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Title	Sphingosine 1-phosphate, a potential target in neovascular retinal disease
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Publication date	2021-05-07
Original Citation	Alshaikh, R. A., Ryan, K. B. and Waeber, C. (2021) 'Sphingosine 1-phosphate, a potential target in neovascular retinal disease', British Journal of Ophthalmology, 106(9), pp.1187-1195. https://doi.org/10.1136/bjophthalmol-2021-319115
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1136/bjophthalmol-2021-319115
Rights	© 2021, the Authors. Published by BMJ Publishing Group Ltd. This article has been accepted for publication in British Journal of Ophthalmology following peer review, and the Version of Record can be accessed online at: http://dx.doi.org/10.1136/bjophthalmol-2021-319115 No commercial re-use. - https://creativecommons.org/licenses/by-nc/4.0/
Download date	2026-09-05 08:16:27
Item downloaded from	https://hdl.handle.net/10468/11391

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Sphingosine 1-phosphate, a potential target in neovascular retinal disease

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ABSTRACT

Neovascular ocular diseases (such as age-related macular degeneration, diabetic retinopathy and retinal vein occlusion) are characterized by common pathological processes that contribute to disease progression. These include angiogenesis, edema, inflammation, cell death and fibrosis. Currently available therapies target the effects of vascular endothelial growth factor (VEGF), the main mediator of pathological angiogenesis. Unfortunately, VEGF blockers are expensive biological therapeutics that necessitate frequent intravitreal administration and are associated with multiple adverse effects. Thus, alternative treatment options associated with lower side effects are required for disease management. This review introduces sphingosine 1-phosphate (S1P) as a potential pharmacological target for treatment of neovascular ocular pathologies. S1P is a sphingolipid mediator that controls cellular growth, differentiation, survival and death. S1P actions are mediated by five G Protein Coupled Receptors ($S1P_{1-5}$ receptors) which are abundantly expressed in all retinal and sub-retinal structures. The action of S1P on $S1P_1$ receptors can reduce angiogenesis, increase endothelium integrity, reduce photoreceptor apoptosis and protect the retina against neurodegeneration. Conversely, $S1P_2$ receptor signaling can increase neovascularization, disrupt endothelial junctions, stimulate VEGF release, induce retinal cell apoptosis and degeneration of neural retina. The aim of this review is to thoroughly discuss the role of S1P and its different receptor subtypes in angiogenesis, inflammation, apoptosis and fibrosis in order to determine which of these S1P-mediated processes may be targeted therapeutically.

Key words

Sphingosine-1 phosphate, Neovascularization, VEGF blockers, Angiogenesis, Age-related macular degeneration, Diabetic retinopathy, Retinal vein occlusion.

INTRODUCTION

At least 2.2 billion people suffer from vision impairment, which in 1 billion cases can be attributed to a preventable or treatable cause. Diabetic retinopathy and age-related macular degeneration account for more than one third of these cases.[1] Neovascular ocular disease including diabetic retinopathy (DR), wet age-related macular degeneration (wet-AMD) and retinal vein occlusion (RVO) have different etiology but result in a similar cascade of pathophysiological events (Table 1). Pathological angiogenesis is key amongst these, and a hallmark of disorders that occurs in response to vascular endothelial growth factor (VEGF), a potent hypoxia-induced angiogenic mediator that triggers the formation of new permeable and unstable blood vessels.[2] Pathological angiogenesis can originate from retinal vasculature which supplies the inner retina and/or choroidal vasculature which supplies the outer retinal and retinal pigment epithelium (RPE). RPE is a monolayer of epithelial cells that represent the main structure of outer blood retinal barrier,[3,4] and prevents retinal invasion of neovascular tissue of choroidal blood vessels.[5] In neovascular ocular disease, angiogenesis is accompanied with disruption of RPE physical and metabolic barrier function (Table 1). This results in continuous leakage of blood or blood components to the surrounding tissues, leading to edema and/or hemorrhage, with possible progression to retinal detachment and irreversible apoptosis of photoreceptors and other retinal cells (Table 1). In addition to angiogenesis, chronic hypoxia causes chronic inflammation and over-production of reactive oxygen species in the retina, triggering cell death and fibrotic cascades (Table 1). Fibrosis in the posterior chamber of the eye has unique characteristics, being characterized by the occurrence of gliosis and epithelial to mesenchymal transition (EMT). Later sections describe the role of S1P in both gliosis and EMT.

Table 1. Common pathological events associated with different neovascular ocular diseases. Most of these have been shown to be affected by S1P signaling.

