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Sphingosine 1-phosphate Chemical Biology

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Abstract

A dozen years ago, the term ‘S1P’ (sphingosine 1-phosphate) was not in the lexicons of scientific literature databases. By early 2008, this query term retrieved well over 1,000 citations from PubMed – about 225 of these appeared in 2007. Indeed, S1P is arguably the most heavily studied lipid molecule at present. What happened to distinguish S1P among many other signaling lipids? We believe that the seminal event was the linking of the investigational drug, FTY720 (fingolimod), to S1P signaling. This realization profoundly altered understanding of S1P biology, revealing both that S1P is prominent in lymphocyte trafficking and that mimicking S1P signaling with an agonist drug can modulate the immune system to considerable therapeutic benefit. Neither fact was known prior to FTY720; indeed, this molecule is testament to the power of chemical biology. In this communication, we attempt to summarize progress to date in S1P chemical biology.

S1P biosynthesis and degradation

In mammals, the long chain base sphingosine is formed by amidase catalyzed hydrolysis of ceramides. Sphingosine is phosphorylated by sphingosine kinase types 1 or 2 (SPHK1, SPHK2) to form S1P, which is either converted back to sphingosine by lipid phosphatases or degraded irreversibly by S1P lyase [1]. S1P synthesis occurs in cells (but see reference [2]), thus the existence of S1P in plasma indicates some efflux system is responsible for S1P’s appearance. A small fraction of long chain bases lack a double bond (sphinganine (dihydrosphingosine), which is the precursor to ceramide in mammalian sphingolipid anabolism) [3]. Sphinganine is a substrate of SPHK and the product, sphinganine 1-phosphate, is for the most part indistinguishable from S1P in its biologic effects (but see reference [4]). The S1P biosynthetic pathway is widespread among mammalian tissues.

S1P concentrations in human and mouse plasma are 200–800 nanoM, where the molecule is nearly all protein-bound. S1P introduced into the mouse vasculature is degraded quickly (T1/2 15 min [5]), which indicates a rapid flux of sphingosine through the pathway outlined above. Mice lacking either SPHK1 or SPHK2 have decreased plasma S1P concentrations [6–8], but the reduction is more pronounced in SPHK1 null animals [6]. Disruption of both *Sphk1* and *Sphk2* gene loci is embryonic lethal in mice [9]. Characterization of the phosphatase(s) that hydrolyze the S1P phosphate monoester has been problematic. Leading candidates for this enzyme are the integral membrane lipid ectophosphatase LPP3 (lipid

A table listing a set of S1P compounds can be found in reference [67].

phosphate phosphohydrolase type 3) [10] and distantly-related members of the same enzyme family that are selective for sphingoid lipids (SPP1, SPP2) [11]. The paucity of selective substrates for, and inhibitors of, these enzymes, as well as the lack of useful mutant mice, leaves the identity of S1P phosphatase uncertain at present.

S1P receptors

S1P signals cells through a set of five, rhodopsin family G-protein coupled receptors named S1P1–5 (formerly EDG1, EDG5, EDG3, EDG6, EDG8) (see reference [12] for review). S1P1, S1P2, and S1P3 are expressed by a wide variety of tissues in mice and humans while S1P4 and S1P5 expression are largely limited to cells of hematopoietic origin. S1P5 is expressed also by oligodendrocytes. The affinity constants of S1P (or dihydro S1P) for the S1P receptor/G-protein complex are mostly in the single digit nanoM range [13]. S1P has a lower affinity for the S1P4 receptor; in strict receptor nomenclature terms, S1P4 is a phytoS1P (rather than S1P) receptor because this minor S1P form (phytosphingosine lacks a 4–5 double bond, rather it has a 4-hydroxyl group) has about 10-fold higher affinity for the S1P4 receptor than S1P [14]. S1P receptors couple to a variety of heterotrimeric G-proteins with the exception of Gαs. The ability of pertussis toxin to interdict many S1P signaling events *in vitro* illustrates the prominence of signaling via Gαi/o. Spiegel has invoked an additional, intracellular S1P receptor (see, for example, [15]), but the identity of this molecule(s) remains unknown.

