Targeting Sphingosine-1-Phosphate Signaling in Lung Diseases

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Abstract

Sphingosine-1- phosphate (S1P), a simple, bioactive sphingolipid metabolite, plays a key role, both intracellularly and extracellularly, in various cellular processes such as proliferation, survival, migration, inflammation, angiogenesis, and endothelial barrier integrity. The cellular S1P level is low and is tightly regulated by its synthesis and degradation. Sphingosine Kinases (SphKs) 1 and 2, catalyze the ATP-dependent phosphorylation of sphingosine to S1P, while the degradation is mediated by the reversible dephosphorylation catalyzed by the S1P phosphatases and lipid phosphate phosphatases and the irreversible degradation to hexadecenal and ethanolamine phosphate by sphingosine-1-phosphate lyase (S1PL). As a ligand for specific G-protein-coupled receptors, $S1P_{1-5}$, which are differentially expressed in different cell types, S1P generates downstream signals that play crucial role in developmental and disease related pathologies. In addition to acting extracellularly on receptors located on the plasma membrane, S1P can also act intracellularly, independently of $S1P_{1-5}$, affecting calcium homeostasis and cell proliferation. The SphKs /S1P /S1PL metabolic pathway is implicated in numerous human pathologies including respiratory disorders, thereby raising the possibility that manipulating intracellular S1P levels could offer therapeutic potential in ameliorating lung diseases. This review focuses on the prospects of targeting S1P signaling and S1P metabolizing enzymes using small molecule inhibitors, receptor agonists, and antagonists in the treatment of lung diseases.

Keywords

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1. Introduction

Sphingolipids constitute a class of lipids that contain a sphingoid base such as sphingosine, sphinganine (dihydrosphingosine), or phytosphingosine backbone linked to long-chain fatty acids (ceramides). The resulting ceramides can be linked to hydrophilic head groups such as phosphorylcholine (sphingomyelin) (SM), carbohydrate moieties (glycosphingolipids), or a phosphate group (ceramide-1-phosphate) (Weete, 1974). SM is located on the exoplasmic surface of the eukaryotic plasma membrane where it plays a paramount role in protecting the cell surface from external agents (Simons & Ikonen, 1997), and also functions as a signaling lipid (Maceyka et., 2012). The first step of sphingolipid *de novo* biosynthesis is the formation of 3-keto-dihydrosphingosine via condensation of L-serine and palmitoyl CoA catalyzed by serine palmitoyltransferase (SPT), the rate limiting enzyme in sphingolipid biosynthesis (Merrill, 2002). 3-Keto-dihydrosphingosine is rapidly reduced to sphinganine (dihydrosphingosine) by ketosphinganine reductase (Stoffel, 1970), followed by ceramide synthase(s) mediated N-acylation to dihydroceramide with different fatty acid chain lengths (Stiban et., 2010). Mammals exhibit six different acyltransferases encoded by lass-genes that show specificities for different fatty acyl CoAs (Futerman & Riezman, 2005). Dihydroceramides can be desaturated to ceramides, which can be channeled to the synthesis of complex sphingolipids such as SM and glycosphingolipids, or phosphorylated by ceramide kinase to ceramide-1-phosphate (Mitsutake et., 2006). Mammalian cells do not convert dihydrosphingosine to sphingosine; however sphingosine can be generated from ceramide by ceramidases (Chalfant & Spiegel, 2005). Also, ceramide can be formed from SM in mammalian cells by sphingomyelinase activation in response to extracellular stimuli such as TNF-α or growth factors (Dbaibo et al., 1993). Sphingosine generated from ceramide is converted to sphingosine-1-phosphate (S1P) by sphingosine kinase (SphK) 1 and/or 2 (Figure 1).

2. Sphingosine-1-phosphate Metabolism and Signaling

Cellular levels of S1P are tightly regulated by its synthesis from sphingosine through the activation of SphKs and degradation through reversible dephosphorylation of S1P to sphingosine by S1P phosphatases (SPPs), lipid phosphate phosphatases (LPPs), or irreversible degradation by a pyridoxal phosphate-dependent S1P Lyase (S1PL) to *2* hexadecenal and ethanolamine phosphate (Saba & Hla, 2004). In unstimulated cells, the balance between S1P production and degradation results in relatively low intracellular levels of S1P. Erythrocytes and platelets have much higher levels of S1P compared to other cells and this is due to lack of S1PL (Ito et al., 2007). S1P is also transported from inside the cell to outside by ABC transporters (Kim et al.,2009; Kobayashi et al., 2009; Mitra et al., 2006; Sato et al., 2007), and the recently identified spinster homolog 2 (Spns2) transporter (Fukuhara et al., 2012; Hisano et al., 2012; Kawahara et al., 2009). In the last two decades, S1P garnered much deserved research attention as it has emerged as a bioactive lipid mediator of diverse cellular processes such as cell growth, and survival (Olivera et al., 1999), motility (Van Brocklyn et al., 2003; Xu et al., 2006; Berdyshev et al., 2011), cytoskeletal organization (Garcia et al., 2001), endothelial permeability (Wang & Dudek, 2009), vascular tone (Levkau, 2008), adherens junctions (Mehta et al., 2005), tight junctions assembly (Lee et al., 1999a; Lee et al., 2006), autophagy (Lavieu et al., 2006; Huang and Natarajan, 2015),

immune regulation (Chi, 2011; Spiegel & Milstien, 2011; Walzer, Chiossone, Chaix, & Calver, 2007) and morphogenesis (Lee et al., 1999a). These pleotropic actions are attributed to its unique inside-out (extracellular), and intracellular signaling, highlighting its role as a signaling sphingolipid. Intracellularly, S1P is known to act as a second messenger and plays a role in calcium homeostasis; however very little is known regarding intracellular targets of S1P. Release of S1P in human lung endothelial cells by the photolysis of caged S1P significantly enhanced endothelial cell (EC) barrier function, which was independent of $S1P_1$, but was dependent on Rac1(Usatyuk et al., 2011). Interestingly, S1P generated in the nucleus by the action of SphK2 is shown to directly target HDACs and an integral component of the HDAC repressor complex (Hait et al., 2009; Fu et al., 2014), but the mechanism and its relevance to disease needs further study. Additionally, S1P has been identified as a missing co-factor required for the E3 ligase activity of TNF receptor-associated factor 2 (TRAF2) (Alvarez et al., 2010). Equally important, S1P is a regulator of mitochondrial assembly and function by binding to prohibitin 2 (Strub et al., 2011), and a modulator of BACE1 activity in Alzheimer's disease (Takasugi et al., 2011). Further, S1P binding to human telomerase reverse trancriptase Tak stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation (Panner Selvam et al., 2015). Taken together, S1P's actions could be autocrine or paracrine or *in situ* where it is generated and thus offers immense therapeutic potential for a number of human diseases including respiratory disorders such as sepsis, asthma, fibrosis, pulmonary hypertension, and lung cancer, which is the focus of this review (Table 1).

S1P signaling has momentous clinical importance as evidenced by recent research, suggesting its pivotal role in regulating diverse immune regulatory networks that contribute to human health and diseases (Maceyka et al., 2012) including respiratory and lung disorders (Brinkmann & Baumruker, 2006). S1P signaling axis is implicated in regulation of many physiological and pathophysiological processes and diseases (Pyne et al., 2009), and targeting S1P levels in pathologies wherein increase or decrease in circulating or tissue S1P levels has been shown to be detrimental (Pyne & Pyne, 2010; Natarajan et al., 2013; Kunkel et al., 2013) which is of significant interest to scientists and pharmaceutical industry. Of note, anti-S1P monoclonal antibody (sphingomab) (O'Brien et al., 2009), which neutralizes extracellular S1P and inhibits its signaling via its receptors, is currently being investigated in pre-clinical and phase I and II trials of tumor growth suppression and age-related macular degeneration (Sabbadini, 2011) (Figure 2).

2.1. Sphingosine Kinases as Drug Targets

SphK1 and SphK2 are the two known isoenzymes that have been identified and characterized in mammals (Kohama et al., 1998; Liu et al., 2000a; Nava et al., 2000). Although both share significant sequence homology, they differ significantly in sub-cellular localization, tissue distribution, and function. Albeit ubiquitously expressed in most tissues, SphK1 expression is substantial in the lung and heart, and SphK2 in the liver and spleen (Melendez et al., 2000). Two functional nuclear export signal sequences (NES) direct SphK1 localization to the cytosol (Inagaki et al., 2003). In contrast, SphK2 has both nuclear import and export sequences, and is found predominantly in the nucleus in many cell types (Igarashi et al., 2003). Both SphK1 and SphK2, when activated by growth and survival

factors, undergo translocation, post-translational modifications, protein-lipid, and protein-protein interactions that ultimately result in elevated S1P levels in the cell (Alemany et al., 2007), which regulates various biological responses. Cytosolic S1P produced by SphK1 enhances cell growth, whereas Sphk2 generated S1P in ER and/or membranes promote apoptosis, as evident from studies using different model systems (Hait et al., 2006; Liu et al., 2003). Despite their differences, *in vivo* studies have shown that SphK1 and SphK2 can compensate for each other, as neither SphK1 nor SphK2 knockout mice models reveal any ostensible phenotype in adulthood. The SphK1/2 double knockout mice are embryonically lethal (Mizugishi et al., 2005). Although S1P has barrier protective effects, and proven ameliorative effect against sepsis-induced lung injury in animal models (Peng et al., 2012), there are limitations to S1P therapy in acute lung injury (ALI) (Natarajan et al., 2013; Wang et al., 2014). Infusion of high concentrations of S1P produces pulmonary edema, stimulates contraction of airway and bronchial smooth muscle cells (Rosenfeldt et al., 2003), and increases airway hyper-responsiveness to allergens in mice (Roviezzo et al., 2007). Several small molecule inhibitors of SphK 1 and SphK2 have been reported and some are listed in Table 2.