Pathological event	**Occurrence in different neovascular ocular disease**					
	AMD	**DR**	**RVO**	**RP**	**ROP**	**Glaucoma**
Retinal and subretinal neovascularization	[6,7]	[7,8]	[7,9]			
Macular edema	[10]	[7,10]	[7,10]			
Disruption of RPE barrier function		[11]				
Apoptosis or degeneration of photoreceptors, RPE and other retinal cells	[12,13]	[14,15]		[16]		
Chronic inflammation	[17]	[18]		[19]	[20]	
Ganglionic cell death and retinal neurodegeneration	[21]	[22]				[23,24]
Retinal and extraretinal fibrosis	[25]	[26]				

AMD is age related macular degeneration, DR is diabetic retinopathy, RVO is retinal vein occlusion, ROP is retinopathy of prematurity and RPE is retinal pigmented epithelium.

Angiogenesis, edema, inflammation, apoptosis and fibrosis contribute to the pathophysiology of DR, wet-AMD and RVO (Table 1). Current therapeutic options largely rely on the blockade of VEGF signaling with biological therapeutics (antibodies, recombinant fusion proteins or pegylated RNA aptamers). They are expensive, with limited stability and are administered by invasive intravitreal injections that can be associated with retinal detachment, subconjunctival hemorrhage, uveitis, and endophthalmitis.[27] It would therefore be beneficial to develop novel therapeutic agents with fewer limitations. The lipid mediator sphingosine 1-phosphate (S1P) is involved in hypoxia-induced angiogenesis. Unlike VEGF, S1P can promote stable blood vessels formation, increase endothelial barrier integrity and positively impact the subsequent pathophysiological steps leading to neovascular ocular diseases.

This review will provide a brief description of S1P metabolism, the distribution and function of S1P receptor subtypes in different retinal tissues, and will then more specifically focus on the documented effects of S1P and the sometimes opposing roles of various S1P receptor subtypes in the processes that contribute to neovascular disease pathogenesis.

S1P PRODUCTION, METABOLISM AND RECEPTOR EXPRESSION IN OCULAR TISSUES

Sphingolipids are lipid-based cell membrane components. In addition to their structural role, they modulate cellular proliferation, migration, differentiation, and survival.[28,29] They are synthesized by serine palmitoyl transferase (SPT) from palmitoyl CoA and serine as summarized in Figure 1. S1P is produced by phosphorylation of sphingosine by one of two sphingosine kinase isoforms, SphK1 and SphK2.[30] S1P can be de-phosphorylated by the action of two phosphatases, or degraded by S1P lyase to produce inactive metabolites (Figure 1).[31]

The regulation of S1P production and release in different body tissues is not yet completely understood. In plasma, S1P is mainly produced by red blood cells (RBCs), endothelial cells, and platelets. Once produced intracellularly, S1P is transported to the extracellular space leading to significantly higher plasma concentrations of the mediator (~1 μM) compared to interstitial fluid levels.[32] Most circulating S1P is not free, but bound to high-density lipoproteins (HDL), albumin, and to lower extent low-density lipoproteins (LDL).

Under normal conditions, SphK2 is the main S1P-producing kinase in rat and mouse retina.[33,34] Under hypoxic or light-induced stress conditions, SphK1 but not SphK2 is upregulated leading to increased intracellular S1P levels in murine retina [33,35]. Little is known about the levels and role of potential S1P carriers in ocular tissues. Albumin can be found in fetal vitreous, the retina and lens.[36] LDL and HDL can be synthesized locally in the retina [37] or diffuse from the systemic circulation through RPE, although HDL diffusion was significantly lower than LDL in rat retina [38] Apolipoprotein E is synthesized by Müller glial cells in neural retina and transported to vitreous humor,[39] with no information describing retinal expression of apolipoprotein A4 or M.

