance in chronic myelogenous leukemia (CML).* SK1/S1P-mediated drug resistance in CML is mediated by S1PR2-mediated PP2A modulation which abolishes proteasomal degradation of Bcr-Abl1 by enhancing its stability, resulting in drug resistance. Pharmacologic inhibitor (SKI-II) or use of molecular approaches (RNAi) by inhibiting SK1 can lead to PP2A-mediated dephosphorylation and degradation of Bcr-Abl1, thereby overcoming drug resistance. *CML* chronic myelogenous leukemia, *PP2A* protein phosphatase 2A, *SK1* sphingosine kinase 1, *S1P* sphingosine 1-phosphate, *S1PR2* S1P2 receptor 2, *SHP1* src homology region 2 domain containing phosphatase 1

we have shown that SK1/S1P and S1PR2 signaling is important for Bcr-Abl1 stability in CML (chronic myeloid leukemia), leading to imatinib resistance. This study showed that SK1/S1P/S1PR2 prevents Bcr-Abl1 dephosphorylation and subsequent degradation by inhibiting PP2A. Inhibition of SK1/S1P/S1PR2 signaling either by pharmacological or molecular approaches restored PP2A-mediated dephosphorylation of Bcr-Abl1 and also enhanced imatinib or nilotinib (drugs used against CML) mediated growth inhibition. Furthermore, allograft tumors derived from 32D cells expressing either wild-type or mutant Bcr-Abl1 genes were more sensitive to nilotinib upon inhibition of SK1/S1PR2 signaling (Fig. 5). These findings suggest that inhibition of SK1/S1P/S1PR2 signaling overcomes drug resistance in CML (Salas et al. 2011). In a separate study, LAMA84 cells which

are resistant to imatinib treatment became sensitized when SK1 is inhibited by F-12509a, a SK1 inhibitor, leading to apoptotic cell death (Bonhoure et al. 2008).

8 SK2 and Drug Resistance

Although SK1/S1P signaling has been well established in drug resistance, only recently SK2/S1P has been shown to confer chemoresistance in cancers. Targeted inhibition of SK2 by molecular/pharmacological agents sensitized cancer cells to chemotherapy. Recently, Antoon's laboratory reported that the SK2 inhibitor ABC294640 decreased estrogen receptor (ER)-positive breast cancer tumor growth by 68.4 % compared to vehicle-treated tumors. Mechanistically, SK2 inhibition decreased estrogen-mediated transcription of ER-regulated genes such as SDF1 and the progesterone receptor (Antoon et al. 2010). Subsequently, inhibition of SK2 by ABC294640 at submicromolar concentration was shown to block proliferation of endocrine therapy-resistant MDA-MB-231 and chemoresistant MCF-7TN-R cells (Antoon et al. 2011). Also ABC294640 diminished NF-κB pro-survival signaling by decreasing activation of Ser536 phosphorylation of the p65 subunit (Antoon et al. 2011). Moreover, ABC294640, which is orally bioavailable, resulted in growth inhibition of xenograft-derived tumors of MCF-7TN-R cells at 50 mg/kg in SCID mice.

Schnitzer et al. (2009) elucidated the role of SK2-mediated chemoresistance in A549 lung cancer cells. In this study, it was demonstrated that hypoxia induces SK2 protein/activity leading to S1P secretion via S1PR1/S1PR3 signaling to activate P42/44 MAPK signaling and confer resistance to etoposide-induced apoptosis (Schnitzer et al. 2009). Recently, Xiao et al. (2012) showed that SK2 confers resistance to sodium butyrate-induced apoptosis in HCT116 colon cancer cells. In fact, sodium butyrate treatment induced the phosphorylation of SK2 by PKD leading to nuclear export, leading to chemoresistance (Xiao et al. 2012).