2.2. S1P Lyase as a Drug Target

S1P Lyase (S1PL) is an endoplasmic reticulum-resident, pyridoxal phosphate dependent, type III membrane protein (Ikeda et al., 2004) that is ubiquitously expressed in all tissues (Serra & Saba, 2010). It catalyzes the terminal step in sphingolipid metabolism by irreversibly cleaving the C2-C3 bond of S1P to generate 2 hexadecenal and ethanolamine phosphate, thereby controlling the S1P concentrations in the cells. Deletion of both alleles of *Sgpl1* gene in mice is lethal and animals do not survive beyond weaning due to congenital anomalies partially attributed to cytotoxic levels of S1P in major organs (Allende et al., 2011); however, partial knock out of *Sgpl1* gene results in small increase in S1P with no effect on the survival (Billich et al., 2013). S1PL is known to be up-regulated and activated in response to various stimuli like the bacterial endotoxin lipopolysaccharide (LPS) (Zhao et al., 2011) and radiation exposure of the mouse lung (Huang et al., 2013). S1PL is likely to undergo various post-translational modifications due to many potential phosphorylation and nitrosylation sites; however, they need to be validated experimentally (Huang et al., 2005; Zhan & Desiderio, 2006). Although S1PL is a novel target in immunomodulation, only a few S1PL small molecular inhibitors have been characterized. The vitamin B6 antagonist, 4′-deoxypyridoxine (4-DP) (Bandhuvula et al., 2007) and the food colorant, 2-acetyl-4-tetrahydroxybutylimidazole (THI) (Ohtoyo et al., 2015) have been widely used to block S1PL activity, *in vitro* and *in vivo*, respectively, and have limited therapeutic application. 4-DP is a non-specific inhibitor of all pyridoxal phosphate dependent enzymes, and may lead to long-term cytotoxicity. THI modulates S1PL activity *in vivo* and not *in vitro* as biotransformation of THI is required for its inhibition of S1PL (Schwab et al., 2005). A recently designed S1PL inhibitor, [(4-benzylpthalazin-1-yl)-2-methylpiperazin-1-yl] nicotinonitrile 5, reduced peripheral T cell numbers after oral administration and conferred protection to experimental autoimmune encephalomyelitis in rats (Weiler et al., 2014). Thus, inhibition of S1PL, which increases intracellular S1P levels, may serve as an alternative pharmacological approach to FTY720 to reduce T cell egress from lymph nodes in treating

certain sub-types of multiple sclerosis. Table 3 lists S1PL inhibitors that may have therapeutic potential.

2.3. S1P receptors as Drug Targets

S1P is a unique lipid mediator that has both intracellular and extracellular targets. S1P generated in the cell is delivered to the extracellular environment by ABC transporters (Kim et al., 2009; Mitra et al., 2006; Sato et al., 2007; Kobayashi et al., 2009) and the recently identified Spns2 (Fukuhara et al., 2012; Kawahara et al., 2009; Hisano et al., 2012) where it acts in an autocrine or paracrine manner as a ligand for G-protein-coupled receptors on the cell surface. Five such S1P receptors (S1PRs) have been characterized so far that are designated $S1P_{1-5}$. S1PRs are differentially expressed in cells and tissues, and are coupled to various heterotrimeric G-proteins, thereby accounting for its pleotropic effects (Hla, 2001). The pattern of S1PR activation differentially regulates downstream signaling effector molecules like small GTPases (Kume et al., 2007; H. Zhou & Murthy, 2004), MAP kinase (Guo et al., 1998; Dikic et al., 1996; Sato et al., 1999), Akt (Tanimoto et al.,, 2004; Baudhuin et al., 2004), and phospholipase C/D (Banno et al., 1999; Okamoto et al., 1998), which in turn mediate various cellular processes including migration, immunity, angiogenesis, and vascular development. The differential expression of S1PRs also accounts for the diversity of the activation of down-stream signaling pathways. For example, binding of S1P to $S1P_1$ or $S1P_3$ results in increase migration of endothelial cells, whereas activation of $S1P_2$ has an opposite effect (Lee et al., 2001; Kimura et al., 2000; Ryu et al., 2002). Moreover, the differential regulation of Rho family of small GTPases, especially Rho and Rac, by S1PRs, is critical for barrier maintenance, cytoskeletal rearrangement, and motility of endothelial and epithelial cells (Lee et al., 1999a; Okamoto et al., 2000). S1P activation of $S1P_1$ enhances barrier integrity by cortical actin rearrangement in endothelial cells via Rac activation but $S1P_3$ activation in epithelial cells results in possible activation of Rho, leading to increased vascular permeability (Garcia et al., 2001; Liu et al., 2000b; Natarajan et al., 2013). $S1P_1$ deletion also hinders downstream Rac activation that leads to defects in vascular maturation, as evident from studies done using $S1P_1$ receptor null mice (Liu et al., 2000b). S1P-mediated multiple immune modulatory functions are also attributed to the differential expression of S1PRs. S1P secreted by mast cells was found to enhance mast cell function by binding to its $S1PR_{1\&2}$ in an autocrine manner (Olivera et al., 2010). While activation of $S1P_1$ by S1P in an autocrine manner causes mast cell migration (Olivera et al., 2010), binding to $S1P_2$ inhibits mast cell migration by down-regulation of Rac activity (Olivera & Rivera, 2005; Sanchez & Hla, 2004). Extracellular addition of S1P to rat islet cells has been shown to inhibit apoptosis, which is also mimicked by di-hydro–S1P addition (Laychock et al., 2013). Similarly, blocking $S1P_1$ & $S1P_3$ by antisense nucleotide attenuated S1P mediated effect on cell survival in endothelial cells (Kwon et al., 2001). Although the differential expression and effects of S1PRs underscores the complexity of S1P signaling, it also presents a valuable approach for targeting S1PRs for drug development. Given the limitation of S1P as a therapeutic agent in treating ALI, there has been a growing interest in the development of analogs of sphingosine and S1P for therapy. One such analog is FTY720, a synthetic derivative of the fungal metabolite, myriocin (Troncoso & Kahan, 1998). FTY720 is phosphorylated to FTY720-P by SphKs, especially by SphK2, *in vitro* and *in vivo* (Paugh et al., 2003). FTY720-P, similar to S1P, also binds to $S1P_1$ and $S1P_3$ (Gräler

& Goetzl, 2004), and modulates endothelial barrier function (Brinkmann et al., 2004). Moreover, FTY720 (Fingolimod) is an anti-inflammatory (Sehrawat & Rouse, 2008) and immune-modulatory agent (Zhou et al., 2009), which has been recently approved by FDA for the treatment of multiple sclerosis (MS) (Chun & Hartung, 2010). Most likely, as in animal models, phosphorylation of FTY720 to FTY720-P by SphK2 is essential for its therapeutic action in MS (Brinkman, 2010). Additionally, FTY720-P enhanced endothelial barrier function via $S1P_1$ and reversed endothelial barrier dysfunction induced by VEGF in endothelial cells (Dudek et al., 2007). In addition to the beneficial effects, both S1P and FTY720-P have therapeutic limitations (Natarajan et al., 2013) as both these mediators induce ubiquitination and proteosomal degradation of $S1P_1$ resulting in increased endothelial permeability after prolonged exposure. However, non-hydrolysable analog of FTY720-P, FTY720-(S)-Phosphonate, which also binds to $S1P_1$ has been shown to exhibit superior efficacy as a barrier protecting agent in cultured ECs and pre-clinical animal models of ALI (Camp et al., 2016; Wang et al., 2014). Although the precise mechanism of action of FTY720-(S)-phosphonate is unclear, its superior efficacy may be attributed to maintenance of $S1P_1$ expression for a prolonged period of time compared to S1P or FTY720-P. Further studies are necessary to delineate the down-stream signaling pathways mediated by S1P, FTY720, and FTY720-P analogs in regulating endothelial barrier function. Several agonists and antagonists that can modulate S1PR activity has been developed as pharmacologic tools to better understand the S1P receptor functions (Table 4 & 5).

2.4. S1P Phosphatases and Lipid Phosphate Phosphatases as Drug Targets

In addition to S1PL, S1P can also be metabolized by SPPs and LPPs to sphingosine (Le Stunff et al., 2002; Jasinska et al., 1999; Zhao et al., 2007; Tang et al., 2015). However, not much is known about the physiological and pathophysiological roles of SPP 1 and 2 and LPP1–3 in lung diseases. Depletion of SPP1 results in unfolded protein response and endoplasmic stress-induced autophagy (Lépine et al., 2011b) and doxorubicin switches protective autophagy in SPP1-depleted cells to apoptosis by calpain-mediated autophagy-related gene 5 cleavage (Lépine, Allegood, Edmonds, Milstien, & Spiegel, 2011a). Also, there is evidence to suggest a role for SPP1 in cancer as depletion of SPP1 with siRNA confers resistance to chemotherapy (Johnson et al., 2003). In contrast to SPP1, SPP2 seems to have a role in inflammation. SPP2 is induced in human umbilical vein ECs (HUVECs) during inflammatory responses and silencing of SPP2 by siRNA significantly reduced TNF-α mediated IL-1β mRNA and protein expression (Mechtcheriakova et al., 2007). LPPs comprising of three major isoforms, LPP1, LPP2 and LPP3, dephosphorylate several bioactive lipids including S1P, LPA, PA, and ceramide-1-phosphate, and modulate the bioavailability of these lipids for cell signaling (Burnett & Howard, 2003; Tang et al., 2015). Very little is known about the role of LPPs in lung diseases; In human endothelial cells, LPP1 has be shown to modify S1P signaling by decreasing extracellular S1P concentrations, thereby lowering the activation of S1P receptors and downstream signaling. In addition, overexpression of LPP1 increases intracellular S1P signaling by promoting intracellular S1P formation (Zhao et al., 2007). Also, studies done using human bronchial epithelial cells showed a physiological role of LPP1 in attenuating LPA-induced IL-8 secretion (Zhao et al 2005). LPP3 may also have a role in tumor growth. LPP3 has been shown to impact glioblastoma progression by amplifying β-catenin and cyclin-D1 activities (Chatterjee et al.,

2011). Several agents such as tetrafluoroaluminate (ALF4), orthovanadate, and N-ethylmaleimide (NEM) are known to inhibit SPPs and LPPs; however, many of these compounds are cytotoxic and have no therapeutic value. Currently, specific small molecule inhibitors to dissect the roles of SPPs and LPPs in human diseases have not been developed.