Germ line disruption of the S1P1 receptor gene is embryonic lethal (E13.5) because of a failure of vascular maturation [16]. This defect is phenocopied by disruption of *S1p1* in the endothelial cell lineage [17] and, satisfyingly, by SPHK1/SPHK2 null mice [9]. S1P2 null mice are seizure-prone [18] and the inner ear does not develop normally, rendering these animals deaf [19,20]. S1P3 null mice are phenotypically unremarkable [21] as are, apparently, S1P5 null mice [22]. S1P4 null mice have not been reported.

FTY720

FTY720 was discovered in the course of a structure-activity relationship (SAR) study using myriocin (ISP-1) as the lead (see Fig. 1). Myriocin, which is a fungal-derived phytosphingosine analog with a connection to Chinese herbal medicine [23], is an inhibitor of serine palmitoyl CoA transferase (SPT, the first enzyme in sphingolipid biosynthesis). Initially studied as a potential anti-fungal drug, myriocin was found to be an immunosuppressant in mice [24]. The impetus for FTY720 discovery was a need to avoid the gastrointestinal toxicity of myriocin and to eliminate the chiral centers in that densely functionalized lead compound. Unlike myriocin, FTY720 does not inhibit SPT. FTY720 prolongs skin allografts in mice while evoking a profound lymphocytopenia [24]. We know now that this hematologic abnormality is a biologic signature of S1P1 receptor agonist drugs.

FTY720 is a potent drug; the ED50 for lymphopenia in mice after oral dosing is about 0.1 mg/kg (mpk). Curiously, FTY720 was found to be without effect on lymphocytes *in vitro* until concentrations in excess of 1 microM are used whereon apoptosis is induced [25]. The anomaly between *in vivo* and *in vitro* potencies was resolved when two groups discovered that FTY720 is phosphorylated rapidly *in vivo* and the product, FTY720-P, is an agonist that is equipotent to S1P at the S1P3, S1P4, and S1P5 receptors and about one log order more potent at S1P1 [13,26]. *In vitro* assays predicted that SPHK2 catalyzes the activation of FTY720 [27] and this prediction was verified when SPHK2 null mice were generated [7,8].

Introduction of either the parent (alcohol) or active (phosphate) drug into rodents results in a rapid equilibrium in blood with the phosphate: alcohol ratio of 3:1 [13]. FTY720 invades the

sphingosine biosynthetic pathway to the extent that it is a substrate for SPHK2 and at least one phosphatase. Interestingly, rat blood *ex vivo* converts FTY720 to FTY720-P, but the reverse reaction does not occur, indicating that whatever phosphatase is responsible for the hydrolysis of FTY720-P, it is not active in blood. The ratio of parent to active drug is a summation of import and export activities as well as SPHK2 and phosphatase activities in a specific tissue environment. Thus, it is to be expected that this ratio will be different in various tissues, biologic fluids (*e.g.* CSF) and inside and outside of cells. In stable renal transplantation patients, the elimination half-life of FTY720 was found to be from 89–157 hours and volume of distribution was 1116 to 1737 liters, indicating a widespread tissue distribution [28].

The use of S1P3 null mice, FTY720 analogs, and receptor type selective agonists (see below 'Other S1P Receptor Agonists' section) led quickly to the realization that agonist activity at the S1P1 receptor is responsible for the lymphopenia [29]. The lymphocyte depletion from blood is the result of sequestration of lymphocytes in secondary lymphoid tissue by inhibition of egress [30]. Current thinking is that effector T-lymphocytes are thus prevented from moving to sites of inflammation such as allografts and autoimmune disease. The blockade of lymphocyte trafficking, whilst sparing lymphocyte activation mechanisms, probably underlies FTY720's minimally immunosuppressant effects (*e.g.* opportunistic infections, malignancies) as compared to, for example, calcineurin inhibitors such as tacrolimus or cyclosporin.