9 S1P/S1PR2 Signaling in Cancer and Drug Resistance

S1P exerts its signaling function in an autocrine or paracrine manner through five G protein-coupled receptors, termed $S1PR_{1–5}$ (formerly referred as the EDG family of receptors). These receptors involve heterotrimeric G proteins such as G_i, G_q, and $G_{12/13}$ to elicit various cellular functions. Importantly, roles of S1P receptors in modulating cancer growth have been studied (Spiegel and Milstien 2003). For instance, S1PR2 has been shown to be important for retinal angiogenesis. When a neonatal mouse was subjected to ischemia-driven retinopathy, the neovascularization event was downregulated in S1P2-null mice compared to wild-type mice. Moreover, the retina of the S1P2-null mouse had significantly reduced proinflammatory Cox-2 mRNA and increased eNOS expression. These findings

indicate the involvement of S1PR2 in retinal angiogenesis and prompt the use of receptor antagonists to counteract ocular neovascularization. Additionally, S1P2 plays an important role in Wilm's tumor, a malignant renal cancer condition. In Wilm's tumor, S1PR2 mRNA was higher in ten cancer specimens compared to noncancerous tissues (Li et al. 2009). Additionally S1PR2 overexpression was shown to induce Cox-2 mRNA and increased prostaglandin E2 synthesis. SiRNA-mediated knockdown or pharmacological inhibition of S1PR2 in WiT49 cells decreased Cox-2 mRNA proving an important role of S1PR2 signaling in driving renal cancer progression (Li et al. 2009). In another study, the migration of WiT49 Wilm's tumor cells was attributed to S1PR1 signaling wherein S1PR1/G_i coupling was shown to induce a promigratory phenotype which depended upon the ratio of S1PR1/S1PR2 and further identified PI3K and Rac1 as downstream regulators of cell motility. S1PR2 was also shown important in esophageal cancer motility and migration, in which TGFβ enhanced S1P-mediated activation of ERK1/2 through S1PR2 through non-Smad signaling (Miller et al. 2008).

In a separate study using a cohort of 304 ER-positive, tamoxifen-treated breast cancer samples, increased SK1 and S1P1 and S1P3 receptor expression was observed. Interestingly cytoplasmic expression of S1P1 and S1P3 was found to be associated with reduced disease-specific survival in patients with ER positive breast cancer (Watson et al. 2010). SK1 also confers resistance towards synthetic retinoid *N*-(4-hydroxyphenyl) retinamide in A2780 ovarian cancer cells; the HPR-resistant cells express more SK1 both at the mRNA and the protein level and became sensitized when treated with SK inhibitor (Illuzzi et al. 2010). Recently, SK1 has been shown to protect androgen-independent LNCaP prostate cancer cells; it does so by increasing S1P signaling through $S1P_{2/3}$ receptors, and the resistance was attenuated by treating with FTY-720, a sphingosine analog that inhibits S1PR signaling (except S1PR2), by inducing proteasome-mediated degradation of SK1 (Tonelli et al. 2010).

These findings suggest that the SK/S1P/S1PR signaling pathway is crucial in sphingolipid-mediated drug resistance in various cancers, and hence targeting this pathway would be an effective anticancer therapeutic strategy to overcome drug resistance.

10 SK/S1P-Mediated Anticancer Therapeutics

Targeting S1P produced by SK1 and SK2 can sensitize cancer cells to therapeutic intervention. Also targeting SK/S1P is attractive because indirect generation of ceramide and sphingosine can have antiproliferative and pro-apoptotic functions. An array of sphingolipid and non-sphingolipid agents has been developed to target SK/S1P and/or S1P receptors (Table 1).

Pan Sphingosine Kinase Inhibitors. *N*,*N*-dimethyl sphingosine (DMS), a pan SK inhibitor, was shown to be effective against a panel of cancer cell lines and exhibited antitumor growth properties in nude mice. Also, L-threo dihydrosphingosine

Table 1 Sphingosine kinase/S1P/S1PR based anti cancer therapeutics

Compound	Target	Cancer type
N,*N*-dimethyl sphingosine (DMS)	SK1, SK2	Leukemia, colon and breast
Safingol (L-threo dihydrosphingosine)	SK1, SK2	Solid tumors
SKI-II	SK1, SK2	Breast
Phenoxodiol	SK1, SK2	Ovarian and prostate
SK1-I	SK1	Glioblastoma, breast and AML
ABC294640	SK2	Breast, prostate and kidney
FTY-720	S1PR	Bladder, prostate, breast and lymphoma
Anti-S1P-mAb	S1P	Lung, ovarian, breast and melanoma