2.5. Acid Sphingomyelinase as a Drug Target

Acid sphingomyelinase (ASM) belongs to a family of enzymes that catalyze the breakdown of SM to ceramide, a bioactive sphingolipid that has been implicated in the pathogenesis of human diseases including cancer, lung disorders, atherosclerosis, and diabetes (Ogretmen & Hannun, 2004; Brodlie et al., 2012; Petrache et al., 2005; Bismuth et al., 2008; Holland et al., 2007). Ceramide and S1P, two interconvertible lipids, form the ceramide-S1P rheostat that controls the cell fate, mediating opposing responses; ceramides are anti-proliferative whereas S1P stimulates cell survival pathways. In humans, deficiency of ASM results in an inherited lipidosis disorder called type A and B Niemann-Pick disease, characterized by accumulation of SM in neural and other tissues (Brady et al., 1966). Ceramide stimulates apoptosis in cells and increased ceramide levels have been observed in the lungs of patients with emphysema (Petrache et al., 2005); however, contradictory findings have been reported about ceramide levels in cystic fibrosis (CF) patients and in mouse model of CF where reduced ceramide levels correlated with defects in fatty acids (Wojewodka et al., 2010). These contradicting results in CF may be due to the various animal models used and the detection of only selected species of ceramides by mass spectrometry. Several pulmonary disorders result from either elevated ASM activity and/or expression. Pharmacological inhibition or genetic knockdown of ASM has been shown to normalize ceramide levels, decrease inflammation and infection, and prevent pulmonary disorders such as fibrosis (Dhami et al., 2010), cigarette smoke-induced emphysema (Petrache et al., 2005), and CF in mice (Becker et al., 2012). Several small molecule inhibitors of ASM such as amitriptyline, trimipramine, desipramine, chlorprothixene, fluoxetine, amlodipine, sertraline, and bisphosphonates may be useful for the treatment of pulmonary disorders such as CF, fibrosis, or emphysema via inhibition of ASM and modulation of ceramides in the lung (Table 6).

3. S1P/SphKs/S1PL Signaling Axis in Respiratory and Lung Diseases

Lung diseases are common medical conditions that affect the function of the lungs with symptoms ranging from mild to life threatening. Lung diseases fall into three broad categories: 1. Airway diseases that affect the tracheal and bronchial tubes resulting in structural changes in the airway, 2. Respiratory diseases that affect the lung structure leading to scarring of tissues, and 3. Pulmonary circulation diseases affect the blood vessels causing clotting and inflammation, which can also affect heart function. Irrespective of the nature of the disease, all the lung disorders affect the ability of the lung to exchange gases, which could lead to acute or chronic disease condition. S1P, being a potent signaling molecule, has been implicated in a number of lung disorders and because of its pleotropic nature it provides us with multiple targets, which could be exploited in developing therapeutics (Brinkmann & Baumruker, 2006). Lung disorders that affect the tubes cause constriction of airways, and airway diseases include asthma, chronic obstructive pulmonary disease

(COPD), and CF (Barnes, 2008; Lyczak et al., 2002). A number of diseases affecting lung tissues alter the tissue morphology by scarring or inflammation, which affects the lungs ability to expand and exchange gases. Diseases that cause restriction of the lung include pulmonary fibrosis, pneumonia, ARDS, and lung cancer (Chapman, 2004). Lung diseases that cause clotting, scarring, and inflammation of the blood vessels affect the normal functioning of the lungs and the heart; pulmonary hypertension is one such disease (Leeman, 1996).

S1P can signal extracellularly (inside out mechanism) by ligation to G-protein-coupled $S1P_{1-5}$ receptors or signal inside the cell, independent of the S1PRs. (Chun et al., 2002). However, the possibility of intracellular action of S1P via intracellularly localized S1PRs is understudied and cannot be ruled out. Recent evidences suggest localization and differential expression of S1PRs in normal and malignant human tissues. All the five S1PRs were expressed in both the cytoplasm and nucleus of benign and malignant tissues from multiple human organs as evidenced by Immunohistochemistry and Immunocytochemistry (Wang et al., 2014). Additionally, in estrogen receptor-positive breast cancer, high cytoplasmic $S1P_1$ and nuclear S1P2 and $S1P_3$ expression and association of these receptors with signaling proteins such as ERK1/2, Akt or SphK1 was reported to be associated with survival or recurrence of estrogen receptor-positive breast cancer (Ohotski et al., 2013). A new paradigm of S1P signaling in the nucleus is emerging that may alter gene expression, in which, SphK2 specific generation of S1P in the nucleus can bind to HDACs 1 & 2 and inhibit HDAC activity (Hait et al., 2009; Fu et al., 2014). Similar to S1P, FTY720-P also seems to bind to HDACs 1 and 2 modulating HDAC activity (Hait et al., 2015). Thus, SphK2/S1P nuclear signaling may represent a novel mechanism of epigenetic regulation of gene expression and cell function. This new role of nuclear S1P signaling may have a potential role in the regulation of human lung pathologies such as inflammation, pulmonary hypertension, and pulmonary fibrosis.

Given the pleotropic nature of S1P signaling, it is evident that targeting S1P signaling and S1P metabolizing enzymes holds enormous therapeutic potential for the treatment of lung diseases. So a diverse array of small molecule inhibitors, agonists, and antagonists targeting SphKs, S1PL, and S1PRs have been identified, characterized, and tested in mice models of lung diseases to unravel the distinct roles of the individual players in S1P signaling. Development of new and specific inhibitors will help to target specific components of the S1P signaling in treating the lung diseases.

3.1a. Acute Lung Injury/ Acute Respiratory Distress Syndrome

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are sudden failure of the respiratory system due to accumulation of fluid in the alveoli. Some of the common causes of ALI/ARDS include sepsis, pneumonia, trauma, radiation, multiple blood transfusion, and aspiration of stomach fluids. Inflammation and endothelial dysfunction are hallmark features of ARDS (Leaver & Evans, 2007) and S1P/SphK1/S1PL signaling axis may have an important role in the pathophysiology of the syndrome (Brinkmann & Baumruker, 2006; Natarajan et al., 2013; Proia & Hla, 2015).

S1P's role as a key regulator of endothelial barrier function is attributed to its signaling through $S1P_1$ & $S1P_3$ that activates downstream Rho GTPases and rearrangement of cytoskeleton (Garcia et al., 2001). Intravenous infusion of S1P has shown to reduce LPS-induced lung vascular permeability and inflammation in murine models (McVerry & Garcia, 2004). The barrier enhancing effects of S1P are generally ascribed to ligation to $S1P_1$, which initiates a series of downstream signaling cascades including Rac activation, cortactin translocation, peripheral myosin light chain phosphorylation, and rearrangement of focal adhesion and adherens junction proteins culminating in increased barrier function in lung endothelium cells *in vitro* (Sun et al., 2009; Abbasi and Garcia, 2013; Takuma et al., 2012; McVerry and Garcia, 2005; Xiong and Hla. 2014). However, elevated concentrations of S1P (>5–10 μM) produces barrier disruption *in vitro* and *in vivo*. Also, Intravenous infusion of S1P at 0.5 mg/kg body wt produces pulmonary edema in mice (Sammani et al., 2010) Ligation of S1P to $S1P_3$ leads to activation of $G_i/G_{11}/G_{12/13}$ coupled signaling pathways, and robust Rho/Rho kinase mediated EC contractile apparatus resulting in cell migration and vascular barrier dysfunction (Sun et al., 2012; Singleton et al., 2007; Li et al., 2015; Ni et al., 2014). Consistent with these findings, mice with genetic knock down of SphK1 were much more susceptible to LPS-induced vascular leak (Wadgaonkar et al., 2009). Similarly, *SphK1$^{-/-}$* mice showed poor recovery from anaphylaxis and delayed histamine clearance, which was improved after S1P injection (Olivera et al., 2010). S1PL expression is enhanced in LPS-induced lung injury mice models, causing decreased S1P levels thereby increasing inflammation and injury; targeting S1PL ameliorated lung injury as it increased intracellular S1P levels and decreased LPS-induced inflammatory cytokines (Zhao et al., 2011). In addition to ALI, SphK1 may play a role in sub-ALI such as radiation-induced lung injury (RILI). Exposure of mice to thoracic radiation (20–25 Gy) for 6 weeks enhanced SphK1 and SphK2 expression and ceramide to S1P ratio in plasma (Mathew et al., 2011). Genetic deletion of SphK1 potentiated susceptibility to RILI, indicating a protective role for SphK1 against RILI (Mathew et al., 2011). Interestingly, pretreatment with myriocin, an inhibitor of SPT, decreased fibrogenesis and inflammation 18 weeks post-radiation exposure, and inhibiting SPT also modulated SphK1 activity and S1P levels in the lung tissue and plasma (Gorshkova et al., 2012). These results support a role for S1P and S1P metabolizing pathways as potential targets against ALI and RILI.

Given the barrier protective role of S1P in ALI, advances have been made in the development and the use of S1PR agonists. Experiments done in HUVECs indicate that at lower doses (0.1–1.0 μM), FTY720 enhance endothelial barrier function; however at higher doses (10 μM –100 μM), it causes barrier disruption and apoptosis (Müller et al., 2011). Similar observations were also recorded in mechanically ventilated mouse model of lung injury (Müller et al., 2011). This complex action of FTY720 in ECs is due to its phosphorylation to FTY720-P by SphK1 or SphK2 that increases its affinity for S1PRs (Billich et al., 2003; Paugh et al., 2003). Though $S1P_1$ specific agonist SEW2871 increased endothelial barrier function in LPS model of lung injury pretreatment with $S1P_1$ antagonist SB-649146 reduced the effect considerably (Sammani et al., 2012), validating the role of $S1P_1$ in preventing vascular leakage. More importantly, studies carried out in murine model(s) of lung injury have highlighted the possibility of developing S1PL as a potential therapeutic option in ameliorating ALI. Mice orally treated with THI (0.05 mg/ml water) for

2 days, post intratracheal LPS instillation showed elevated S1P levels in the lung tissues and BAL fluids but not in the plasma (Zhao et al., 2011). Moreover, THI treatment also blocked neutrophil infiltration to the alveolar space and attenuated IL-6 secretion, thus clearly offering protection against LPS induced lung injury (Zhao et al., 2011). Targeting S1PL using siRNA in human lung microvascular endothelial cells (HLMVECs) challenged with LPS, paralleled with the murine studies show diminished barrier disruption, IL-6 secretion, and LPS-induced p38 MAPK phosphorylation, emphasizing that targeting S1PL could be a viable therapeutic option (Zhao et al., 2011).

