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Targeting Sphingosine Kinases for the Treatment of Cancer

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Abstract

Sphingosine kinases (SK1 and SK2) are key, druggable targets within the sphingolipid metabolism pathway that promote tumor growth and pathologic inflammation. A variety of isozyme-selective and dual inhibitors of SK1 and SK2 have been described in the literature, and at least one compound has reached clinical testing in cancer patients. In this chapter, we will review the rationale for targeting SKs and summarize the preclinical and emerging clinical data for ABC294640 as the first-in-class selective inhibitor of SK2.

1. POTENTIAL DRUG TARGETS IN THE SPHINGOLIPID METABOLISM PATHWAY

Sphingolipid metabolism is a key pathway in cancer biology (Hait & Maiti, 2017; Lee & Kolesnick, 2017; Newton, Lima, Maceyka, & Spiegel, 2015; Ogretmen, 2018; Siddique, Li, Chaurasia, Kaddai, & Summers, 2015), in which ceramides, sphingosine, and sphingosine 1-phosphate (S1P) regulate tumor cell death, proliferation, and drug resistance, as well as host angiogenesis, inflammation, and immunity. As indicated in Fig. 1, ceramide is produced by the hydrolysis of sphingomyelin in response to several growth stimulatory (e.g., growth factors and oncoproteins) and inflammatory (e.g., cytokines and radiation) signals. Alternately, ceramide can be synthesized de novo proceeding through the precursor dihydroceramide, which is converted into ceramide by dihydroceramide desaturase (DES1). Ceramide induces apoptosis in tumor cells without disrupting quiescent normal cells (Kolesnick & Fuks, 2003). Ceramide is hydrolyzed by ceramidases to produce sphingosine, which is phosphorylated by sphingosine kinases (SK1 and SK2) to produce S1P. S1P is dephosphorylated by S1P phosphatase 1 and 2 and degraded by S1P lyase, which cleaves S1P yielding phosphoethanolamine and hexadecenal. In addition to intracellular targets, S1P binds to and activates a family of G protein-coupled receptors, i.e., S1P-receptor 1–5 (S1PR1–5), which mediate at least some of the biological activities of this lipid.

Studies in many cancer cell lines indicate that S1P induces proliferation and protects against ceramide-induced apoptosis. Therefore, a critical balance, i.e., a ceramide/S1P rheostat, has

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been hypothesized to determine the fate of tumor cells (Spiegel & Milstien, 2002). Sphingolipids also regulate the sensitivities of tumor cells to anticancer drugs (Hendrich & Michalak, 2003; Sietsma, Veldman, & Kok, 2001). For example, ceramide increases apoptosis induced by paclitaxel (Lucci, Han, Liu, Giuliano, & Cabot, 1999), etoposide (Perry & Kolesnick, 2003), and gemcitabine (Guillermet-Guibert et al., 2009; Modrak, Cardillo, Newsome, Goldenberg, & Gold, 2004). Therefore, inhibition of ceramidase or SK is expected to increase tumor chemosensitivity by elevating ceramide levels in the cells. In addition to their direct effects on tumor cells, SKs regulate deleterious inflammation from cytokines such as tumor necrosis factor-alpha (TNFα) and IL-6 (Aoki, Aoki, Ramanathan, Hait, & Takabe, 2016; Chiurchiu, Leuti, & Maccarrone, 2018; Gomez-Munoz et al., 2016; Pettus, Chalfant, & Hannun, 2004; Snider, Orr Gandy, & Obeid, 2010). In particular, S1P is critical for the activation of granulocytes that escalate inflammatory processes in many cancers, especially during chemo- or radiotherapy. Therefore, manipulation of sphingolipid metabolism to elevate ceramide levels and/or to reduce S1P production is an increasingly important approach to the treatment of hyperproliferative and inflammatory diseases, including cancers.

Among the enzymes and receptors that metabolize or interact with sphingolipids, most drug development efforts have focused on inhibition of ceramidases, SKs, or S1PRs. Recent reviews discuss the roles and pharmacology of ceramidases in detail (Coant, Sakamoto, Mao, & Hannun, 2017; Saied & Arenz, 2016; Tan, Pearson, Feith, & Loughran, 2017). Additionally, S1PR biology and a diverse set of compounds that modulate S1PR signaling have been well discussed in several recent reviews (Hait & Maiti, 2017; Huwiler & Zangemeister-Wittke, 2017; Juif, Kraehenbuehl & Dingemanse, 2016; Mao-Draayer, Sarazin, Fox, & Schiopu, 2017; Patmanathan, Wang, Yap, Herr, & Paterson, 2017; Pyne, El Buri, Adams, & Pyne, 2017). Similarly, a number of excellent recent publications describe the molecular properties and functions of SKs (Haddadi, Lin, Simpson, Nassif, & McGowan, 2017; Pyne, Adams, & Pyne, 2016; Siow & Wattenberg, 2011; Song, Zhou, & Sheng, 2017) and provide comprehensive reviews of SK inhibitors (Aurelio et al., 2016; Cao et al., 2018; Hatoum, Haddadi, Lin, Nassif, & McGowan, 2017; Lynch, Thorpe, & Santos, 2016; Pitman, Costabile, & Pitson, 2016; Plano, Amin, & Sharma, 2014; Pyne, Adams, & Pyne, 2017; Pyne, Bittman, & Pyne, 2011; Sanllehi, Abad, Casas, & Delgado, 2016; Santos & Lynch, 2015). This chapter will not duplicate these contributions but rather will discuss some of the key issues that spotlight the potential utility of inhibiting SK activity in cancer patients and describe the preclinical and early clinical data relating to ABC294640, which is the first SK2-targeted drug to reach clinical testing in cancer patients.

2. SPHINGOSINE KINASES AS TARGETS FOR ANTICANCER DRUGS

SKs are important new targets for anticancer drugs for several reasons. First, conversion of sphingosine into S1P is a key site for manipulation of the ceramide/S1P rheostat that regulates tumor cell proliferation and death. Second, the production of S1P in response to inflammatory cytokines is dependent on SK activity (Billich et al., 2005; Hanna et al., 2001; Maines et al., 2008; Mastrandrea, Sessanna, & Laychock, 2005; Nayak et al., 2010; Radeff-Huang et al., 2007; Snider et al., 2010; Xia et al., 1998), typically through nuclear factor-kappa B (NF-kB) (Xia et al., 1998). Because inflammation is a driving force in many types

of cancer, suppression of patient systemic or local inflammation is expected to reduce tumor growth. Finally, sphingolipids are likely to play central roles in regulating tumor immunology, although this potential has been sparsely addressed to date (Molino, Tate, McKillop, & Medin, 2017; Rodriguez et al., 2016; Sensken, Nagarajan, Bode, & Graler, 2011). In particular, we hypothesize that inhibition of SK activity will alter signaling through the PD-1/PD-L1 pathway such that the host immune response to the cancer is improved. This postulate is based on the observations that PD-L1 expression in tumor cells is induced by IFN-γ (Cheng et al., 2007; Dondero et al., 2016; Iwai et al., 2002; Muhlbauer et al., 2006), and IFN-γ signaling is dependent on sphingolipid metabolism (Bajwa et al., 2017; Ottenlinger et al., 2016; Seo, Alexander, & Hahm, 2011; Wakita, Nishimura, Tokura, Furukawa, & Takigawa, 1996). Furthermore, Akt- (Abdelhamed, Ogura, Yokoyama, Saiki, & Hayakawa, 2016; Atefi et al., 2014; Dong et al., 2016; Lastwika et al., 2016; Song et al., 2013; Yang et al., 2017; Zhao et al., 2017), NF-kB- (Gowrishankar et al., 2015), and TNFα- (Wang et al., 2017) signaling induce PD-L1 expression, and all of these pathways are regulated by sphingolipid signaling. Therefore, manipulation of tumor immunology using SK inhibitors is an unexplored potential new approach to improved cancer therapy.