The molecular mechanism whereby FTY720 inhibits lymphocyte egress remains uncertain. One proposal is that FTY720-P, although a receptor agonist, acts as a 'functional (physiologic) antagonist', that is the drug causes desensitization of S1P1 signaling pathways in lymphocytes. These cells are thus rendered effectively S1P1 null and are unable to sense the gradient of S1P across lymphoid tissue that is supposedly necessary for proper egress. This idea is supported by the behavior of S1P1 null thymoctyes, which fail to egress properly from lymphoid tissue [30,31]. Further, an S1P lyase inhibitor evokes lymphopenia (see below, 'S1P Lyase Inhibitors' section), perhaps because S1P accumulates within lymphoid tissues and thus disrupts the S1P gradient [32]. Counter to this argument is the inability of S1P1 receptor antagonists to evoke lymphopenia (although very few S1P receptor antagonists have been described). Further, the model presumes the chemoattractant nature of S1P for lymphocytes yet there is a paucity of evidence for the S1P/S1P1 axis functioning in lymphocyte migration; indeed, S1P is a rather anemic chemoattractant of T lymphocytes. A competing idea is that S1P1 receptor agonists influence 'stromal gates' that control lymphocyte egress [33]. Readers wishing to explore the intricacies of this debate are referred to two excellent recent reviews on the topic [34,35].

FTY720 is efficacious in a wide variety of autoimmune disease and allograft models (see [12] for review). Although the drug failed to meet primary endpoints in phase III renal transplantation studies (*i.e.* was not significantly better than the comparison drug, mycophenolate mofetil, in combination with cyclosporine [36,37]), FTY720 has exhibited remarkable efficacy in a placebo controlled, phase II trial of relapsing remitting multiple sclerosis [38]. The adverse events associated with FTY720 in humans are a transient bradycardia (common), dyspnea (4% of patients) and macular edema (rarely). In rodents, the bradycardia is not observed with S1P3 receptor-sparing agonists and it was not observed in S1P3 receptor null mice [29,39]. However, the use of S1P3 receptor-sparing agonists in humans or non-human primates has not been reported, thus the relative roles of various S1P receptor types in controlling heart rate in humans is not known.

Finally, the remarkable generosity of Novartis' scientists in distributing FTY720 to academic laboratories has catalyzed numerous studies. In addition to the disease models

mentioned above, FTY720 has been documented to be efficacious in models of neoplastic disease [40], atherosclerosis [41], renal ischemia reperfusion injury [42–44], pain [45], angiogenesis [46], acute lung injury [47], and others. It is worth noting that FTY720 might have actions independent of its phosphorylation to FTY720-P. These include antagonism at the CB1 cannabinoid receptor [48] and activation of protein phosphatase 2A [49].

Other S1P Receptor Agonists

The motivation for identifying additional S1P receptor compounds is two-fold. First, more selective compounds will help to parse the plethora of FTY720's actions to smaller sets of S1P receptors. Second, more selective S1P receptor agonists might recapitulate the efficacy of FTY720 while avoiding some adverse events such as bradycardia. Regarding the latter goal, S1P1 receptor selective agonists modulate lymphocyte trafficking and this biology might be solely responsible for the therapeutic efficacy of FTy720. However, FTY720 is a 'dirty' drug in that it is an agonist at four S1P receptors. It is important to be mindful that all the clinical data, and nearly all the animal model data, have been developed with this compound. One of the few animal models run with a more selective compound, AUY954, (an amino carboxylate that is a selective S1P1 agonist) demonstrated that this molecule was efficacious in a rat heart transplant model [50]. It will be interesting to learn whether an S1P1 receptor selective agonist can replicate the success of FTY720 in the clinic.

S1P1 receptor agonists have been relatively easy to identify by screening chemical libraries. For example, a modest-sized (<100,000 entities) library yielded multiple S1P1 receptor agonists [51]. In addition, systematic chemical manipulation of S1P analogs has led to discovery of additional S1P1 compounds with enhanced potency and duration *in vivo* [52]. These S1P1 receptor agonists evoke lymphopenia in rodents, but do not cause bradycardia; however, their efficacy in animal disease models has not been reported. In contrast to S1P1, the S1P2 receptor has proved uniquely resistant to 'drugging'. Indeed, only a single antagonist compound (see below, 'S1P Receptor Antagonists' section) has been identified for this receptor type. Agonist compounds that are selective for the other individual S1P receptors have not been published, although an S1P4/5 selective agonist has been identified [53].