3.1b Pseudomonas *aeruginosa* mediated lung inflammation and injury

Pulmonary infections remain a significant global health concern, as it is associated with significant morbidity and mortality in neonates and adults. *Pseudomonas aeruginosa* (*PA*) is a common, gram negative, environmental, opportunistic pathogen that causes clinical pneumonia in humans. *PA* mediated pulmonary infections are prevalent in people with CF, COPD, and in the clinical setting of mechanical ventilation. *PA* infection activates host NADPH oxidase (Nox) proteins (Fu et al., 2013), and acid SM (Managò et al., 2015), leading to the generation of reactive oxygen species (ROS) and ceramides, respectively. Excessive ROS and ceramide production have been implicated in lung inflammation, cell death, and susceptibility to infection in CF (Seitz et al., 2015). However, recent studies also suggest a role for ceramides and sphingosine in mounting lung defense against bacterial pathogens as targeting sphingolipid pathways suppresses the recruitment of neutrophils and other inflammatory immune cells into the lung, thereby reducing lung inflammation (ManagòAntonella et al., 2015). *PA* infection of mouse lung enhanced global acetylation of histones and genetic deletion of SphK2, but not SphK1, mitigated *PA*-mediated histone acetylation, and generation of pro-inflammatory cytokines such as IL-6 and TNF-α production both in mouse lungs and mouse lung epithelial cells (Fu et al., 2014). Further, infection of mouse alveolar epithelial cells with heat inactivated *PA* or flagellin, a principle component of bacterial flagella, induced SphK2 phosphorylation, and its translocation to the nucleus and inhibition of SphK2 with ABC 294760 attenuated histone H3 and H4 acetylation and these data suggest a novel role for SphK2 in regulating HDACs/HATs and expression of pro-inflammatory cytokines in response *to PA* infection (Fu et al., 2014). The *PA* mediated H3 and H4 histone acetylation was also attenuated by Nox4 siRNA and SphK2 inhibitor ABC 294760 blocked Nox4 dependent ROS generation (Fu et al., 2014). Thus, *PA* infection of mouse lungs induces chromatin modification via SphK2/S1P/Nox4 nuclear signaling. The role of nuclear SphK2/S1P signaling mediating chromatin modification and lung inflammation warrants further investigation (Fig. 3).

3.2 Asthma

Inflamed airways, as a result of exposure to pollutants, allergens, and infection are known to trigger asthmatic symptoms (Nelson et al., 2003); Elias et al., 1999). This causes recurrent wheezing and shortness of breath, accompanied by coughing that could worsen overtime resulting in asthmatic attack. Both in allergic and non-allergic asthma, T-helper cells are recruited to the airways, which secrete cytokines that in turn causes airway inflammation, and promoting leukocytosis (Cohn et al., 2004; Halim et al., 2012). Inflammatory cells such as mast cells, macrophages, and eosinophils that are recruited to the site, exacerbate the

inflammation, and work in concert to airway remodeling of the airway. While existing drugs, β2-Adrenergic receptor agonists, leukotriene analogs, and glucocorticoids (Bai, 1992; Chowdhury & Dal Pan, 2010; Hamid et al., 2003; Montuschi & Peters Golden, 2010; Barnes, 2012) can alleviate the clinical manifestations of asthma, there is no permanent cure for this complex airway disorder.

The role of S1P in the development of asthma by regulating inflammatory responses was first established by a study using rat mast cell line that showed Sphk activation and increase in S1P levels in response to FcεRI stimulation (Choi et al., 1996). Regulation of mast cell activation and degranulation has been credited to cytoplasmic S1P-interceded MAPK activation, while sphingosine was shown to have an opposite effect (Prieschl et al., 1999). Moreover, differential roles of SphK1 and SphK2 have been observed in a human mast cell line; SphK1 stimulated TNF-α, IL-6, and inflammatory mediators, and both SphK1 and SphK2 were required for TNF- α secretion (Oskeritzian et al., 2008). Elevated levels of S1P in BAL fluids from allergic asthmatic patients was shown to act on airway smooth cells, prompting airway remodeling in a Rho-Kinase dependent manner (Fuerst et al., 2014). Role of S1P in regulating macrophage function was validated in SphK1 knockdown mouse model of allergic asthma, where reduction in macrophage number and IL-4 and IL-5 secretion was observed (Lai et al., 2008). Evidences additionally point out that eosinophil infiltration into the airway and their adhesion to pulmonary endothelium is mediated by S1P via RhoA/Rho-kinase pathway (Sashio et al., 2012). Elevated levels of S1P in airway smooth muscle cells also increase COX-2 mediated PGE2 production thereby repressing β-2 adrenergic activity (Rumzhum et al., 2016). S1P also induces IL-8 secretion in a dose-dependent manner in BEAS-2B cells, mediated by $S1P_2$, and nuclear factor kB (NF-kB) (O'Sullivan et al., 2014). In rodent asthma models, S1P induced airway hyper responsiveness is mediated by $S1P_3$ as evident from $S1P_3$ agonist based study (Trifilieff & Fozard, 2012). The differential roles of $S1P_2$ and $S1P_3$ in asthma may be due S1P action on lung epithelial $S1P_2$ and pulmonary artery smooth muscle cell $S1P_3$. Given the deleterious effects of SphK1 and SphK2 in aggravating asthmatic conditions, blocking SphK activity and/or S1P2 or $S1P_3$ may be beneficial in ameliorating asthmatic allergic inflammation.

Use of specific SphK1 inhibitor SK1-I in murine allergic asthma models revealed attenuation of inflammation due to the suppression of NF-kB (Price et al., 2013). Oral administration of FTY720-P, a potent agonist of all S1PRs except $S1P_2$, increased airway hyper-reactivity in rodents, whereas AUY954, a highly selective $S1P_1$ agonist did not, suggesting the potential role of $S1P_3$ in S1P induced hyper-reactivity (Trifilieff & Fozard, 2012). However, in repeat allergen exposure models, FTY720 abrogated airway inflammation and hyper -responsiveness by inhibiting Th-2 associated transcription factors (Karmouty-Quintana et al., 2012). In mice subjected to antigen-induced allergic bronchial asthma, pretreatment with SKI-II, a non-selective SphK inhibitor, diminished airway hyper-responsiveness but not inflammation (Chiba et al.,2010b). In antigen-challenged mice, use of W123, JTE-013, and BML-241 – S1PR antagonists selective for $S1P_{1-3}$ respectively, resulted in down regulation of S1PRs and further substantiated the role of $S1P_2$ involvement in bronchial smooth muscle contractility, a distinctive feature of asthma (Chiba et al., 2010a). Consistent with this study, subcutaneous administration of S1P to Balb/C mice have shown to increase bronchial hyper-responsiveness and lung resistance in a dose-and time-

dependent manner (Roviezzo et al., 2010). Further, this study revealed an increase in mast cell number and elevated secretion of IL-4, IL-13, and IL-17 secretion levels, thereby demonstrating the importance of S1P signaling in asthma (Roviezzo et al., 2010). Inhaled delivery of SphK1 inhibitors dimethyl sphingosine and SKI-II also decreased asthmatic symptoms by preventing eosinophil inflammation in OVA administered mice (Nishiuma et al., 2008).

3.3 Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) is a lung disease of the premature neonates treated for respiratory distress syndrome by mechanical ventilation (Gien & Kinsella, 2011). Babies with BPD have lung tissue scarring and inflammation that leads to interruption of lung development. BPD is characterized by thickened interstitium, alveolar simplification, abnormal pulmonary vasculature, and increased pulmonary resistance and in some cases, pulmonary hypertension (Coalson, 2003). There is no effective treatment for BPD and therapeutic approaches to alleviate symptoms are not efficacious.

A study that characterized sphingolipid profile in newborn mice exposed to hyperoxia showed transient increase in ceramide levels together with alveolar damage and lung function abnormalities. Nonetheless, ceramide levels decreased after treatment with D-sphingosine that improved alveolar histology, suggesting a role for ceramide in BPD (Tibboel et al., 2013). However, recent investigations in the neonatal murine model showed that enhanced S1P generation in mice exposed to hyperoxia is injurious and is associated with BPD (Harijith et al., 2013). Interestingly, *in vivo* studies using specific knockout mice revealed that SphK1, but not SphK2, appears to have an adversarial role in hyperoxia induced BPD (Harijith et al., 2013). Further, SphK1 deficient mice exposed to hyperoxia revealed significantly less ROS formation and lung injury compared to SphK2 knockout mice under similar conditions of exposure, whereas S1PL heterozygous knockout mice showed increased lung inflammation and injury compared to the wild type neonatal mice exposed to hyperoxia (Harijith et al., 2013). Exogenous addition of S1P to human lung microvascular endothelial cells (HLMVECs) triggered reactive oxygen species (ROS) production whereas knocking down SphK1 using siRNA blocked hyperoxia-induced ROS generation (Harijith et al., 2013). The hyperoxia-induced ROS generation was mediated by Nox2 and involved S1P / SphK1 / Spns2 / S1P$_{1\&2}$ signaling axis in the endothelium.

SKI-II, a small molecule inhibitor of both the isoforms of SphK, attenuated hyperoxia-induced ROS in HLMVECs (Harijith et al., 2013), further confirming that blocking S1P may confer protection in BPD. Mechanism(s) of S1P-mediated activation of ROS generation in BPD is unclear but may involve Nox family members, which require future investigations.

3.4 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease that causes obstructive airflow from the lungs. Cigarette smoke is the leading cause of COPD, followed by irritating gases and pollutants. COPD includes two conditions, emphysema-the damage of the air sacs and chronic bronchitis-the inflammation of air way lining, that obstructs the airflow to lungs causing shortness of breath(Yoshida & Tuder, 2007). One of the noteworthy

mechanisms of lung tissue damage in COPD is the disruption of the balance between apoptosis and generation of new structural cells in the lung. The pro-apoptotic effect of ceramide is implicated in alveolar cell apoptosis and oxidative stress in emphysema development, as evident from various *in vivo* (Petrache et al., 2005) and *in vitro* (Tepper et al., 1997; Ravid et al., 2003; Sawada et al., 2002) studies carried out in the past decade. Studies aimed to antagonize the effect of ceramides to tilt the balance against apoptosis were effective by increasing intracellular S1P levels, which is anti-apoptotic and pro-proliferative (Cuvillier et al., 1996; Osawa et al., 2005). S1PR profiling in the lungs of COPD patients showed significant down regulation $S1P_5$, which could be a possible target for COPD pharmacotherapy (Cordts et al., 2011). A potential link between $S1P_5$ and S1P metabolizing enzymes and defective alveolar macrophage function in COPD has been reported (Barnawi et al., 2015). A significant increase in mRNA levels of SphK1/2, $S1P_2$, and S1PL in alveolar macrophages from COPD patients compared to control subjects was observed while $S1P_5$ and SGPL1 expression was unaffected by smoking status, suggesting a COPD "disease effect" rather than smoke effect per se (Barnawi et al., 2015). Further, significant associations were noted between $S1P_5$ and both lung function and phagocytosis. Cigarette smoke extract significantly increased mRNA expression of SphK1, SphK2, $S1P_2$, and $S1P_5$ in THP-1 macrophages, confirming the results from patient-derived macrophages, and antagonizing SIP_5 significantly improved phagocytosis of the macrophages (Barnawi et al., 2015).