2.1 Sphingosine Kinase 1-Derived S1P

SK1 is activated by numerous growth factors and cytokines including epidermal growth factor (EGF), platelet-derived growth factor, vascular endothelial growth factor, and TNFα (Yester, Tizazu, Harikumar, & Kordula, 2011), and by cross-linking of the high affinity receptor for IgE (FcεRI). Receptor stimulation results in ERK1/2-mediated phosphorylation of Ser225 of SK1, thereby increasing its activity and allowing its translocation to the plasma membrane (Pitson et al., 2003). Calcium and integrin-binding protein 1 bind the activated SK1 and facilitate this translocation (Jarman, Moretti, Zebol, & Pitson, 2010) to the plasma membrane where it interacts with phosphatidylserine (Stahelin et al., 2005). The S1P produced there can be exported from the cell through ATP-binding cassette (ABC) transporters where it can act as an extracellular autocrine or paracrine signaling lipid. S1P binds five different G protein-coupled receptors named S1PR1–5.

S1PR1 couples exclusively to $G\alpha_i$ eliciting a number of responses. Importantly, signaling through S1PR1 is required for lymphocyte egress from lymph nodes and inhibition of SIPR1 function by FTY720 (fingolimod, Gilenya) via internalization sequesters lymphocytes in the lymphoid organs (Matloubian et al., 2004) and provides therapeutic benefit to patients with multiple sclerosis. Cross-linking of FcεRI in response to binding of IgE activates SK1 leading to autocrine signaling of S1P through both S1PR1 and S1PR2. Signaling through S1PR1 in response to FcεRI cross-linking leads to cytoskeleton rearrangement and migration toward the impetus (Jolly et al., 2004).

S1PR2 couples to multiple Gα subunits, specifically i, q, and 12/13 (Windh et al., 1999). S1PR2 has frequently been associated with vascularization, and S1PR2 null mice exhibit disturbances in the stria vascularis, which forms the vasculature of the inner ear (Kono et al., 2007). S1PR2 was linked to pathological angiogenesis of the eye following ischemic injury by inhibiting the production of endothelial nitric oxide synthetase (eNOS) and promoting the expression of cyclooxygenase-2, thus driving the inflammatory process (Skoura et al., 2007).

S1PR2 has been linked to inflammatory processes in other ways as well. Signaling through S1PR2 leads to mast cell degranulation, which leads to anaphylaxis and resultant pulmonary edema in mice. Pretreatment of mice with an S1PR2 antagonist, JTE-013, drastically attenuated anaphylaxis in response to stimuli due to an inhibition of mast cell degranulation (Oskeritzian et al., 2010). Conversely, S1PR2 also seems to be important for recovery from anaphylaxis. SK1 null mice and S1PR2 null mice have delayed clearance of histamine following anaphylaxis. In addition, they had severe hypotension during that time indicating that SK1-derived S1P signaling through S1PR2 impacts blood pressure control and recovery from anaphylaxis (Olivera et al., 2010).

Like S1PR2, S1PR3 couples to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$. Activation of S1PR3 in response to the glucocorticoid dexamethasone in human fibroblasts results in activation of the PI3K/Akt prosurvival pathway, as well as inhibiting apoptosis through increased expression of the antiapoptotic Bcl-2 member Bcl-xL (Nieuwenhuis, Luth, & Kleuser, 2010). Like the other S1P receptors, S1PR3 has been implicated in the inflammatory process. S1P is released from the cell in response to signaling through the protease-activated receptor 1, and this leads to amplification of proinflammatory signaling through S1PR3 (Niessen et al., 2008). S1P signaling through S1PR3 has also been shown to impact vasoconstriction by increasing intracellular Ca^{2+} concentrations and signaling through Rho kinase in human coronary artery smooth muscle cells (Murakami et al., 2010).

S1PR4 couples to $G\alpha_i$ and $G\alpha_{12/13}$ in response to S1P. Less is known about S1PR4, but unlike the other S1P receptors, it has been shown to have immunosuppressive effects. Specifically, signaling through S1PR4 was shown to inhibit the proliferation of T cells and their secretion of effector cytokines while stimulating the release of IL-10, a suppressive cytokine (Wang, Graeler, & Goetzl, 2005).

S1PR5, like S1PR4, couples to both $G\alpha_i$ and $G\alpha_{12/13}$ in response to S1P. S1P signaling through S1PR5 was shown to block the migration of oligodendrocyte precursor cells suggesting that S1P is important in cellular communication events involved in brain development (Novgorodov, El-Alwani, Bielawski, Obeid, & Gudz, 2007). Signaling through S1PR5 also inhibits the motility of esophageal cancer cells (Hu et al., 2010). Conversely, signaling through S1PR5 has been shown to be necessary for the egress of natural killer cells from the bone marrow (Mayol, Biajoux, Marvel, Balabanian, & Walzer, 2011). Furthermore, S1P signaling through S1PR5 has been shown to trigger endoplasmic reticulum (ER) stress in the PC3 prostate cancer cell line leading to autophagic cell death (Huang et al., 2014).

SK1-derived S1P also has distinct intracellular functions. TNFa binding to TNF receptor 1 recruits TNF-associated factor 2 (TRAF2) to the intracellular domain of the receptor. TRAF2 can then bind SK1. The S1P produced by SK1 acts as a cofactor for TRAF2 activating its E3 ubiquitin ligase function. TRAF2 mediates the K63 polyubiquitination of receptor interacting protein 1 (RIP1). The polyubiquitination of RIP1 serves as a scaffold for TGFβ-activated kinase 1 and the IkB kinase complex allowing for the activation of NF-kB (Spiegel & Milstien, 2011). Interestingly, TNFα has also been shown to induce the degradation of SK1 in a cathepsin B-dependent manner (Taha et al., 2005).

2.2 Sphingosine Kinase 2-Derived S1P

Like SK1, SK2 catalyzes the phosphorylation of sphingosine; however, SK2 can also phosphorylate two additional sphingoid bases: dihydrosphingosine and phytosphingosine (Gault, Obeid, & Hannun, 2010). Mice with genetic deletion of SK1 ($SK1^{-/-}$) or SK2 ($SK2^{-/-}$) develop normally indicating that the isozymes are able to compensate for one another to some degree. However, knocking out both genes simultaneously is embryonic lethal (Mizugishi et al., 2005). There are five reported splice variants of SK2, and SK2 has been reported to localize to several subcellular compartments including the cytosol (Okada et al., 2005), the ER (Maceyka et al., 2005), and the mitochondria (Strub et al., 2011). However, the primary localization seems to be the nucleus due to a nuclear localization sequence (NLS) within SK2 (Igarashi et al., 2003). Because of the differences in subcellular localization of SK1 and SK2, pools of S1P produced by SK1 and SK2 are likely to have distinct biological functions.