An alternate strategy for S1P drug discovery is to use FTY720 as a lead compound to realize more selective agents. This 'pro-drug' strategy requires that the compounds are both substrates for an SPHK and, following phosphorylation, exhibit reasonable (*i.e.* nanomolar) affinity for some S1P receptor. As depicted in Fig. 2, FTY720 can be conceptualized as consisting of a 'warhead', 'linker', and 'tail' regions. The head region is largely constrained by the requirements of SPHK2; that is, only one spatial arrangement around the amino carbon is allowed (this center is pro-chiral in FTY720, SPHK2 yields *S*-FTY720-P [54]) and only small alkyl substitutions are permitted at the amino carbon [24]. However, lack of an alkyl substitution at the amino carbon results in compounds that are too rapidly dephosphorylated *in vivo* (our unpublished data). Within a given series, the length and position of the alkyl tail about the phenyl ring influence efficacy at the S1P1 and S1P3 receptors (see below, 'S1P Receptor Antagonists' section). The linker region (phenyl ethyl in FTY720) has proved amenable to manipulation to yield pro-drug compounds with useful activity profiles, particularly when the linker region is conformationally constrained. For example, a compound (VPC01091) with a phenyl cyclopentyl linker is an SPHK2 substrate and the phosphorylated form retains potency as an S1P1 agonist but has negative efficacy at the S1P3 receptor (*i.e.* inverse agonist) [55]. Thus, VPC01091 is an S1P3 receptor antagonist and does not cause bradycardia in experimental animals (unpublished data). Curiously, one isomer of VPC01091 is extremely long-lived *in vivo*; a single oral dose (3 mpk) renders rodents lymphopenic for more than two weeks [55].

S1P Receptor Antagonists

Prior to the realization that FTY720 is an S1P receptor agonist pro-drug, it was widely assumed that if an S1P signaling-directed drug was to find utility, it would be in blocking, not mimicking, S1P signaling. This long-standing idea has yet to be reduced to practice because of a lack of useful S1P receptor antagonists or SPHK inhibitors (see below, 'Sphingosine Kinase Inhibitors' section). In the ensuing discussion, we restrict the term 'antagonist' to the pharmacologic sense (receptor antagonist) rather than the broader 'functional (physiologic) antagonist' appellation assigned to FTY720-P as a result of its propensity to de-sensitize S1P1 receptor signaling.

An early generation of FTy720 analogs replaced the phenyl ethyl linker (see Fig. 3) with a phenyl amide functionality. In the course of performing an SAR analysis of this scaffold, a subset of molecules was found in GTPγ[^{35}S] binding assays to be inverse agonists at the S1P1 and S1P3 receptors [56]. While most molecules in the phenyl amide series are S1P receptor agonists (or inactive), when the 'tail' is no longer than eight carbon atoms, *meta* (1,3) or *ortho* (1,2) to the amide linker, and the amino carbon is in the *R* configuration, the molecules are S1P1/3 receptor antagonists. The lead molecule in the series, VPC23019, behaves as a competitive antagonist and exhibits Ki values of about 25 and 300 nanoM at the S1P type 1 and type 3 receptors, respectively [56].

Because the phosphate moiety is cleaved rapidly *in vivo* (the alcohol forms of the phenyl amide scaffold are not SPHK substrates, which negates the pro-drug approach in this case), metabolically-stable phosphonate analogs were synthesized. Both hexyl (S1P *R*) [57] and octyl (VPC44116) [58] phenyl amide phosphonates have been characterized. Fortunately, in these cases, receptor affinity did not suffer with the substitution of a phosphonate moiety for phosphate. The hexyl phosphonate compound has been shown to have a greater separation in affinities between the S1P1 and S1P3 receptors and thus is more S1P1-selective than the octyl compound [58]. To date, these phenyl amide compounds are the only published S1P1 receptor antagonists. It is curious that while screening chemical libraries has yielded a clutch of S1P1 agonists, that approach apparently has not been productive in finding S1P1 antagonists.

Now that an S1P1 antagonist has been found, was it worth finding? As a tool compound, the answer is certainly 'yes'. For example, S1P *R* and VPC44116 do not evoke lymphopenia in mice, which runs counter to a prediction of the 'functional antagonist' model of FTY720 action. Rather, the antagonist molecules block the lymphopenic activity of at least some S1P1 agonists [57,58]. As potential therapeutic agents, the answer is more nuanced. Both S1P *R* and VPC44116 cause increased leakage of Evan's blue dye in lung after a single dose in mice [57,58]. S1P1 agonists such as FTY720 oppose the pulmonary edema provoked by intra-tracheal administration of a pro-inflammatory cytokine [47]. The increased lung vasculature leakage evoked by the S1P1 receptor antagonists – albeit after a single dose of one compound type – indicates that an 'S1P tone' functions to control capillary permeability. Interestingly, there is not an obvious S1P agonist tone affecting lymphocyte egress because the antagonists do not cause lymphocytosis. On a positive note, daily administration of VPC44116 (10 mpk) to rats after vascular insult (balloon catheterization of the carotid artery) markedly lessened the degree of injury [59].