Strategies to mimic S1P signaling are known to be effective in improving COPD. Triggering S1P signaling using FTY720 or S1P receptor agonist SEW2871 increased SphK1 expression and activity, and attenuated lung parenchyma apoptosis in emphysema (Diab et al., 2012). Administration of sphingosine activated pro-survival pathways and increased the S1P to ceramide ratio, thereby restricting alveolar space enlargement. (Ghidoni et al., 2015). Hence, increasing S1P to ceramide ratio and signaling could be a therapeutic option in attenuating lung apoptosis and could offer protection against COPD.

3.5 Cystic Fibrosis

Cystic fibrosis (CF) is a genetic disorder that causes lung damage and affects the cells that secrete mucus, sweat, and digestive juices. Rather than acting as lubricant, the thick and sticky secretions, the products of the defective CFTR gene, clog the airways that interfere with normal function of the lung (Wine, 1999).

The pro-inflammatory cytokine TNF-α-mediated down regulation of CF transmembrane conductance regulator (CFTR) negatively impact S1P signaling in resistance arteries (Meissner et al., 2012); however, a recent investigation suggests a feedback link of S1P, wherein it inhibits CFTR activity through adenosine monophosphate-activated kinase (Malik et al., 2015). Low levels of S1P in BAL fluids detected in CFTR knockout mice partly affect dendritic cell recruitment, which plays a crucial role in chronic infections in CF (Xu et al., 2013).

Altered S1P signaling has been shown to affect dendritic cell function in CF lung. Augmenting cystic fibrotic BALF with S1P recovered the expression of major histocompatibility complex class II molecules (MHCII), CD 40, and CD 86 in both wild

type lung and blood dendritic cells (Xu et al., 2013). This finding argues that S1P could restore the innate immune function in CF. It will be interesting to test the effect of FTY720 and its analogs in dendritic cell recruitment, mucus secretion, and lung infections in CFTR knockout mice.

3.6 Pulmonary Hypertension

Pulmonary hypertension is a lung disorder where the blood pressure in the pulmonary arteries rises above normal levels, as a result of constricted arteries that carry blood from the heart to the lungs. This strains the right ventricle of the heart, weakening it to an extent that it loses its ability to pump blood to the lungs (Rubin, 1997).

Influence of S1P and its receptors in vascular remodeling in pulmonary hypertension has been documented through multiple studies. S1P induces vascular inflammation in pulmonary hypertension through $S1P_1$ by activation of p38MAPK and JNK/SAPK pathways mediated by EGFR and PDGFR transactivation (Yogi et al., 2011). $S1P_2$ is identified as the primary cell surface receptor that mediates S1P-induced vasoconstriction of lungs in mice and $S1P_2$ -dependent downstream activation of Rho-Kinase signaling alters pulmonary vascular tone (Szczepaniak et al., 2010). In contrast, $S1P_4$ is implicated in S1P-mediated vasoconstriction in hypertensive rat lungs (Ota et al., 2011). However, in experiments using pulmonary artery smooth muscle cells, SphK1 generated S1P signals through both $S1P_2$ and $S1P_4$, activating downstream Rho kinase causing hypoxic pulmonary vasoconstriction (Tabeling et al., 2015). Recent investigation on the role of S1P signaling in excessive pulmonary vascular remodeling demonstrated that the pro-proliferative effects of SphK1 comes into play to promote pulmonary artery smooth muscle cell (PMSMC) proliferation in hypoxia–dependent pulmonary hypertension mouse model (Chen et al., 2014).. S1P levels were increased in lungs of patients with pulmonary arterial hypertension and pulmonary arteries from rodent models of hypoxia-induced pulmonary hypertension (Chen et al., 2014). Unlike $SphK2^{-/-}$ mice, $SphK1^{-/-}$ mice were protected against hypoxia-induced pulmonary hypertension, whereas $Sgpl1^{+/-}$ mice were more susceptible (Chen et al., 2014). In hPASMCs, PDGF induces SphK1 expression via Egr-1, a PDGF-associated transcription factor, to promote pulmonary artery SMC proliferation (Sysol etal., 2016). Pharmacologic inhibition of SphK1 prevented the development of hypoxia-induced pulmonary hypertension in rodent models of pulmonary hypertension. Overexpression of SphK1 and stimulation with S1P, potentially via ligation of $S1P_2$, promoted PASMC proliferation *in vitro*, whereas SphK1 deficiency inhibited PASMC proliferation (Chen et al., 2014).

Use of S1P receptor agonists and antagonists in pulmonary hypertension models uncovered the prominent roles of S1PRs and the downstream signaling pathways. $S1P_4$, initially thought to be restricted in cardiovascular system, was found to mediate S1P-mediated vasoconstriction in normotensive lungs when treated with $S1P_4$ agonists VPC23153 and phytosphingosine-1-phosphate, while dual $S1P_1$ and $S1P_3$ agonist VPC24191 did not elicit such response (Ota et al., 2011). JTE013, a putative inhibitor of $S1P_2$, has been shown to attenuate the S1P induced vasoconstriction in intact mouse lung (Szczepaniak et al., 2010). Further, intraperitoneal treatment of JTE013 prevented hypoxia-mediated pulmonary hypertension in mice, emphasizing a role for $S1P_2$ in pulmonary vascular remodeling (Chen

et al., 2014). The collective body of evidence accumulated from the above studies underlines a broader role for S1P signaling in the pathophysiology of pulmonary hypertension and targeting S1P signaling axis could ameliorate hypertension.

3.7 Lung Cancer

Carcinoma of the lung is the uncontrolled growth of lung tissue that is malignant in nature. There are three types of lung cancer: Non-small cell lung cancer (NSCLC), Small cell lung cancer (SCLC) and Lung carcinoid tumor (Cai et al., 2015). Exposure to cigarette smoke is the principal risk factor for lung cancer development (Hecht, 2002). Lung cancer is the leading cause of cancer deaths in US and worldwide (Jemal et al., 2008).

S1P is a potent angiogenic factor (Argraves et al., 2004; Lee et al., 1999b; Liu et al., 2000b), and mitogenic signaling of S1P comes in the forefront of deciding cell survival and proliferation (Augé et al., 1999; Panner Selvam & Ogretmen, 2013). The ceramide/S1P rheostat is a prominent feature of human cancer and perturbed sphingolipid metabolism has been associated with increased risk of lung cancer in a nested-control study. The study also concluded that increased S1P and ceramide levels could be markers for latent lung cancer(Alberg et al., 2013). The reason for increased ceramide levels associated with increased lung cancer risk was attributed to precancerous and cancerous cells producing larger pools of ceramide resulting in the conversion to S1P eventually tilting the balance towards S1P (Alberg et al., 2013). A recent study using non-small cell lung cancer (NSCLC) cells identified intracellular S1P playing a principal role in enhancing cell migration and extracellular S1P in apoptosis (Bradley et al., 2014). These diverse effects of S1P are in part due to the altered expression of Spns2, the S1P transporter and impaired sphingolipid metabolizing enzymes (Bradley et al., 2014). Marked increase of SphK1 expression observed in NSCLC cells activates PI3K/Akt/NF-kB pathways, resulting in tumor progression and poor survival (Fumarola, et al., 2014). In lung adenocarcinoma cells, $S1P_3$ upregulation enhances EGFR expression, increasing proliferation and anchorage independent growth, thereby contributing to tumor progression (Hsu et al., 2012). SphK2 generated S1P is implicated in chemo resistance of A549 lung cancer cell line exposed to hypoxia, where increase in SphK2 protein expression and activity correlated with increased S1P concentration (Schnitzer et al., 2009); however, knockdown of SphK2 using siRNA only resulted in partial protection. The role of S1PRs in tumor growth is not clearly established; $S1P_1$ in ECs promotes angiogenesis and tumor growth, whereas $S1P_2$ in ECs and bone marrow derived cells (BMDC) reveal an opposing effect (Du et al., 2010). Very recently, SphK2 generated S1P was found in association with the catalytic subunit of telomerase in normal fibroblasts and lung cancer cells, promoting cell proliferation and tumor growth (Panneer Selvam et al., 2015).

Targeting S1PRs suggests SphK2 generated S1P is responsible for offering chemo resistance in hypoxic A549 cells (Schnitzer et al., 2009). When A549 cells were incubated with $S1P_1/S1P_3$ antagonists, VPC 23019, and JTE013 (specific for $S1P_2$), only VPC 23019 exhibited a protective effect by blocking ERK1/2 signaling (Schnitzer et al., 2009). A combination therapy using SphK2 specific inhibitor ABC294640, and human recombinant TNF-α related apoptosis-induced ligand (TRAIL) heightened the activity of caspase 3/8 and

increased the expression levels of death receptors in NSCLC (Yang et al., 2015). A recent study that distinguished the role of S1P in telomerase stabilization in lung cancer cell lines used ABC294640 to delineate the role of SphK2 in hTERT stability in A549 cells. Inhibiting SphK2 with ABC294640 increased the ubiquitination-mediated degradation of hTERT, indicating that SphK2-generated S1P is responsible for the stability of hTERT (Panneer Selvam et al., 2015). Thus, blocking SphK1 and/or SphK2 activity may have beneficial effect in treating lung cancers.

3.8 Mesothelioma

Mesothelioma is a rare yet aggressive cancer that affects the membrane lining the lungs, with asbestos exposure being the principal risk factor (Abakay et al., 2015). Recent evidence points to the pro-apoptotic role of sphingosine in mesothelioma cells, promoting cell cycle arrest at G_0/G_1 phase by inhibiting PKC δ (Okuwa et al., 2012). The pro-proliferative effects of SphK1, as observed in malignant pleural mesothelioma (MPM) tumor tissues and MPM cell lines, is ascribed to its ability to alter the chromatin landscape by modulating the expression levels of histone acetyl transferases (HATs) and cell cycle checkpoint genes (Kalari et al., 2012). Studies using SKI-II (10μM) to inhibit SphKs in mesothelioma cell lines revealed attenuation of Histone H3 and Histone H4 acetylation and phosphorylation of H3 at serine 10 (Kalari et al., 2012). Also, inhibiting SphKs blocked the phosphorylation of CDK-2 that is vital for G1/S transition, obstructing mesothelioma cell division. Further investigation revealed that in response to SKI-II, there is an increase transcription of CDK-2 inhibitors, P21^{Cip1}, and P27^{Kip1}. Supplementary experiments targeting SphK1/SphK2 using siRNA established SphK1 and not SphK2 as the principal player in the development of malignant mesothelioma (Kalari et al., 2012). These studies suggest that SphK1 could be a therapeutic target for the treatment of mesothelioma; however, the role of SphK2 in malignant mesothelioma cannot be ruled out and warrants further investigation.