SK2 has been reported to paradoxically inhibit or promote cell growth. SK2 was first reported to suppress growth and promote apoptosis when it was overexpressed. This activity is attributed to a putative BH3 domain within SK2 that is absent in SK1 and which can bind with Bcl-xL preventing its suppression of proapoptotic Bcl-2 family members such as Bax and Bak (Liu et al., 2003). Mutation of a single leucine residue in the BH3 domain of SK2 drastically decreased apoptosis. Alternately, knockdown of SK2 also results in increased apoptosis in glioblastoma cells (Van Brocklyn et al., 2005), colorectal carcinoma cells, and MCF7 breast cancer cells (Sankala et al., 2007). These seemingly conflicting effects are likely attributed to artifacts from the overexpression of SK2 thereby disrupting the regulation of apoptosis and the subcellular distribution of S1P (Sankala et al., 2007).

Like SK1, SK2 is activated by phosphorylation by Erk1/2 (Hait, Bellamy, Milstien, Kordula, & Spiegel, 2007). In the mitochondria, SK2-derived S1P binds to prohibitin 2, which regulates the assembly of complex IV (cytochrome-c oxidase) of the electron transport chain (Strub et al., 2011). S1P derived from SK2 has also been associated with the formation of amyloid plaques in Alzheimer's disease. The major component of these plaques, amyloid-β, is a cleavage product of the amyloid-β precursor protein (APP). Cleavage of APP is carried out by the β-secretase β-site APP cleaving enzyme 1 (BACE1). SK2-derived S1P has been shown to bind the transmembrane domain of BACE1, which allosterically modifies the catalytic domain increasing its activity (Takasugi et al., 2011). Thus, overexpression of SK2 activity could contribute to Alzheimer's disease.

Caspase 1-mediated cleavage of SK2 in cells undergoing apoptosis allows for the translocation of a still catalytically active fragment of SK2 to the plasma membrane where it binds phosphatidylserine and mediates the phosphorylation of sphingosine (Weigert et al., 2010). As with SK1-derived S1P, this S1P can be exported from the cell and serve as a chemoattractant for monocytes and macrophages by interacting with S1P receptors (Gude et al., 2008).

The unique nuclear localization of SK2 provides it with a role in epigenetic regulation of gene expression. For example, SK2 has been shown to bind histone H3 and impact its acetylation in the MCF7 breast cancer cell line (Hait et al., 2009). siRNA knockdown of

SK2 was reported to decrease the acetylation of H3–K9, H4–K5, and H2B–K12, and addition of S1P to isolated nuclei of siSK2 cells resulted in restoration of acetylation of these three residues. This same study showed that S1P and dihydro-S1P were able to inhibit histone deacetylases 1 and 2, and that siRNA knockdown of SK2 enhanced histone deacetylase (HDAC) activity. Additionally, the authors showed that activation of SK2 by phorbol 12-myristate 13-acetate (PMA, a potent activator of protein kinase C, upstream of ERK1/2-medi-ated SK2 phosphorylation) enhanced the colocalization of SK2 with HDAC1. PMA is a known inducer of the transcription of the cyclin-dependent kinase inhibitor p21 and the transcription factor c-fos. When combined with siRNA knockdown of SK2, PMA failed to induce the transcription of p21 and c-fos. This correlated with a reduction in acetylation of H3 associated with the promoters of p21 and c-fos (Hait et al., 2009). This agreed with this lab's earlier findings showing knockdown of SK2 prevented doxycycline-induced p21 expression (Sankala et al., 2007). These results are supported by Igarashi et al. who reported that overexpression of SK2 or SK1 with a fused NLS, but not native SK1, causes an inhibition of DNA synthesis (Igarashi et al., 2003). SK2-derived S1P has, thus, been demonstrated to impact gene transcription through regulation of HDAC activity.

Studies of the role of SK2 in carcinogenesis have also provided discordant results, with some research groups finding SK2 to be a tumor suppressor and others a mediator of tumor growth. For example, treatment of $SK2^{-/-}$mice with the carcinogen azoxymethane and the inflammation-inducing toxin dextran sodium sulfate was reported to result in more numerous, larger, and more aggressive tumors than wild-type mice receiving the same treatments. This was reported to coincide with an increase in STAT3, NF-kB, and IL-6 expression in hematopoietic cells of the knockout mice, which drove the colitis and associated cancer. Conversely, SK2 has been described as oncogenic in acute lymphoblastic leukemia (ALL) because knockdown of SK2 or pharmacological inhibition using the inhibitor ABC294640 (discussed below) both attenuated development of leukemia in ALL mouse models (Wallington-Beddoe et al., 2014). This was attributed to a reduction in the expression of c-Myc (and thereby c-Myc target genes) by way of reduced acetylation levels of H3, linking SK2 to the expression this important oncogene. This same study also showed the importance of SK2 in the development of ALL. When BCR/ABL-transduced B-cell progenitor cells were implanted in 29 sublethally irradiated mice, 22 developed ALL with a median survival of 42 days. When the same BCR/ABL translocation was introduced to $SK2^{-/-}$cells, which were then implanted in 29 sublethally irradiated mice, only 16 developed ALL and these experienced a median survival of 58 days. Cells recovered from the $SK2^{-/-}$mice had reduced c-Myc expression compared with their wild-type counterparts (Wallington-Beddoe et al., 2014). The expression of SK2 was linked to actin rearrangement in MCF7 cells with expression promoting a structural rearrangement of actin into membrane ruffles/lamellipodia indicative of a more migratory phenotype (Lim, Sun, Bittman, Pyne, & Pyne, 2011). SK2 expression was also shown to negatively correlate with disease-free survival and overall survival in nonsmall cell lung cancer (NSCLC) patients (Wang et al., 2014). Additionally, inhibition of SK2 has delayed tumor growth in mouse xenograft models of pancreatic, kidney, liver, and colon cancers (Beljanski, Knaak, Zhuang, & Smith, 2011; Beljanski, Lewis, & Smith, 2011; Chumanevich et al., 2010).

Overall, the propensity of data indicates that SK2 is a key mediator of enhanced growth of cancer. It is likely that studies using $SK2^{-/-}$mice do not reflect the normal biology of the critical SK node and clearly do not mimic the pharmacologic inhibition of SK2 because of the absence of the protein, which in and of itself is proapoptotic due to its endogenous BH3 domain. Similarly, studies in which SK2 is overexpressed by transfection are compromised by inappropriate subcellular distribution of SK2 (and its S1P product) and excessive proapoptotic pressure from the elevated presence of BH3 domains.