SK1 sphingosine kinase 1, *SK2* sphingosine kinase 2, *S1P* sphingosine 1-phosphate, *S1PR* S1P receptor, AML acute myeloid leukemia

(Safingol) inhibits SK and is currently in phase I clinical trials (Schwartz et al. 1997). Both DMS and Safingol inhibit SK competitively and displayed inhibitory effects towards ceramide kinase and PKC and activate sphingosine-mediated targets such as PI3K and casein kinase 2 (Igarashi et al. 1989; Kedderis et al. 1995; King et al. 2000; McDonald et al. 1991; Megidish et al. 1998; Sugiura et al. 2002). Some of the reported off-target effects of DMS include hemolysis and hepatotoxicity (Kedderis et al. 1995). Interestingly, phenoxodiol, a synthetic analog of plant isoflavone genistein, exhibits anticancer and antiangiogenenic functions by inhibiting SK. Phenoxodiol exerts its anticancer function by inhibiting endothelial cell function both in vitro and in an in vivo model of angiogenesis, and currently phenoxodiol is in clinical trials against ovarian and prostate cancers (De Luca et al. 2005; Gamble et al. 2006). French et al. (2003) have demonstrated the use of non-lipid selective inhibitors of SK and showed inhibition against a panel of cancer cell lines, with greater selectivity to SK than other protein kinases; additionally these SK inhibitors were noncompetitive and displayed antiproliferative functions (French et al. 2003). Furthermore, French et al. (2006) showed SKI-II (4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol), a noncompetitive inhibitor at nanomolar to submicromolar concentration in vitro, and displayed excellent oral bioavailability with antitumor functions in vivo (French et al. 2006). Additionally, other compounds such as F12509a, a sesquiterpene quinone-based competitive inhibitor of SK, and B5354c, a noncompetitive inhibitor isolated from a marine bacterium, were found to inhibit SK in some cancers, but their efficacy and specificity are still under investigation (Kono et al. 2000a, b, 2002).

SK1 Selective Inhibitors. SK1-I (2R, 3S, 4E)-*N*-methyl-5-(4′-pentylphenyl)-2-aminopent-4-ene-1, 3-diol is a SK1 selective inhibitor and has shown efficacy against orthotopic as well as xenograft glioblastoma or AML xenograft tumors (Kapitonov et al. 2009; Paugh et al. 2008). Recently, Nagahashi et al. (2012) demonstrated decreased breast cancer progression in an 4T1-Luc orthotopic mice and also showed decreased lymph node and lung metastasis upon SK1-I treatment. Interestingly SK1-I decreased serum S1P levels and caused mammary tumor cells to undergo apoptosis (Nagahashi et al. 2012). Moreover, novel SK1-specific inhibitors such as 6ag, 9ab, and12aa were synthesized and tested in vitro, but the

in vivo efficacy still needs to be validated (Xiang et al. 2009). There is a need for the development of SK1-specific small molecule inhibitors to be utilized as effective anticancer agents.

SK2 Selective Inhibitors. ABC294640 is a novel SK2-specific inhibitor with excellent oral bioavailability and toxicology properties. ABC294640 displays antiproliferative effects against a variety of cancer cell lines at nanomolar up to submicromolar concentrations. This SK2 selective compound induced autophagic cell death in breast, prostate, and kidney xenograft tumors in vivo (Antoon et al. 2010; Beljanski et al. 2010, 2011; French et al. 2010). ABC294640 is currently in phase I clinical trials against solid tumors. Moreover, sphingoid analogs such as SG12 and SG14 displayed specificity towards SK2, and in particular SG14 did not inhibit PKC (Kim et al. 2005).