3.9 Pulmonary fibrosis

Pulmonary fibrosis is a condition that causes scarring of lung tissues, marked by alveolar epithelial injury, and accumulation of myofibroblasts (Wynn & Ramalingam, 2012); (Selman & Pardo, 2012). The stiffened lung tissue makes breathing difficult and reduces oxygen supply in the blood. A number of factors including chronic inflammation, infections, environmental pollutants, radiation exposure, and autoimmune disease contribute towards pulmonary fibrosis, but if an identifiable cause of the disease cannot be pinpointed then it is termed idiopathic pulmonary fibrosis (IPF) (Bourke, 2006). Lung damage caused by pulmonary fibrosis is permanent and in certain cases a lung transplant, the only viable treatment, is recommended (Mason et al., 2007); (George, Arnaoutakis, & Shah, 2011). Recently, FDA has approved two drugs for the treatment of IPF but it is too early to determine the efficacy of these drugs in ameliorating the disease (Rangarajan et al., 2016; Bonella et al., 2015).

S1P and S1P signaling may contribute to the development and progression of IPF and pulmonary fibrosis in animal models. Increased expression of SphK1/SphK2 correlated negatively with lung function and survival, as analyzed by microarray in peripheral blood mononuclear cells of IPF patients (Huang et al., 2013). Consistent with this observation, SIP

levels were found to be elevated in BAL fluids of IPF patients compared to control groups that correlated with poor lung prognosis in IPF patients (Milara et al., 2012). Elevated S1P levels in a murine model of bleomycin-induced pulmonary fibrosis resulted from enhanced SphK1, but not Sphk2 expression (Milara et al., 2012), which exacerbated lung fibrogenesis by increasing expression levels of TGF-β, fibronectin, and lung collagen. Genetic knockdown of SphK1, but not SphK2, offered protection against bleomycin-induced pulmonary fibrosis in mice (Huang et al., 2013). SphK1 levels are found to be elevated in human and mouse lung fibroblast following TGF- β stimulation, causing myofibroblast differentiation by activating $S1P_{2\ \&\ 3}$ (Huang et al., 2013). Similarly, in IPF patients, increased SphK1 levels contributed to epithelial to mesenchymal transition through $S1P_{2\ \&\ 3}$ in alveolar type II cells (Milara et al., 2012). In radiation-induced fibrosis murine model, impairing *de novo* synthesis of SphK1 delayed the onset of fibrosis and reduced pulmonary inflammation (Gorshkova et al., 2012). Diverse roles of S1P formed after TGF- β stimulation has also been reported in lung fibroblasts, with S1P inhibiting α-smooth muscle actin through $S1P_1$ and stimulating via $S1P_3$ (Sobel et al., 2013). Targeting SphK1 with specific inhibitors decreased pulmonary fibrosis and increased survival in bleomycin-induced lung fibrosis mice models (Huang et al., 2013). Buildup of extracellular matrix proteins (ECM), a characteristic feature of fibrosis, has also been attributed to S1P signaling via $S1P2_{\ \&\ 3}$ agonist-based studies in human lung fibroblasts (Sobel et al., 2013). Consistent with the previous study, $S1P_3$ knockout mice showed decreased inflammation and fibrosis in bleomycin-induced murine model of fibrosis (Murakami et al., 2014). Furthermore, manipulating the intracellular levels of SphK1 by over expressing S1PL attenuated lung fibrosis post bleomycin challenge, suggesting a critical role of S1PL as a natural suppressor of lung fibrosis (Huang & Natarajan, 2015; Huang et al., 2015). These studies suggest a major role for S1P signaling in the development of lung fibrosis, where $S1P-S1P_1$ signaling appears to be protective. Conversely, $S1P-S1P_{2\ \&\ 3}$ signaling seems to promote fibrosis and could be potential therapeutic targets.

Extensive studies carried out using bleomycin and radiation–induced pulmonary fibrosis murine models underscore the therapeutic potential of S1P signaling in IPF. Mice administered with SphK inhibitor SKI-II on day 8-post-bleomycin challenge decreased S1P levels in lung tissue and increased survival (Huang et al., 2013). Use of TGF-β and S1PR agonists S1P, FTY720, Ponesimod, and SEW2871 to investigate the molecular pathway revealed divergent pathways involved in ECM synthesis in normal human lung fibroblasts (Sobel et al., 2013). While TGF- β signals through Smad pathway, S1PR agonists trigger P13K/Akt and ERK 1/2 –dependent pathways to induce ECM (Sobel et al., 2013). Besides, non-specific S1P receptor agonists S1P and FTY720 induced potent ECM synthesis, while highly selective S1P agonists Ponesimod, and SEW 2871 did not, underlining the pro fibrotic role of $S1P_2/S1P_3$ (Sobel et al., 2013). However, in a separate bleomycin model study, prolonged exposure to FTY720 and $S1P_1$ specific agonist AUY954 resulted in fibrosis, suggesting $S1P-S1P_1$ pathway could also be a therapeutic target (Shea et al., 2012).

4. Conclusion and Future Directions

Novel therapies are needed for the treatment of lung diseases, as most of them are fatal with no effective cure in sight. S1P, a potent bioactive lipid mediator, regulates plethora of

signaling pathways, which have been implicated in the development of a number of lung pathologies. S1P signals intracellularly or extracellularly via G-Protein-coupled receptors and there has been considerable interest and progress made in developing novel and specific antagonist for the S1PRs. Additionally, the recent observations that S1P generated in the nucleus by nuclear SphK2 functions as a epigenetic co-regulator, has opened a new area of nuclear S1P signaling in chromatin modification and epigenetic role of sphingolipids in inflammation and lung injury. Current evidence emerging from studies using small molecule inhibitors in rodent models of lung diseases suggests that SphKs/S1P/S1PR signaling axis could be potential target(s) to ameliorate respiratory ailments. However, decreasing S1P levels in circulation or lung tissue might have some adverse effect in vascular system as it is a potent angiogenic factor. Therefore, localized delivery of SphK or S1PR inhibitors might mitigate any adverse vascular effects of systemic route of altering S1P levels. While SphK1 and to a limited extend SphK2 and S1PRs have been extensively investigated as potential therapeutic targets in a number of lung disorders, targeting S1PL, the enzyme that catalyzes the terminal degradation of S1P in the sphingolipid metabolic pathway, has received less attention. Given its role in regulating intracellular levels of S1P, both blocking and activating S1PL holds immense potential as a therapeutic target in treating lung diseases. Recently, a few specific S1PL inhibitors have been synthesized and their efficacy validated in autoimmune disease models; however, their therapeutic potential in combating lung diseases will require future studies in pre-clinical animal models of lung inflammation. Similarly, blocking ASM with small molecule inhibitor(s) seems to offer some protection against lung inflammation, an area that has potential for drug development. Last but not the least, targeting ceramide synthases with small molecule inhibitors may have beneficial outcome in COPD/emphysema and development of novel inhibitors specific for the six isoforms of the ceramide synthase is essential for *in vivo* studies in rodent models. Despite lack of Phase I or Phase II clinical trials with many of the sphingolipid inhibitors, there is tremendous potential to develop and test new and novel inhibitors of sphingolipid metabolizing enzymes and S1PRs to ameliorate respiratory disorders.

Acknowledgments

Abbreviations

4-DP	4′-deoxypridoxine
ABC transporter	ATP-binding cassette transporter
Akt	Protein kinase B
ALF4	Tetrafluoroaluminate
ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
ASM	Acid Sphingomyelinase

BACE1	beta-site APP-cleaving enzyme
BAL	Bronchoalveolar Lavage
BMDC	Bone marrow derived cells
BPD	Broncho Pulmonary Dysplasia
CDK-2	Cyclin Dependent Kinase
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CoA	Co Enzyme A
COPD	Chronic Obstructive Pulmonary Disease
COX-2	Cyclooxygenase-2
EC	Endothelial Cell
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinase
FcεRI	High-Affinity Receptor for IgG
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HLMVEC	Human Lung Microvascular Endothelial Cells
hTERT	Human Telomerase Reverse Transcriptase
HUVEC	Human Umbilical Vein Endothelial Cell
IL-1β	Interleukin-1-beta
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IPF	Idiopathic Pulmonary Fibrosis
JNK	c-Jun N-terminal kinase
LPA	Lysophosphatidic Acid
LPP	Lipid Phosphate Phosphatase
LPS	Lipopolysaccharide

MAPK	Mitogen-Activated Protein Kinase
MPM	Malignant Pleural Mesothelioma
mRNA	Messenger RNA
NEM	N-ethylmaleimide
NES	Nuclear Export Sequences
NOX	NADPH-oxidase
NSCLC	Non-Small Cell Lung Cancer
P21^{Cip1}	Cyclin-Dependent Kinase Inhibitor 1A
P27^{Kip1}	Cyclin-Dependent Kinase Inhibitor 1B
PASMC	Pulmonary Artery Smooth Muscle Cell
PDGFR	Platelet-Derived Growth Factor Receptor
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3-Kinase
PKCẟ	Protein Kinase C-delta
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
S1PL	Sphingosine-1-Phosphate Lyase
S1PR	Sphingosine-1-phosphate receptor
SAPK	Stress-Activated Protein Kinase
SCLC	Small Cell Lung Cancer
siRNA	Short Interfering RNA
SM	Sphingomyelin
SphK1	Sphingosine Kinase 1
SphK2	Sphingosine Kinase 2
SPT	Serine Palmitoyltransferase
Spns2	Spinster Homolog 2
SPP	Sphingosine-1-phosphate Phosphatase
TGF-β	Transforming Growth Factor- beta
Th-2	T helper 2, THI, 2-acetyl-4-tetrahydroxybutylimidazole

| **TNF-α** | Tumor Necrosis factor- alpha |
| **TRAF-2** | TNF Receptor-Associated Factor 2 |