3. SPHINGOSINE KINASE INHIBITORS

Because of the importance of S1P in growth signaling, a great deal of research has been done with the aim of inhibiting S1P signaling. Numerous therapeutic strategies have been developed that target either one or both SKs, as well as those that inhibit S1PR signaling. As indicated above, these SK inhibitors have been extensively reviewed elsewhere, so only a few compounds are highlighted here. The earliest example of an SK inhibitor is N,N-dimethylsphingosine (DMS), a naturally occurring N-methyl derivative of sphingosine that inhibits both SK1 and SK2 with K_is of ~ 16 and ~ 14 μM, respectively (Gao, Peterson, Smith, & Smith, 2012). Dimethylsphingosine inhibits SK activity in human platelets preventing platelet aggregation in response to exogenous S1P (Yatomi et al., 1996) and induces apoptosis in leukemic and colonic carcinoma cell lines (Jendiroba, Klostergaard, Keyhani, Pagliaro, & Freireich, 2002; Sweeney et al., 1996). Two additional sphingosine analogs, L-*threo*-dihydrosphingosine and N,N,N-trimethyl-sphingosine, have also been described as SK inhibitors; however, they also inhibit protein kinase C, which may be responsible for the observed phenotype (Canals & Hannun, 2013). The first nonlipid SK inhibitors were described in 2003, and the compound called SKI-II in particular has been widely used to probe SK involvement in many cell processes (French et al., 2003). SKI-II inhibits both isoforms with K_is of 16 μM (SK1) and 8 μM (SK2) (Gao et al., 2012), but it has also been shown to inhibit DES1 (Cingolani et al., 2014). Treatment of prostate cancer and breast cancer cells with SKI-II induces apoptosis in vitro (Antoon, Meacham, et al., 2011; Leroux et al., 2007) and in vivo (French et al., 2006). The compound PF-543, which is over 100-fold more selective for SK1 than SK2, has a K_i of 3.6 nM for SK1 (Schnute et al., 2012). Though it does not affect the growth of 1483 HNSCC cells, it was able to inhibit the formation of S1P in human whole blood ex vivo by >90% compared to control (Schnute et al., 2012). SK1-I is a specific competitive inhibitor of the ATP-binding site of SK1 with a K_i of 10 mM that has been shown to induce apoptosis in the U937 and Jurkat leukemic cell lines (Paugh et al., 2008). Treatment of these cells with SK1-I decreased ERK1/2 and Akt phosphorylation. Additionally, treatment of AML xenograft bearing mice with SK1-I reduced tumor growth (Paugh et al., 2008). SK1-I also decreased Akt activation and increased apoptosis in glioblastoma cell lines and slowed glioblastoma xenograft growth (Kapitonov et al., 2009). Importantly, in a murine model of breast cancer, treatment with SK1-I reduced metastasis and decreased overall tumor burden (Nagahashi et al., 2012), which was attributed to diminished angiogenesis caused by the suppression of circulating S1P. FTY720 is a structural analog of sphingosine. Following its phosphorylation by SK2, the resultant phospho-FTY720 can inhibit SK1 or exit the cell via ABC transporters to bind S1P receptors (except S1PR2). Binding results in the internalization of the receptor and

prolonged receptor downregulation muting extracellular S1P signaling (Nagahashi et al., 2014). Prevention of S1P receptor signaling, mainly that of S1PR1, has a dramatic clinical impact in preventing the release of lymphocytes from lymphoid tissue thereby attenuating the autoimmune response responsible for multiple sclerosis (Allende, Dreier, Mandala, & Proia, 2004; Nagahashi et al., 2014). An analog, (R)-FTY720 methyl ester, was reported to have specific inhibitory activity against SK2, which prevented actin rearrangement in MCF7 cells in response to S1P thereby inhibiting motility (Lim et al., 2011).

3.1 Preclinical Development of ABC294640, the First-in-Class Inhibitor of SK2

Developed by Apogee Biotechnology Corporation, 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide (ABC294640, Fig. 2) is an inhibitor of SK2 that is competitive with respect to sphingosine and therefore does not have off-target effects on protein kinases (French et al., 2010; Gao et al., 2012). In tissue culture, ABC294640 reduces S1P levels (French et al., 2010; Gao et al., 2012; Maines, French, Wolpert, Antonetti, & Smith, 2006; Maines et al., 2008), suppresses the proliferation of many tumor cell lines, and inhibits tumor cell migration concomitant with loss of microfilaments. ABC294640 depletes S1P and elevates ceramide in tumor cells, suppresses signaling through pERK, pAkt, and NF-kB, and promotes autophagy and/or apoptosis in a broad range of cancer cells (Antoon, White, et al., 2011; Beljanski, Knaak, & Smith, 2010; Ding et al., 2016; French et al., 2010; Gao et al., 2012; Qin et al., 2014; Schrecengost, Keller, Schiewer, Knudsen, & Smith, 2015). Importantly, ABC294640 downregulates the expression of c-Myc in a variety of tumor cell lines (Lewis, Voelkel-Johnson, & Smith, 2016; Schrecengost et al., 2015; Venant et al., 2015; Venkata et al., 2014; Wallington-Beddoe et al., 2014) and also reduces androgen receptor expression in prostate cancer cells (Schrecengost et al., 2015; Venant et al., 2015). In addition to SK2, ABC294640 inhibits DES1, which accounts for the marked increases in dihydroceramides in cells treated with the drug (French et al., 2010; Venant et al., 2015). ABC294640 is synergistically cytotoxic with gemcitabine toward pancreatic cancer cell lines and results in decreased expression of both RRM2 and MYC, decreased S780 phosphorylation of Rb, and increased acetylation of H3-K9 and p21 (Lewis et al., 2016). The reduction in Rb phosphorylation is consistent with studies by Kraveka et al. who demonstrated that inhibition of DES1 is associated with reduction of Rb phosphorylation and suppression of cell cycle progression (Kraveka et al., 2007). Fig. 2 describes the current model in which ABC294640 dually inhibits SK2 and DES1 resulting in inhibition of multiple signaling pathways that promote tumor growth.

As summarized below, ABC294640 has therapeutic activity in diverse mouse tumor models both alone and in combination with other anticancer drugs (Antoon, White, Driver, Burow, & Beckman, 2012; Antoon et al., 2010; Antoon, White, et al., 2011; Beljanski et al., 2010; Beljanski, Knaak, et al., 2011; Beljanski, Lewis, et al., 2011; Dai et al., 2017; Dai, Smith, Foroozesh, Miele, & Qin, 2018; French et al., 2010; Qin et al., 2014; Schrecengost et al., 2015; Venant et al., 2015; Venkata et al., 2014; Wallington-Beddoe et al., 2014; Xu et al., 2018; Xun et al., 2015; Zhou, Chen, & Yu, 2018), as well as a number of rodent inflammation models (Fitzpatrick, Green, Frauenhoffer, et al., 2011; Fitzpatrick, Green, Maines, & Smith, 2011; Liu et al., 2012; Maines et al., 2008; Maines, Fitzpatrick, Green, Zhuang, & Smith, 2010; Maines et al., 2006; Poti et al., 2012; Shi et al., 2012) not discussed

here. Additionally, ABC294640 diminished tumor incidence and multiplicity in the azoxymethane/dextran sulfate sodium model of colon carcinogenesis (Chumanevich et al., 2010). Antitumor activity is associated with accumulation of ABC294640 in the tumors, reduction of tumor S1P levels, and induction of apoptosis (French et al., 2010). Intratumoral concentrations of ABC294640 can exceed the IC_{50} for tumor cell cytotoxicity by as much as 25-fold (French et al., 2010), and ABC294640 reduces plasma S1P levels ~ 50% at therapeutically efficacious doses (Beljanski, Lewis, et al., 2011). Preclinical studies demonstrated an excellent oral bioavailability and safety profile for ABC294640, with no hematologic or major organ toxicity (French et al., 2010).

The in vivo anticancer activity of ABC294640 was first shown by inhibition of tumor growth in a syngeneic JC (breast cancer) xenograft model in BALB/c mice (French et al., 2010). In additional studies in breast cancer models, ABC294640 has been shown to bind to the antagonist ligand-binding domain of the estrogen receptor thereby inhibiting the growth of the estrogen receptor positive breast cancer cell line MCF7 both in vitro and in vivo (Antoon et al., 2010). ABC294640 was also effective at inducing apoptotic cell death in triple-negative MCF7 cells (MCF7-TN-R) by diminishing prosurvival signaling through the NF-kB pathway (Antoon, White, et al., 2011). In vitro levels of apoptosis were further increased by addition of either etoposide or doxorubicin after pretreatment with ABC294640. Additionally, Ki67 staining was decreased in MCF7-TN-R orthotopic tumors treated with ABC294640 when compared to control. However, ABC294640 is ineffective against the luminal, endocrine-resistant MDA-MB-361 breast cancer cell line, although the drug did reduce Bcl-2 in MCF7 tumors (Antoon et al., 2012).