S1P Receptor Selective Compounds. FTY-720, a sphingosine analog, phosphorylated by SK2, acts through S1P receptors (S1PR1, 3, 4, 5) to cause receptor endocytosis and rapid lymphocyte egress in multiple sclerosis (Brinkmann et al. 2010, 2002). FTY-720 has been efficacious against refractory multiple sclerosis and approved by FDA as an anti-MS drug (Brinkmann et al. 2010). FTY-720 also inhibited SK1, ceramide synthase, phospolipase A2, and SGPL1, and it was shown to activate tumor suppressor PP2A (Bandhuvula et al. 2005; Berdyshev et al. 2009; Lahiri et al. 2009; Matsuoka et al. 2003; Neviani et al. 2007; Payne et al. 2007; Vessey et al. 2007). FTY-720 has been reported to inhibit colon and breast cancer cell lines in situ through S1P receptor independent effects. In addition, phosphorylated-FTY720 (P = FTY-720) was found to be important for antiangiogenesis in an in vitro spheroid model, indicating a receptor-dependent function, which can also be mimicked by SEW2871, a S1PR1-specific antagonist (Nagaoka et al. 2008; Schmid et al. 2007). Further investigation is required to delineate the receptor-dependent and receptor-independent functions of FTY-720 against cancer growth and/or proliferation. Recently, (R)-FTY-720-OMe, a stereo-specific analog of FTY-720, showed SK2-specific inhibition and caused actin rearrangement in MCF-7 cells (Lim et al. 2011), suggesting that this novel enantiomer can be used against breast cancer. Other receptor antagonists such as VPC4416, VPC2309, VPC25239, and W146 against S1PR1/3 and S1PR1, respectively, have shown promising results in situ (Davis et al. 2005; Sanna et al. 2006).

Antibody-Based Therapeutics. An anti-S1P-mAb that specifically targets and neutralizes S1P has been shown to be effective against lung A549, ovarian SKOV3, breast MDA-MB-231, and melanoma F16/B10 cancer models in situ and in vivo. Anti-S1P-mAb functions as a molecular sponge to neutralize S1P signaling and cause tumor regression in both xenograft and allograft models or to inhibit lung metastasis (Ponnusamy et al. 2012). Sphingomab (LT1002) and its humanized form (LT1009) neutralize bFGF- and VEGF-induced angiogenesis and block S1P-induced endothelial cell tube formation and migration in numerous in vitro assays (Visentin et al. 2006). Interestingly, LT1009 (sonepcizumab, Lpath Inc.) is currently in phase I/II clinical trials as an anticancer drug (Sabbadini 2011).

11 Conclusion and Future Perspectives

Recent developments in sphingolipid biology, especially the discovery of the roles of SK/S1P/S1PR2 signaling in the regulation of cell proliferation, angiogenesis, drug resistance, and metastasis, have added to our understanding of these processes in various cancers. Importantly emerging evidence suggests that sphingolipids have various functions based on their subcellular localization, relative distribution in tissues, fatty acyl chain-length specificities, and their direct downstream targets via lipid-protein binding. Importantly, chain length-specific ceramides were found to be altered in cancers; thus—from a therapeutic perspective—reconstitution of ceramide generation by use of small molecule inhibitors or ceramide analogs/mimetics could be effective anticancer techniques. In contrast, SK and S1P were elevated in various cancers and inhibitors; antagonists and monoclonal antibodies against SK/S1P/S1PR signaling might hold promise for decreasing cancer growth, proliferation, and metastasis. Recent identification of SPNS2 as a novel S1P transporter (Kawahara et al. 2009) will help delineate the mechanism of S1P transport from various cell types, altering systemic S1P accumulation, which then plays a role in inducing tumor metastasis (Ponnusamy et al., 2012). Moreover, identification of the S1PR1 crystal structure can point towards more potent antagonists of S1PR-specific compounds to mediate anticancer functions (Hanson et al. 2012). Although recent research in sphingolipid metabolism and biology has yielded significant mechanistic information towards cancer pathogenesis and therapeutics, limitations exist regarding our understanding of their roles in varying cancer subtypes, subcellular compartmentalization, and direct downstream targets, which often yield context-dependent effects in response to changes in their generation/accumulation. Moreover, the inherent obstacle in studying membrane-bound enzymes and their lipid products involved in metabolism must be overcome to identify molecular and structural details of their functions in the regulation of cancer growth, proliferation, metastasis, and for the development of novel anticancer therapeutics.

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