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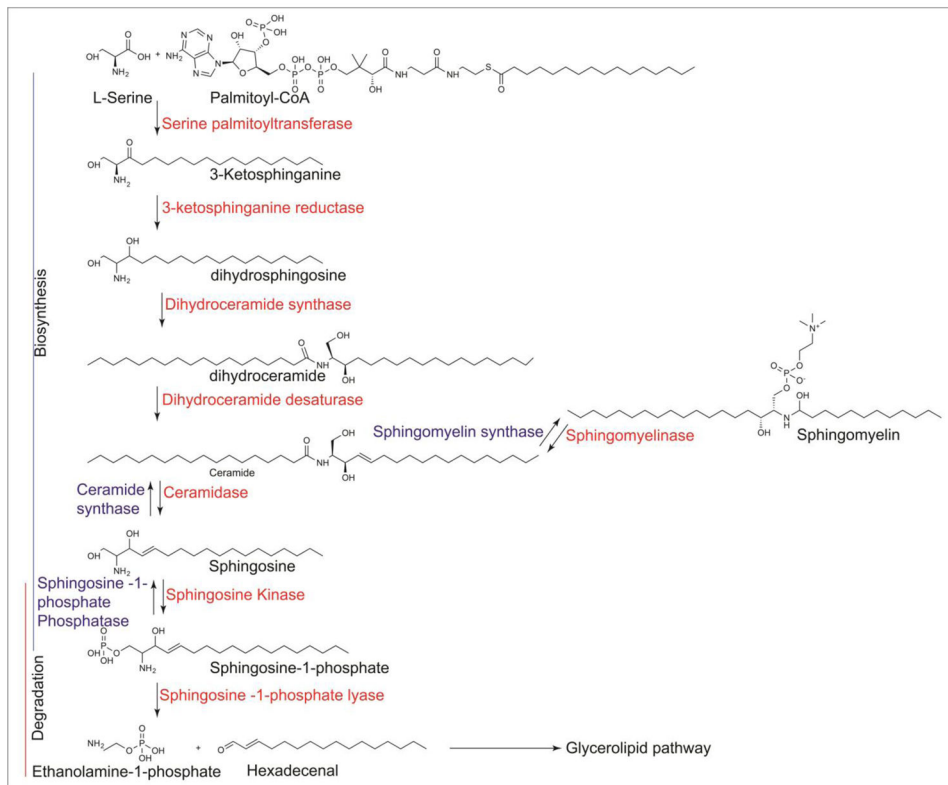
Fig 1. *De novo* **Sphingolipid Metabolism in mammalian cells**
Illustration of the key enzymatic steps in the biosynthesis, degradation and recycling of sphingoid bases.
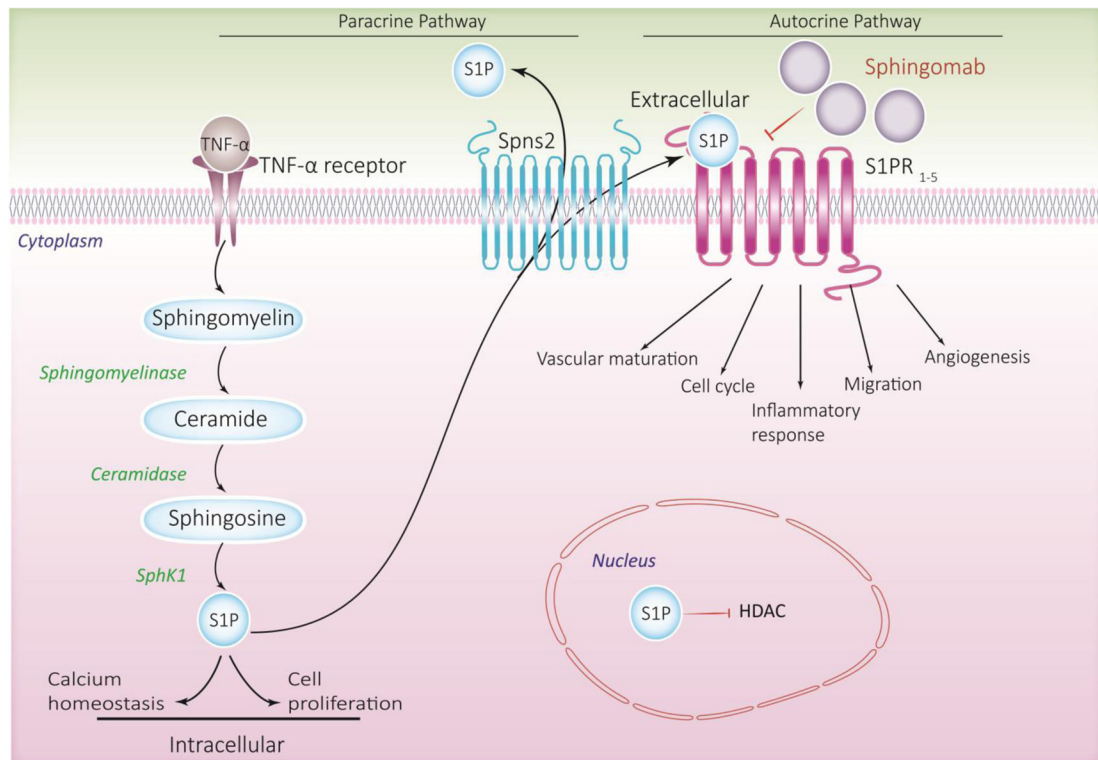
Fig 2. S1P signaling Axis

This scheme depicts the inside-out signaling of S1P. S1P produced by Sphingosine kinase 1 or 2 can act intracellularly on unknown targets in the cytoplasm; S1P generated by sphingosine kinase 2 in the nucleus inhibits HDAC activity. S1P is also exported out of the cell by specific transporter SpnS2 and act extracellularly on S1P receptors in an autocrine or paracrine manner. Extracellular S1P can be targeted by specific monoclonal antibody (sphingomab) to neutralize its effects.
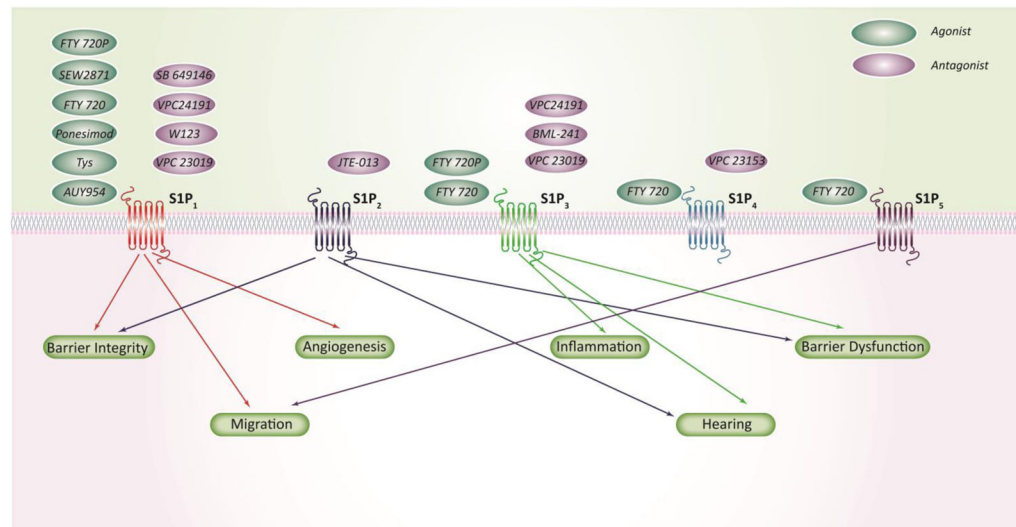
Fig 3. S1P receptors as drug targets

Extracellular S1P act as a ligand for specific G-protein coupled S1P receptors (S1PR $_{1-5}$) and regulate multiple cellular processes such as barrier integrity, migration, angiogenesis and inflammation. S1P receptors expression and activity can be modulated by well characterized S1P receptor agonists or antagonists that offer immense therapeutic potential in the treatment of lung disorders.
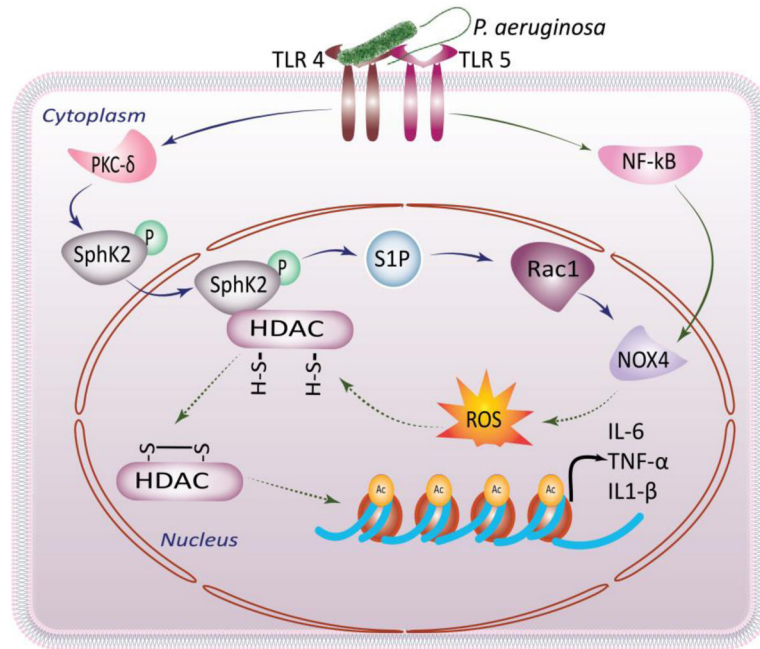
Fig 4. Nuclear S1P signaling mediating chromatin modification in *Pseudomonas aeruginosa*-induced Lung inflammation and injury

Nuclear S1P generated by activated Sphk2 in response to *P. aeruginosa* infection activates Nox 4 and generates nuclear ROS. SphK2/S1P signaling also induce histone acetylation and block HDAC activity in *P. aeruginosa*-induced lung inflammation.