While S1P can act as an intracellular second messenger and as an extracellular mediator, the latter effects, mediated by five G-protein coupled receptors ($S1P_{1-5}$) predominate.[35] $S1P_{1-3}$ receptors are expressed in almost every body tissue, while $S1P_4$ and $S1P_5$ expression is largely restricted to the lymphatic and nervous systems.[40,41] S1P receptor expression in retina varies depending on cell type and pathophysiological status (see Table 2 for a summary). Under healthy conditions, $S1P_1$ receptors predominate in retina, while photoreceptors mainly express $S1P_2$ receptors (Table 2).[33,35] Retinal pigmented epithelium (RPE) cells show robust $S1P_{1-3}$ receptor expression, with different subtypes predominating in different cell lines.[42,43] Retinal

vasculature endothelial cells, isolated from human donor tissues predominantly express $S1P_2$ and $S1P_3$ receptors (Table 2).[44] New single cell RNA sequencing (scRNA-seq) data show that $S1P_1$ and $S1P_3$ genes are the most strongly expressed in retinal endothelial cells,[45] while choriocapillaris endothelial cells mostly express $S1P_1$, followed by $S1P_3$.[46] Muller glial cells express $S1P_1$ and $S1P_3$ receptors, with $S1P_3$ receptor expression most evident in peripheral rather than foveal Müller glial cells.[47] S1P receptors densities are altered under pathological conditions. $S1P_2$ and $S1P_3$, but not $S1P_1$ receptors, are upregulated following light-induced retinal damage.[33] Likewise, a 5-fold increase in the retinal $S1P_2$ receptors expression is seen in response to hypoxia.[48] Recent reports also highlight the role of $S1P_2$ receptors in laser-induced choroidal neovascular lesions (CNV).[49] Additionally, scRNA-seq reveal noticeable downregulation of both $S1P_1$ and $S1P_3$ receptor expression in the choroidal endothelial cells pooled from an AMD patient.[46]

Growing evidence suggests that S1P plays a significant role in normal retinal development. $S1P_{1-3}$ receptors loss leads to significant defects in the retinal vascular network of post-natal mice.[50] Additionally, S1P has essential functions in photoreceptor development, proliferation, differentiation and survival.[51] A link between sphingolipids and ocular disease was first suspected following the observation of ocular abnormalities in sphingolipidoses, a group of lysosomal storage disorders characterized by build-up of certain sphingolipids in which retinal degeneration, neovascularization and blindness are common manifestations.[52,53] Hereditary and sensory autonomic neuropathy type 1 (HSAN1) is characterized by a mutated SPT. The defective enzyme synthesizes deoxysphingolipids lacking the hydroxyl group at C1, which is essential for synthesis of other sphingolipids. Deoxysphingolipids are cytotoxic, suggesting the involvement of these mediators in the neuropathy of HSAN1.[54] Deoxysphingolipids are also

seen in macular telangiectasia. Patients with this condition have normal SPT, but significantly lower serum serine levels, resulting in the use of alanine instead of serine as a substrate for SPT, ending in formation of deoxysphingolipids. Deoxysphingolipid serum levels in these patients is positively correlated to the disease severity.[55,56] However, a role for S1P and its receptors in these diseases has not been reported and they will not be discussed further in this review.

Table 2. S1P receptors expression in different tissues of the ocular posterior segment.

Posterior segment structure	Experimental condition (cell line/ animal used)	Dominant receptor expression	Other receptors expressed	Detection method	References
Retina	Adult Sprague Dawley rat eye tissue	$S1P_1$	- $S1P_3$ has the highest expression after $S1P_1$ - $S1P_2$, and $S1P_5$ had minimal expression	qRT-PCR	[33]
	Post-natal mice eye tissue	$S1P_1$	- $S1P_3$ has the highest expression after $S1P_1$ -$S1P_2$ had minimal expression	qRT-PCR	[33]
Photoreceptors	661W photoreceptor cell line	$S1P_2$	- $S1P_1$ and $S1P_3$ were detected in significantly lower levels - $S1P_4$ and $S1P_5$ were detected in negligible concentrations	qPCR	[35]
RPE	ARPE-19 cell line	$S1P_2$	- $S1P_5$ and $S1P_1$ exhibit higher expression after $S1P_2$	qPCR	[57]

			- $S1P_3$ was barely detected while $S1P_4$ was not detected.		
	ARPE-19 cell line	$S1P_3$	- $S1P_1$, $S1P_2$, $S1P_4$ and $S1P_5$ were all expressed at lower levels.	Semi quantitative RT-PCR	[43]
	Primary human RPE	$S1P_1$, $S1P_2$, $S1P_3$	- $S1P_4$ almost undetectable - $S1P_5$ has minimal expression	RT-PCR	[42]
RPE-choroid	Adult Sprague Dawley rats	$S1P_3$	-	qRT-PCR	[33]
Retinal vasculature endothelial cells	Primary Human Retinal Endothelial Cells (HREC) isolated from donor tissue	$S1P_2$	- $S1P_3$ was expressed in slightly lower but comparable levels to $S1P_2$. - $S1P_1$ was expressed in significantly lower levels. - $S1P_4$ and $S1P_5$ were not significantly expressed	qRT-PCR	[44]