A growing body of evidence indicates that ABC294640 may be particularly effective for the treatment of prostate cancer. Initially, ABC294640 was also shown to inhibit proliferation in androgen-resistant PC-3 and LNCaP prostate cancer cell lines (Gestaut, Antoon, Burow, & Beckman, 2014). Schrecengost et al. (2015) then showed that ABC294640 abrogates signaling pathways requisite for prostate cancer growth and proliferation. Of particular importance, ABC294640 treatment of early-stage and advanced prostate cancer models caused downregulation of c-Myc and androgen receptors (both full-length in LNCaP cells and the splice variant in 22Rv1 cells) expression and activity. This corresponded with significant inhibition of growth, proliferation, and cell-cycle progression. Finally, oral administration of ABC294640 was found to dramatically impede xenograft tumor growth. Studies by Venant et al. provided further confirmation of activity toward prostate cancer cells in association with decreased expression of both c-Myc and androgen receptors (Venant et al., 2015). Furthermore, that study confirmed that ABC294640 inhibits DES1 activity resulting in marked increases in dihydroceramides both in tissue culture and in xenografts. Studies by McNaughton et al. examined the effects of SKI-II and ABC294640 in androgen-independent LNCaP-AI prostate cancer cells and demonstrated that these compounds suppress both SK1 and DES1 by inducing their proteolysis (McNaughton, Pitman, Pitson, Pyne, & Pyne, 2016). Overall, the combined effects of ABC294640 on SK1, SK2, DES1, and androgen receptors in androgen-sensitive and castrate-resistant prostate cancer cells and xenografts build a strong rationale for examining the therapeutic effects of ABC294640 in prostate cancer patients.

ABC294640 has been shown to promote autophagic cell death in xenografts of A-498 (kidney cancer) cells in SCID mice as evidenced by in-creases in Beclin-1 and the cleavage of the microtubule-associated proteins 1A/1B light chain 3A (LC3). This same study demonstrated a decrease of MAPK and Akt pathway activation in response to ABC294640 in vivo (Beljanski et al., 2010). Similarly, ABC294640 caused pronounced apoptosis, cell-cycle arrest, and inhibition of NSCLC growth in vitro and in vivo (Dai et al., 2018). This is consistent with a previous report that ABC294640 enhances the antitumor effects of TRAIL in NSCLC cells, likely by enhancing the translocation of death receptor 4/5 to the cell membrane (Yang et al., 2015). Leili et al. demonstrated that ABC294640 enhances doxorubicin-stimulated apoptosis in NSCLC cells, and that this is associated with suppression of the expression of survivin (Leili, Nasser, Nadereh, Siavoush, & Pouran, 2018). Also using lung cancer cells in vitro, Guan et al. demonstrated that apoptosis induced by ABC294640 can be suppressed by overexpression of glucosylceramide synthase (GCS) or enhanced by the GCS inhibitor PDMP (Guan, Liu, Yan, & Zhou, 2016). Studies by Xun et al. using transformed and primary colorectal cancer cells demonstrated that these cells are also sensitive to growth inhibition by ABC294640 that could be amplified by combination with 5-fluorouracil and cisplatin and attenuated by cotreatment with a JNK inhibitor (Xun et al., 2015). Similarly, ABC294640 promoted apoptosis in skin squamous cell carcinoma cells in vitro and inhibited tumor growth in vivo associated with inhibition of Akt and JNK signaling (Zhou et al., 2018).

The potential use of ABC294640 in treating cancers of hematopoietic origin has also been demonstrated. Treatment of primary effusion lymphoma (PEL) cells positive for Kaposi's sarcoma-associated herpesvirus (KSHV) (BCBL-1 cell line) with ABC294640 induced dose-dependent apoptosis that corresponded with decreases in phosphorylation of ERK1/2, Akt, and NF-kB (Qin et al., 2014). When these cells were injected into NOD/SCID mice, ABC294640 treatment delayed disease progression as indicated by spleen size, weight gain, and ascites volume. ABC294640 treatment vastly attenuated the pathogenesis of $KSHV^+$ PEL (Qin et al., 2014). Mechanistically, elevation of dihydroceramides was shown to induce apoptosis in KSHV-infected PEL cells through activation of KSHV lytic gene expression (Dai et al., 2015). Finally, impressive activity of ABC294640 toward virally induced cancers both in vitro and in vivo was also demonstrated by Dai, et al. who found that KSHV long-term-infected immortalized endothelial cells are efficiently killed by ABC294640, whereas noninfected cells were not (Dai et al., 2017).

ABC294640 has anticancer activity in models of multiple myeloma (MM). Treatment of both immortalized MM cell lines and freshly isolated $CD138^+$ myeloma cells from myeloma patients resulted in inhibition of proliferation (Venkata et al., 2014). ABC294640 increased caspase 3 activation and caspase 9 cleavage in OPM1 MM cells indicative of apoptotic cell death. Treatment of MM cell lines with ABC294640 also increased mRNA and protein expression of the proapoptotic Bcl-2 family member Noxa, which is regulated by p53. The same study additionally showed that ABC294640 promotes proteosomal degradation of myeloid cell leukemia 1 (Mcl-1), a Bcl-2 family protein overexpressed in the majority of MM patients. Thus, ABC294640 shifts the balance of these apoptotic mediators in the direction of apoptosis. ABC294640 also enhanced the proteosomal degradation of c-Myc in the MM cells. Additionally, the authors demonstrated that ABC294640 induced apoptosis of

MM cells even in the presence of bone marrow stromal cells, which support the growth of MM cells. These findings prompted in vivo studies using a luciferase expressing MM.1S cell line xenograft where ABC294640 suppressed tumor growth (Venkata et al., 2014).

ABC294640 has also been evaluated in preclinical testing against ALL (Wallington-Beddoe et al., 2014). ABC294640 inhibited proliferation and induced cell death in ALL cell lines and ALL patient samples but, importantly, not bone marrow mononuclear cells. Cell death in this instance was attributed to a mixed etiology, being caspase-independent but not completely autophagic. Next, a gene expression profile of the impact of ABC294640 on gene expression was compiled, which revealed an inhibition of c-Myc and c-Myc-regulated genes. *MYC* downregulation in response to ABC294640 treatment was confirmed by qRT-PCR. This same study showed that inhibition of SK2 by ABC294640 prevented S1P-mediated inhibition of HDAC activity resulting in a reduction in the association of c-Myc with histone 3 acetylated on lysine 9 and thereby a reduction in c-Myc expression. Xenografts of ALL in NOD/SCID mice treated with ABC294640 had significantly less disease than vehicle control following 21 days of treatment (Wallington-Beddoe et al., 2014).