Table 1

SphK expression and S1P levels in Lung Diseases

Lung Disease	Expression of SphK1/SphK2	S1P Levels	Therapy Option
Sepsis	*Decreased SphK1 Increased S1P Lyase*	*Low*	Activation of SphK1
BPD	*Increased SphK1*	*Elevated*	Inhibition of SphK1 activity
IPF	*Increased SphK1*	*Elevated*	Inhibition of SphK1 activity
PH	*Increased SphK1*	*Elevated*	Inhibition of SphK1 activity
VILI	*Decreased SphK1 Increased S1P Lyase*	*Elevated*	Inhibition of SphK1 activity
Lung Cancer	*Increased SphK1/SphK2*	*Elevated*	Inhibition of SphK1/SphK2 activity
RILI	*Increased SphK1/SphK2*	*Elevated*	Inhibition of SphK1/SphK2 activity

Table 2

Sphingosine Kinase Inhibitors

Inhibitor	Chemical name	Structure	Specificity
SKI-I	(2R,3S,4E)-N-methyl-5-(4′ - pentylphenyl)-2-aminopent-4-ene-1,3- diol. HCl		SphK1
SKI-I	5-(2-Naphthalenyl)-1H-pyrazole-3- carboxylic acid 2-[(2-hydroxy-1- naphthalenyl)methylene]hydrazide		SphK1 & SphK2
SKI-II	2-(p-Hydroxyanilino)-4-(p- chlorophenyl)thiazole		SphK1
Safingol	(2S,3S)-2-amino-1,3-octadecanediol		SphK1 & SphK2
ABC294640	(1s,3r,5R,7S)-3-(4-chlorophenyl)-N- (pyridin-4-ylmethyl)adamantane-1- carboxamide		SphK2
DMS	(E,2R,3S)-2-(Dimethylamino)-octadec- 4-ene-1,3-diol		SphK1 & SphK2
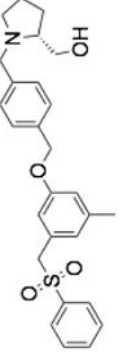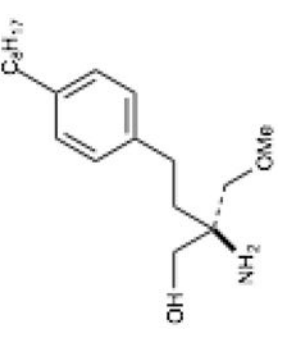
Inhibitor	Chemical name	Structure	Specificity
PF-543	*1-[[4-[[3-methyl-5- [(phenylsulfonyl)methyl]phenoxy]meth yl]phenyl]methyl]-2R- pyrrolidinemethanol*		SphK1
FTY-720 Methyl ether	*2-amino-2-(methoxymethyl)-4-(4- octylphenyl)butan-1-ol*		SphK2

Table 3

Sphingosine-1-Phosphate Lyase inhibitors
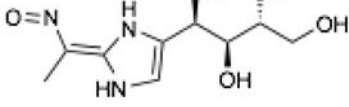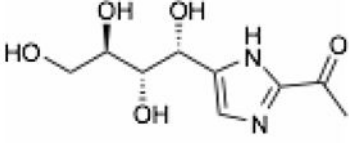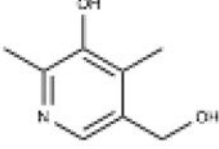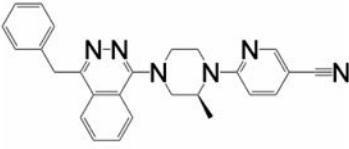
Inhibitor	Chemical name	Structure	Specificity
LX-2931	*(1R,2S,3R)-1-[(2Z)-2-(1- nitrosoethylidene)-1,3- dihydroimidazol-4-yl]butane-1,2,3,4- tetrol,*		S1PL
THI	*1-[5-[(1R,2S,3R)-1,2,3,4- tetrahydroxybutyl]-1H-imidazol-2-yl]-ethanone*		S1PL
4-Deoxy pyridoxine	*5-(Hydroxymethyl)-2,4- dimethylpyridin-3-ol*		S1PL
Compound 31 (Novartis)	*(4-benzyl-phthalazin-1-yl)-2-methyl- piperazin-1-yl]-nicotinonitrile 5*		S1PL

Table 4

S1P receptor Agonists
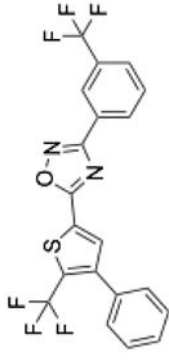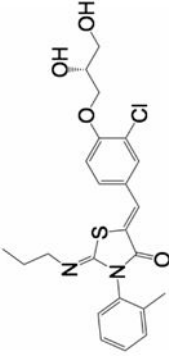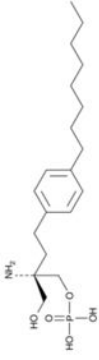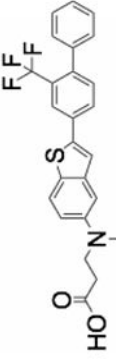
Agonist	Chemical name	Structure	Specificity
SEW2871	*5-[4-phenyl-5-(trifluoromethyl)-2- thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole*		S1PR1
Ponesimod	*(2Z,5Z)-5-{3-Chloro-4-[(2R)-2,3- dihydroxypropoxy]benzylidene}-3-(2- methylphenyl)-2-(propylimino)-1,3- thiazolidin-4-one*		S1PR1
FTY 720-P	*2-amino-2[2-(4-octylphenyl)ethyl]-1,3- propanediol, mono dihydrogen phosphate ester*		S1PR1, S1PR3, S1PR4, S1PR5
AUY954	*3-[[2-[4-phenyl-3- (trifluoromethyl)phenyl]-1- benzothiophen-5- yl]methylamino]propanoic acid*		S1PR1

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Table 5

S1P receptor Antagonists
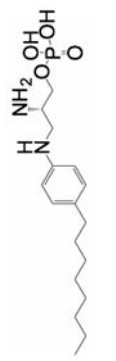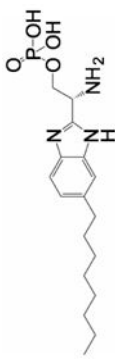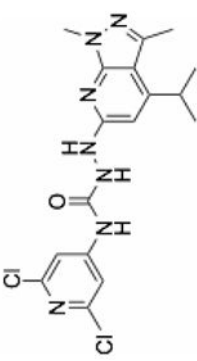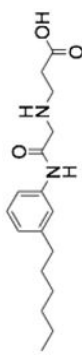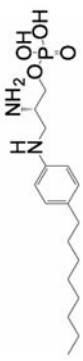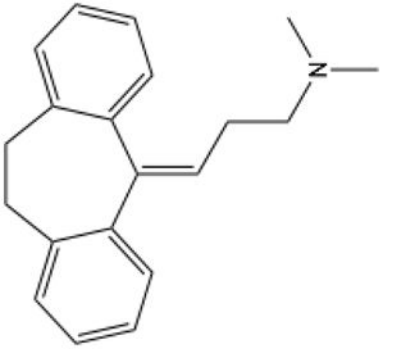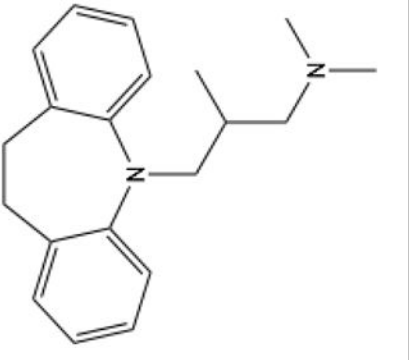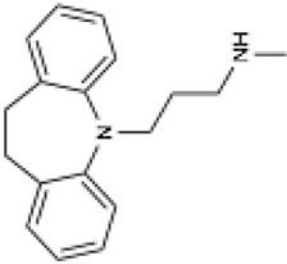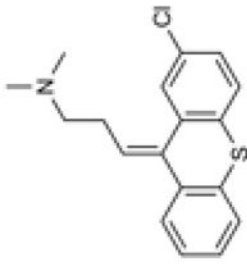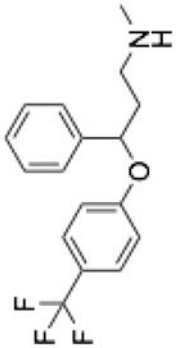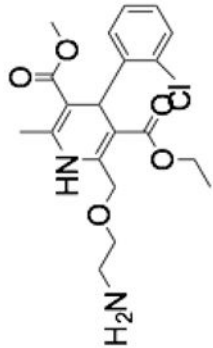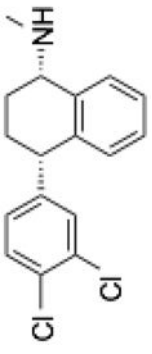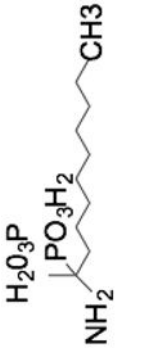
Antagonist	Chemical name	Structure	Specificity
VPC 23019	*(R)-2-amino-3-((3-octylphenyl)amino)- 3-oxopropyl dihydrogen phosphate*		S1PR1 & 3
VPC 23153	*(R)-phosphoric acid mono-[2-amino-2- (6-octyl-1H-benzoimiazol-2-yl)-ethyl] ester*		S1PR4
SB 649146		Structure not known	S1PR1
BML-241	*2-undecyl-thiazolidine-4-carboxylic acid*		S1PR3
JTE 013	*N-(2,6-dichloro-4-pyridinyl)-2-[1,3- dimethyl-4-(1-methylethyl)-1H- pyrazolo[3,4-b]pyridin-6-yl]-hydrazinecarboxamide*		S1PR2
W123	*3-(2-(3-hexylphenylamino)-2- oxoethylamino) propanoic acid*		S1PR1
VPC 24191	*(S)-phosphoric acid mono-[2-amino-3- (4-octyl-phenylamino)-propyl] ester*		S1PR1 & 3

Table 6

Acid Sphingomyelinase inhibitors

Inhibitor	Chemical Name	Structure	Specificity
Amytripytline	*3-(10,11-Dihydro-5H-dibenzo [a,d]cycloheptene-5-ylidene)-N,N- dimethylpropan-1-amine*		Acid Sphingomyelinase
Trimipramine	*(±)-3-(10,11-dihydro-5H-dibenz o[b,f]azepin-5-yl)-N,N,2- trimethylpropan-1-amine*		Acid Sphingomyelinase

Inhibitor	Chemical Name	Structure	Specificity
Desipramine	*3-(10,11-Dihydro-5H- dibenzo(b,f]azepin-5-yl)-N- methylpropan-1-amine*		Acid Sphingomyelinase
Chlorprothixene	*(Z)-3-(2-chlorothioxanthen-9- ylidene)-N,N-dimethyl-propan-1- amine*		Acid Sphingomyelinase
Fluoxetine	*N-methyl-3-phenyl-3-[4- (trifluoromethyl)phenoxy]propan-1- amine*		Acid Sphingomyelinase
Amlodipine	*(RS)-3-ethyl 5-methyl 2-[(2- aminoethoxy) methyl]-4-(2- chlorophenyl)-6-methyl-1,4- dihydropyridine-3,5-dicarboxylate*		Acid Sphingomyelinase

Inhibitor	Chemical Name	Structure	Specificity
Setraline	(1S,4S)-4-(3,4-dichlorophenyl)-N- methyl-1,2,3,4- tetrahydronaphthalen-1-amine		Acid Sphingomyelinase
Bisphosphanate			Acid Sphingomyelinase