Optic nerve	Post-natal mice eye tissue	$S1P_3$	- $S1P_1$ has the highest expression after $S1P_3$. - $S1P_2$ was expressed in minute amounts.	qRT-PCR	[33]

qRT-PCR is quantitative reverse transcriptase polymerase chain reaction, qPCR is quantitative real-time polymerase chain reaction and RT-PCR is reverse transcriptase polymerase chain reaction

The altered expression and differential effects of various S1P receptor subtypes under stress conditions offers unique opportunities to target these receptors with selective agonists or antagonists in ocular disorders. The following sections describe the role of individual S1P receptor subtypes in the relevant pathological processes. In this context, it is worth bearing in mind the uncertain specificity of the pharmacological agents used in the studies described in this review.[58] Better characterized clinical candidates, or clinically used S1P receptor drugs have been developed primarily for the management of multiple sclerosis (MS).[59,60] These include approved S1P receptors modulators fingolimod (FTY720), siponimod (BAF312) and ozanimod (RPC1063). Fingolimod binds to all S1P receptors except $S1P_2$, siponimod and ozanimod are selective $S1P_1$ and $S1P_5$ modulators.[61] To the best of our knowledge, these agents have not been used to characterize the role of S1P signaling in ocular pathophysiology. Ocular pathologies and macular edema are associated with fingolimod therapy in MS patients,[62] but may result from MS progression rather than fingolimod treatment.[63]

ANGIOGENESIS

Angiogenesis is the process by which new blood vessels develop from pre-existing ones. The process is carefully regulated by a balance of stimulatory (e.g., VEGF, hypoxia inducible factor (HIF), transforming growth factor-β (TGF-β)) and inhibitory signaling, which maintains a minimal turnover of endothelial cells. In adults, physiological angiogenesis is transient and only occurs during menstrual cycle and wound healing.[64] Under specific conditions (e.g., hypoxia, inflammation, acidosis), quiescent endothelial cells show massive proliferation and migration in a phenomenon called "the angiogenic switch", where the influence of angiogenesis activators exceeds that of the inhibitors.[64]. The vessel formation is then initiated by the release of pro-angiogenic mediators and growth factors.[65] These factors trigger transcriptional responses in the endothelium, with specialized endothelial cells becoming "tip cells" which guide vessel branching toward the angiogenic stimulus. This is followed by enzymatic (e.g., metalloproteineases) lysis of the basement membrane and extracellular matrix, while other endothelial cells proliferate and follow the lead of the tip cells. These cells differentiate into stalk cells responsible for lumen formation, basement membrane deposition, growth and quiescence of new endothelial cell and expression of intercellular junction proteins. This is accompanied by migration of pericytes and vascular smooth muscle cells to support the young blood vessels. Once blood is flowing in the new vessel, fluid shear forces act as an inhibitor of angiogenesis, a process in which $S1P_1$ receptors play a key role.[66]

Under pathological conditions (e.g., diabetic retinopathy), persistent tissue hypoxia results in sustained release of angiogenic stimulators and exhaustive endothelium activation.[67] This triggers erosion of basement membrane in multiple locations, substantial tip cell formation and continuous endothelial proliferation and migration. Due to the high concentration of angiogenic mediators, endothelial activation occurs even in newly formed vessels. This leads to fragile

endothelium with no time for maturation and subsequently no ability to restore blood flow,[64] further exacerbating hypoxia, leakage of blood components to the surrounding tissues and release of more angiogenic mediators in a vicious cycle that contributes to disease progression in many pathologies including neovascular ocular disease.[68]

ROLE OF S1P IN RETINAL AND CHOROIDAL ANGIOGENESIS AND BLOOD VESSEL INTEGRITY

While S1P can be described as a hypoxia-induced pro-angiogenic mediator, this oversimplifies its complex role in vascular growth and stability.[69,70] The effect of S1P in angiogenesis is mediated mainly via $S1P_1$ and $S1P_2$ receptors, which are both highly expressed in retinal endothelium. The number and integrity of the vessels formed in response to this lipid depend on which receptor subtype is principally involved. To add further complexity, the nature of the endothelial response to S1P depends on which carrier protein (albumin, apolipoprotein A4, or M) presents the lipid to its target receptor.[71] Similar observations were made in RPE cells.[49] Finally, it is important to note that $S1P_1$ receptors are an essential component of fluid shear stress sensing and can be activated in the absence of S1P.[66]