Combination of ABC294640 with other anticancer drugs has shown promising results. Combination of ABC294640 with sorafenib resulted in synergistic cell killing in A-498 kidney carcinoma and BxPC-3 pancreatic adenocarcinoma cells by means of apoptosis, and the combination attenuated the growth of xenografts of each cell line in SCID mice to a greater degree than either drug alone (Beljanski, Knaak, et al., 2011). This same combination proved effective in two hepatocellular carcinoma xenografts with the mode of cell death being autophagic (Beljanski, Lewis, et al., 2011). As with the A-498 and BxPC-3 cells, ABC294640 decreased the levels of p-Erk1/2 provoking the idea that ABC294640-mediated cell death could come as a result of diminished MAPK signaling. Similarly, combination of ABC294640 with sorafenib caused synergistic apoptosis of cholangiocarcinoma cells, and this was associated with marked suppression of STAT3 phosphorylation (Ding et al., 2016). Combination of ABC294640 with the microtubule stabilizer paclitaxel was shown to greatly increase caspase-9 signaling and apoptosis when compared with either drug alone (White, Chan, Antoon, & Beckman, 2013). ABC294640 has also been combined with doxorubicin, vincristine, imatinib, and bortezomib in ALL cells (Wallington-Beddoe et al., 2014). Only additive effects were observed with the DNA intercalating agent doxorubicin and the mitosis inhibitor vincristine. However, synergistic cell death was produced with the protea-some inhibitor bortezomib and the multityrosine kinase inhibitor (i.e., Bcr-Abl inhibitor) imatinib when either was combined with ABC294640 (Wallington-Beddoe et al., 2014). ABC294640 treatment of cervical carcinoma cells promoted apoptosis that was amplified by combination with the BCL-2 inhibitor GDC-0199 both in vitro and in vivo (Xu et al., 2018).

3.2 Phase I Clinical Trial of ABC294640

A phase I clinical trial of ABC294640 in patients with advanced solid tumors has been completed (Britten et al., 2017). ABC294640 was administered orally on a continuous schedule, with 28 days constituting a Cycle. Tumors were reimaged every two Cycles (8

weeks), and patients were allowed to continue receiving the drug if there was no disease progression by RECIST tumor imaging criteria. Twenty-one patients received the drug in this phase I trial. The first patient on study at 250 mg qd developed grade 4 hyperglycemia in the setting of rapidly progressing pancreatic cancer; however, no other patients experienced possibly drug-related hyperglycemia. Among the four patients enrolled at 750 mg bid, one ovarian cancer patient had dose-limiting grade 3 nausea and vomiting, and two patients were unable to complete Cycle 1 due to diverse possibly drug-related toxicities. One patient at the expanded 500 mg dose level was dose reduced at Cycle 3 due to grade 1 adverse events. The other five patients at 500 mg bid tolerated the drug well, and the 500 mg bid dose level was established as the recommended phase II dose. Across all dose levels, the most common drug-related toxicities were nausea, fatigue, vomiting, and diarrhea. In addition, 2 patients experienced psychiatric dis-orders including agitation/anxiety, mood changes, and/or hallucinations; and 5 patients experienced grade 1–2 nervous system disorders, including dizziness, dysarthria, dysgeusia, dysesthesia, memory loss, muscle spasms, paresthesia, somnolence, and/or spasticity that resolved on discontinuation of ABC294640.

Among the patients evaluable by RECIST, 1 (6%, a patient with refractory cholangiocarcinoma) had a Partial Response, 6 (38%) had Stable Disease, and 9 (56%) had Progressive Disease as their best response. Subjects included a patient with advanced hepatocellular carcinoma who lived for 10 months after completing 4 cycles; a patient with recurrent metastatic bladder cancer who received 12 cycles; and a patient with advanced cholangiocarcinoma who received 18 cycles.

Pharmacokinetic (PK) profiling was conducted on Days 1 and 28 of Cycle 1, and data from patients treated with 500 mg of ABC294640 are shown in Fig. 3. After oral dosing, plasma levels of ABC294640 typically peaked at 1–2 h, and then declined with a halftime of clearance of ~ 4 h. The peak plasma concentrations (C_{max}) and AUCs within a cohort were generally similar. Notably, the C_{max} and AUC values for most patients were not significantly different between Day 1 and Day 28, indicating a lack of metabolic adaptation to the drug. Importantly, C_{max} levels of ABC294640 in patients receiving 500 mg were in the range expected to have therapeutic activity. Specifically, antitumor activity in mouse models occurs at ABC294640 doses of 25–50 mg/kg, which results in plasma C_{max} levels of ~3.5 μg/mL (French et al., 2010). Patients receiving a 500 mg dose of ABC294640 had C_{max} levels averaging 16.4 μM (Fig. 3), with 9 of 12 profiles exceeding the threshold of 9 μM. Patients given 250 mg of ABC294640 exceeded the C_{max} threshold approximately 50% of the time, whereas all patients receiving 750 mg of ABC294640 reached this level. Because the $t_{1/2}$s for clearance are equal in mice and humans, the drug exposure in patients at 500 mg and higher dose levels is expected to be sufficient to inhibit SK2 in the tumors.

The most direct pharmacodynamic biomarkers for inhibition of SK2 and DES1 by ABC294640 are S1P and dihydroceramide levels in the tumor. However, tumor biopsies were not obtained in the phase I clinical trial, so sphingolipids in the plasma were measured as a biomarker for ABC294640. In the patients, ABC294640 treatment caused rapid decreases in plasma S1P levels with a parallel increase in plasma dihy-droC_{16}-ceramide levels (Fig. 4). In general, plasma S1P reached a minimum approximately 12 h after ABC294640 treatment and recovered to baseline by 24 h. This is consistent with the

clearance profile of the drug and supports the BID dosing schedule going forward into phase II clinical trials. The maximum S1P decreases for the 250, 500, and 750 mg cohorts were 51 ± 6% (n = 10), 46 ± 9% (n = 5), and 54 ± 14% (n = 2), respectively, indicating that ABC294640 exposures attained with the 250 mg dose are sufficient to optimally inhibit S1P generation in the patients.

4. CONCLUSIONS

Continuing research on the sphingolipid metabolism pathway is providing increasing support for targeted disruption of key enzymes in this pathway for the treatment of cancer and inflammatory diseases. Important differences in the biology and pharmacology of SK1 and SK2 have been revealed by insightful basic and translational research and provide rationale for continuing the movement of novel inhibitors into clinical testing. Ultimately, the efficacies and safety of these new drugs will reveal whether or not targeting sphingolipid metabolism provides therapeutic benefit to patients. Clinical testing of ABC294640 is continuing with trials in patients having MM, cholangiocarcinoma, or hepatocellular carcinoma currently underway.

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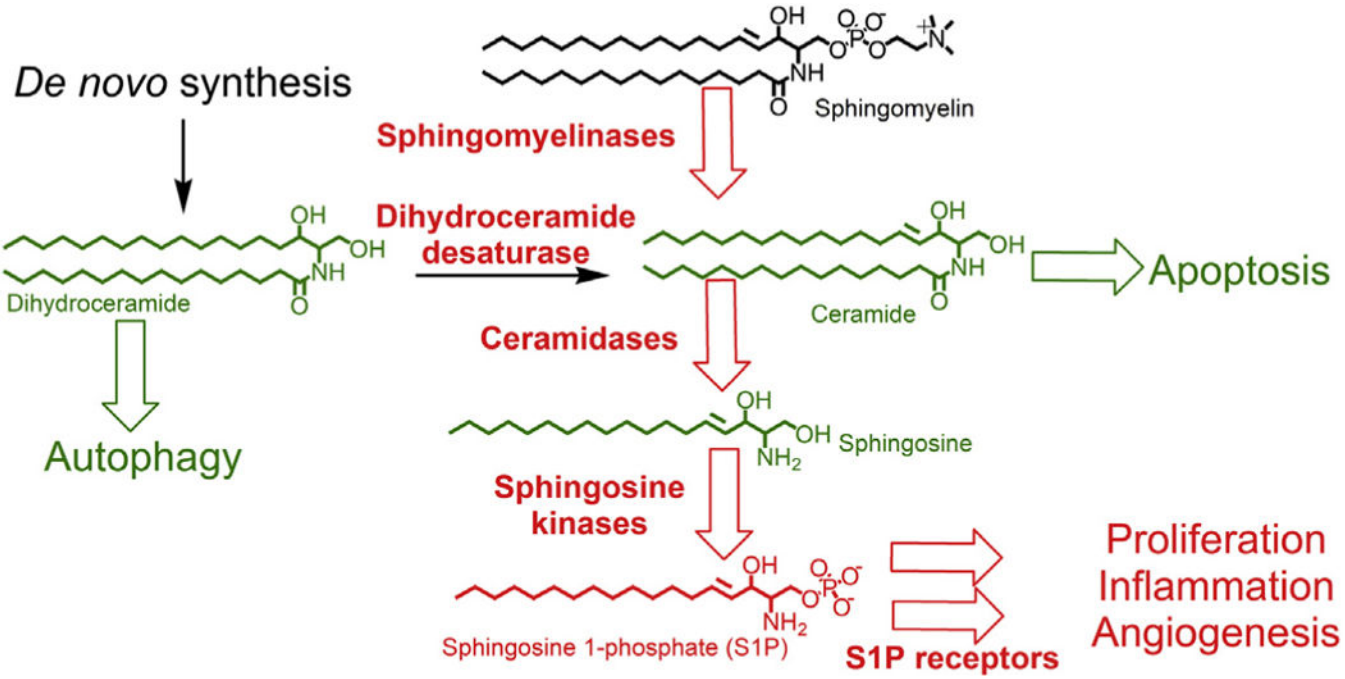