Although S1P2 receptor-selective compounds have proved very difficult to find, a single S1P2 antagonist, JTE-013, has been described [60]. Although commercially available, the compound is rather expensive and, in our hands, not very potent, which limits its utility. It is unfortunate that a set of chemical biology tools are not yet available to buttress the interesting findings made with S1P2 mouse genetics (see, for example, [61]).

An alternative to receptor antagonists are ‘chemical antagonists’ such as an S1P selective antibody. The concept is to introduce a high-affinity S1P binding agent so as to alter the competition of S1P binding proteins, and thus reduce the S1P ‘activity’. Indeed, such a reagent has been described. Although not widely available, one group has reported this biologic agent to be efficacious in tumor models [62]. It will be interesting to learn experience with this reagent should it be brought into the clinic.

Sphingosine Kinase Inhibitors

Interest in SPHK chemical biology is driven by the desire to determine the biologic effects of interdicting some, or all, S1P synthesis and a desire to understand the SAR of SPHK substrates so as to engineer S1P pro-drugs. SPHK1 is far more selective than SPHK2 regarding substrates. For example, SPHK2 will phosphorylate two isomers of sphingosine, while SPHK1 phosphorylates only the naturally-occurring D-*erytho* isomer [63]. In our experience, sphingosine analogs that are active as SPHK1 substrates are difficult, but not impossible [7], to find. This selectivity is recapitulated with the few SPHK inhibitors that have been described. Although N,N dimethyl sphingosine and the commercially-available SKI II and SKI V [64] have been deployed as SPHK1 inhibitors, we have found in broken cell assays that these molecules do not inhibit either human or mouse SPHK1 at concentrations of up to 100 microM in competition with 2–5 microM sphingosine (Kharel, Y., Macdonald, T.L. and Lynch, K.R. unpublished data). Further, a set of sphingosine analogs that contained numerous SPHK2 inhibitors was nearly devoid of SPHK1 inhibitors [65]. However, very recently a D-*threo*-sphingosine analog (SK1-I ((2*R*,3*S*,4*E*)-N-methyl-5-(4’-pentylphenyl)-2-aminopent-4-ene-1,3-diol)) was reported to be a selective human SPHK1 inhibitor (Ki ≈ 10 μM) [66]. In using SPHK-directed compounds, it is important to realize that there exists a wide variation in activity of SPHK substrates and inhibitors at mouse vs. human SPHK2 receptors.

S1P Lyase Inhibitors

A single S1P lyase inhibitor has been described [28]. This compound, 2-acetyl-4-tetrahydroxybutylimidazole (THI), is a food coloring component that induces lymphopenia in mice. This effect is antagonized by dietary co-administration of vitamin B6, and is mimicked by the vitamin B6 antagonist, 4-deoxypyridoxine. These observations led to investigations of S1P lyase, which requires a pyridoxal phosphate co-factor. S1P lyase is the only route of irreversible S1P catabolism, thus inhibition of this enzyme is expected to result in accumulation of S1P. The explanation proffered for the lymphopenic effect is that treatment with these inhibitors raises S1P levels inside lymphoid tissues and thus lymphocytes are less able to sense the S1P gradient that is said to be necessary for egress [32]. The development of additional S1P lyase inhibitors probably awaits the development of a facile biochemical assay of this enzyme.

Conclusion

Although FTY720 has proved to be a complicated drug, this molecule has revolutionized the S1P field because it validated S1P signaling as a *bona fide* drug target and raised S1P signaling from merely interesting to potentially important. Once considered merely a step in ceramide catabolism, S1P now has the attention of investigators ranging from neurologists to synthetic chemists. It will be exciting to learn over the next decade whether this attention results in S1P-directed drugs.

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