Figure 1. A simplified model of sphingolipid metabolism.

Enzymes and processes that promote tumor growth are shown in red, whereas lipids and processes that inhibit tumor growth are shown in green. Proteins that are currently under consideration as targets for new anticancer drugs include sphingomyelinases, dihydroceramide desaturase (DES1), ceramidases, sphingosine kinases, and sphingosine 1-phosphate (S1P) receptors.

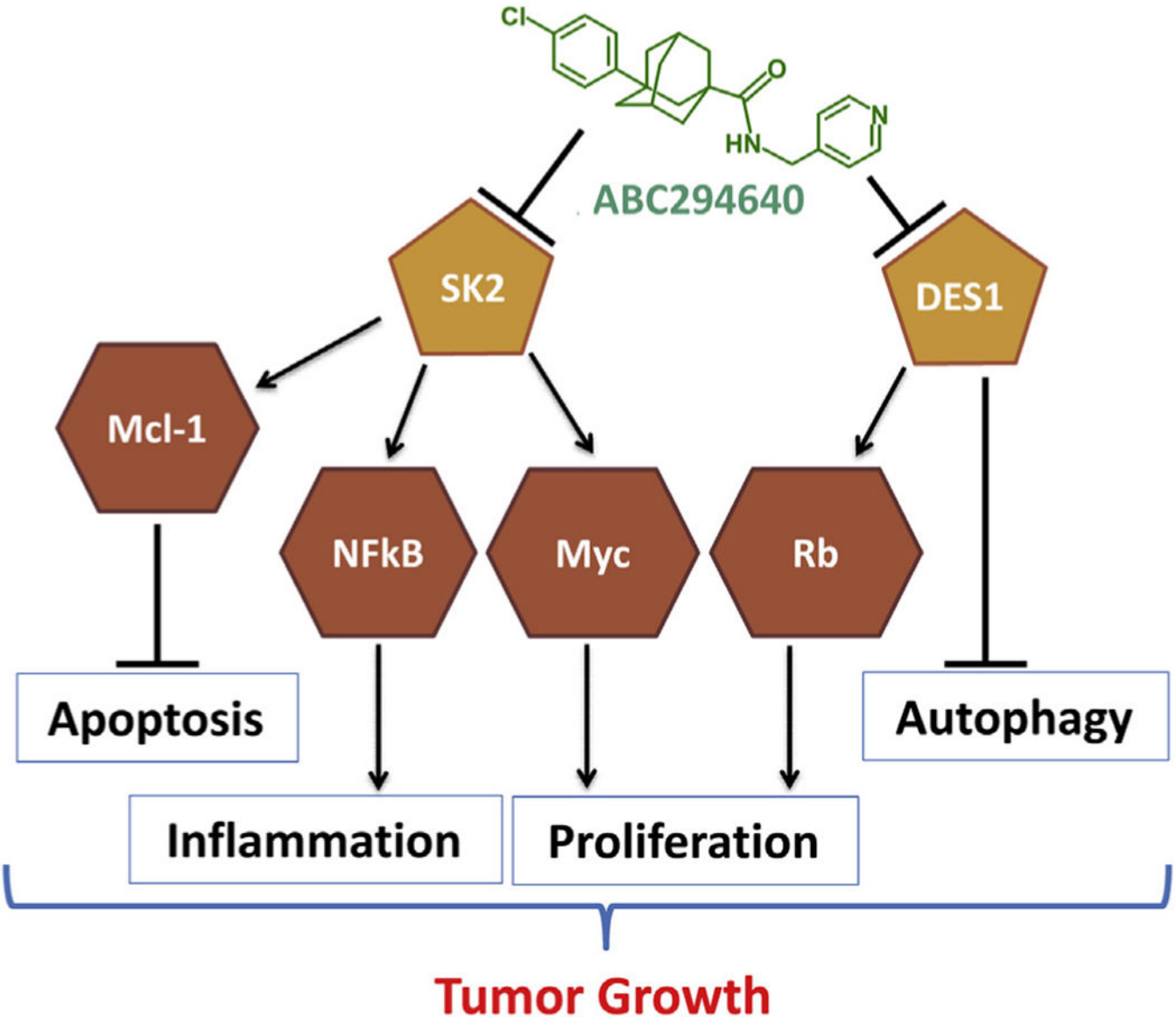

Figure 2. Mechanism for Suppression of Tumor Growth by ABC294640.

ABC294640 has similar potency toward sphingosine kinase (SK2) and dihydroceramide desaturase (DES1). Inhibition of SK2 activity causes downregulation of the expression of myeloid cell leukemia 1 (Mcl-1) and c-Myc and suppresses the activation of nuclear factor-kappa B (NF-kB), thereby promoting apoptosis and inhibiting proliferation and inflammation. In parallel, inhibition of DES1 by ABC294640 promotes autophagy and promotes Rb phosphorylation resulting in suppression of proliferation.

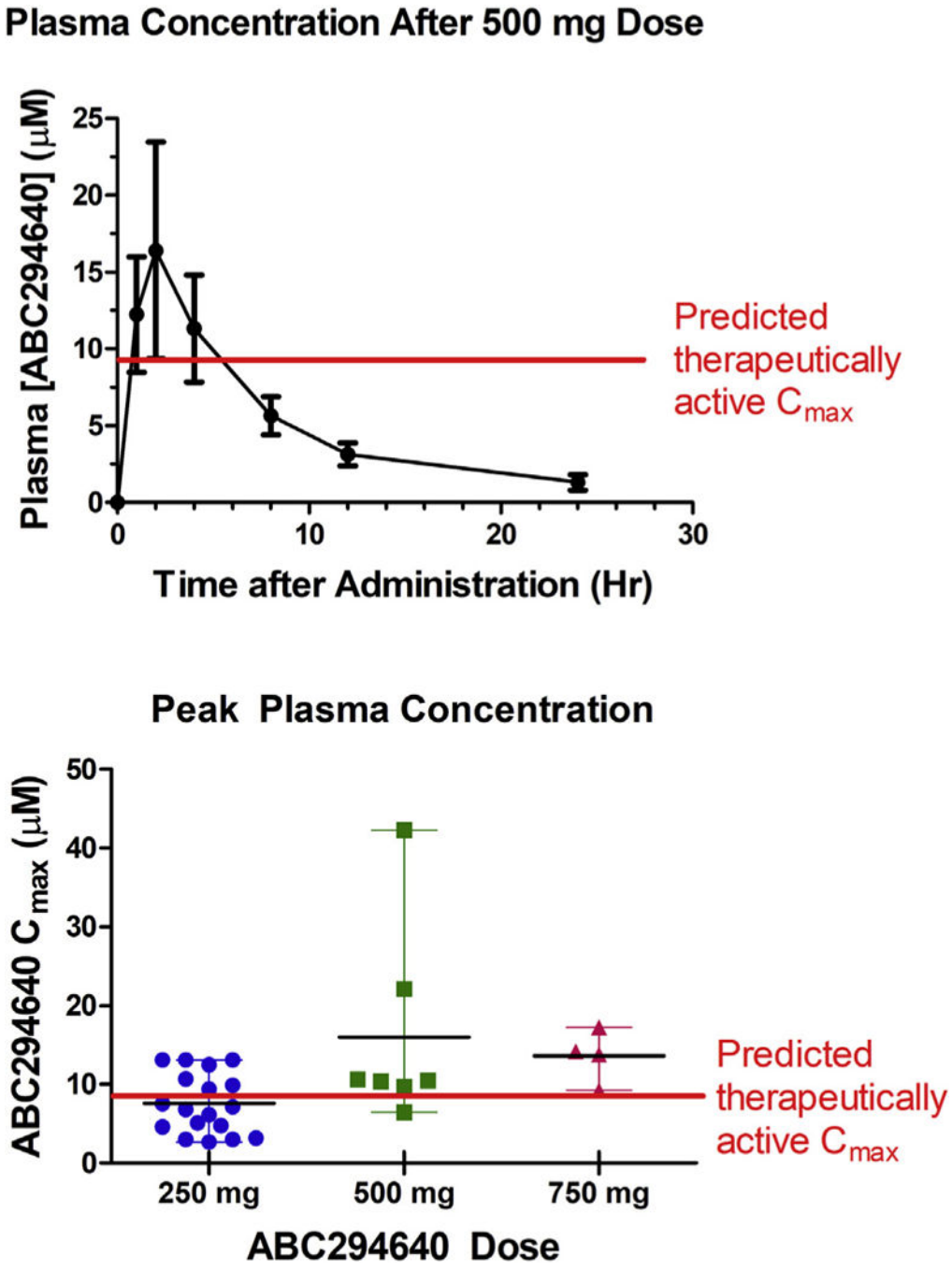

Figure 3. Pharmacokinetics of ABC294640 in humans.

Top Panel. Plasma was isolated from patients given 500 mg of ABC294640 on either Day 1 or Day 28 of Cycle 1 and levels of ABC294640 were quantified using a GLP LC/MS method. Values represent the mean ± SEM for 7 data sets. Antitumor activity in mouse models occurs at ABC294640 doses ≥ 25 mg/kg, which provides plasma C_{max} levels of 9 μM. The horizontal line indicates the C_{max} predicted to have antitumor activity based on the mouse tumor models. Bottom Panel. C_{max} values are shown for patient samples collected on either Day 1 or Day 28 (there is no change in C_{max} or AUC in this period). The horizontal

line indicates the Cmax predicted to have antitumor activity based on the mouse tumor models.

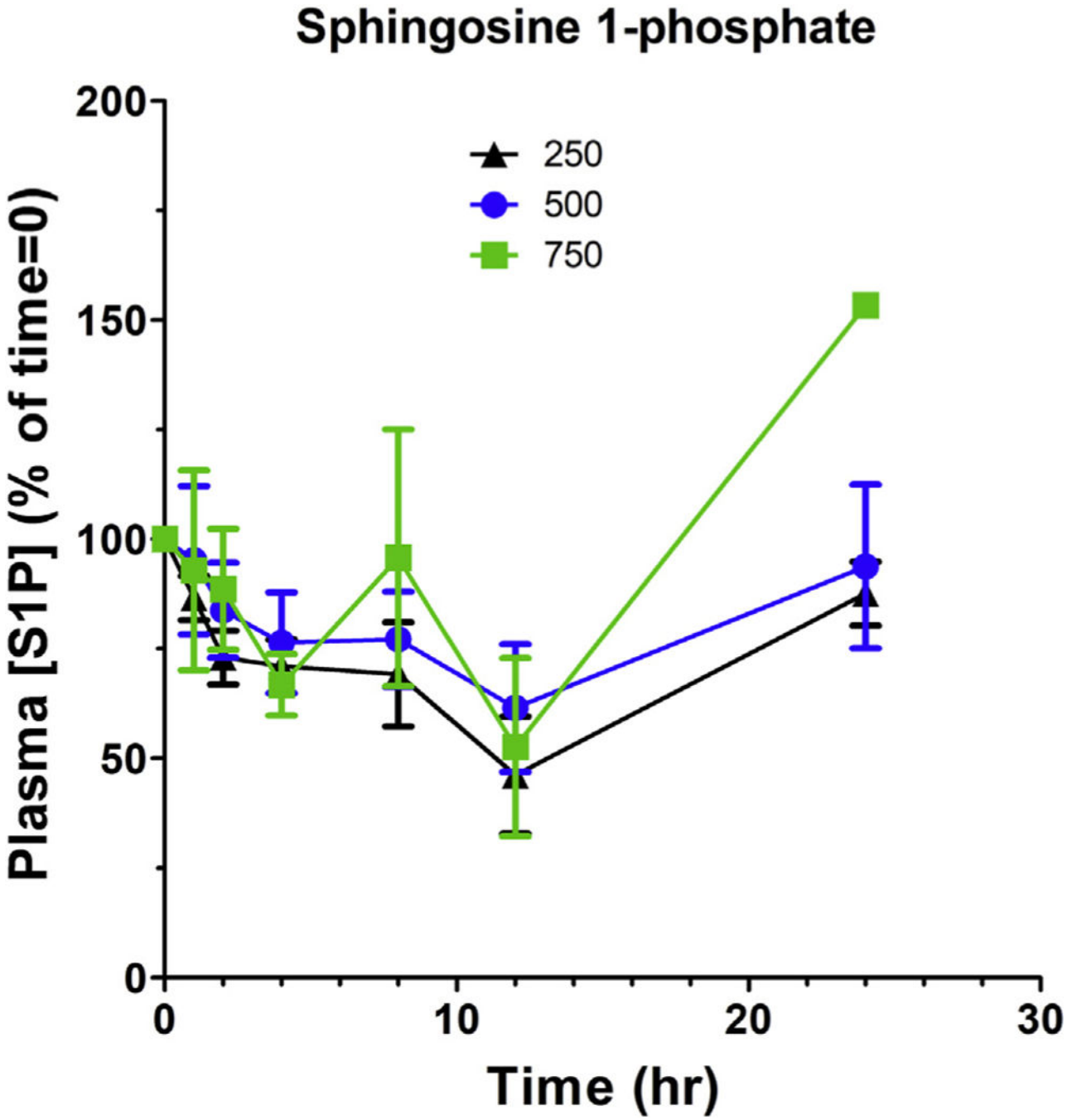

Figure 4. Effect of ABC294640 treatment on plasma sphingosine 1-phosphate (S1P) levels.

S1P concentrations were normalized to the Time 0 levels for individual patients receiving 250 mg (▲), 500 mg (●), or 750 mg (■) of ABC294640.