$S1P_1$ receptors

$S1P_1$ receptors trigger a distinctive and controlled angiogenic pattern where only a limited number of blood vessel sprouts are formed.[66,72–74] These sprouts undergo full maturation and acquire the characteristic of mature endothelium forming a competent vascular network.[66,75,76] S1P-mediated enhancement of endothelial integrity is abolished in $S1P_1$-knockdown endothelium,[76] while $S1P_1$ gene deletion increases tip cell formation in the retinal vasculature, leading to hyper-sprouting, which is associated with disrupted intercellular junctions, reduced capillary perfusion and hypoxia in surrounding retinal tissues, with VEGF over-expression in

retinal endothelium.[66] Similar results are obtained in postnatal mice retinas by administering the $S1P_1$ antagonist W146.[74] Conversely, $S1P_1$ receptor overexpression reduces the number of tip cells and vessel branch points in retinal vasculature of mice embryos,[66] while $S1P_1$ receptor activation with the $S1P_1$ agonist SEW2871 results in angiogenesis characterized by fewer but longer blood vessel branches and reduces the VEGF angiogenic effect on human umbilical vein endothelial cells (HUVEC) or mouse microvascular endothelial cells.[74] As pathological neovascularization is characterized by higher VEGF levels, the ability of $S1P_1$ receptor activation to antagonize VEGF angiogenic effect is of pathological relevance.[74] Finally, the levels of cell junction proteins are reduced following S1pr1 silencing by siRNA, targeted S1pr1 gene deletion in endothelial cells, or VEGF stimulation.[74]

In conclusion, while $S1P_1$ receptor stimulation is generally pro-angiogenic, it results in the formation of fewer blood vessel sprouts, that develop competent endothelium. This can restore blood flow to the hypoxic retina without resulting in edema or hemorrhage (Figure 2).

$S1P_2$ receptors

$S1P_2$ receptors activate an angiogenetic response similar to that of VEGF,[77] in which continuous, uncontrolled blood vessel sprouting occurs in response to hypoxia. Consequently, vascular maturation is defective due to the sustained endothelial cell proliferation and migration. This results in leaky vascular architecture, with interrupted adherens junctions and inadequate blood flow (Figure 2). Indeed, hypoxia upregulates $S1P_2$ receptors in retinal endothelium and leads to formation of blood vessel sprouts with leaky basement membranes and limited perfusion.[48] In $S1P_2$ knockout mice, hypoxia-induced angiogenesis is characterized by formation of competent blood vessel sprouts that have comparable blood flow to the mature vasculature.[48] Similar results are seen with an $S1P_2$ receptor antagonist, as laser-induced choroidal neovascularization is

reduced after intravitreal injection of JTE013.[49] The effect of $S1P_2$ receptors on angiogenesis may vary in different endothelial cell types, as JTE013 administration increases S1P-mediated abdominal subcutaneous angiogenesis in mice.[78] In addition to angiogenic effects on retinal vasculature, $S1P_2$ activation with albumin-bound S1P results in disruption of barrier integrity and increased RPE permeability. Preincubation of RPE with JTE013 results in the repair of disrupted epithelium and reduced vascular leakage. Additionally, RPE shows increased VEGF release in response to S1P, an effect which is inhibited by JTE013, whereas the $S1P_{1/3}$ receptor antagonist VPC23019 has no effect.[49]

The net effect of S1P on vascular endothelium depends on the balance between $S1P_1$ and $S1P_2$ receptors. In vivo evidence show predominant expression of $S1P_2$ receptors in mouse CNV lesions,[49] upregulation of $S1P_2$ and $S1P_3$ receptors in light-induced damage in rat retinas,[33] and upregulation of $S1P_2$ receptors in a mouse model of retinal ischemia.[48] Additionally, $S1P_2$ knockout mice show enhanced retinal vascularization with normal vascular morphology following ischemic insult compared to *S1P*$_2$+/+ mice[48]. Likewise intravitreal injection of $S1P_2$ antagonist JTE013 significantly reduced CNV lesion areas in mice.[49] This evidence explains why administration of anti-S1P antibodies reduces choroidal neovascularization and vessel leakage.[44,79] an action thought to be mediated by $S1P_2$ receptors. The type of S1P carrying molecules may also matter, as ApoM-bound S1P elicits a favorable activation of $S1P_1$ receptors resulting in reduced vascular leakage and increased expression of junction proteins in RPE, while albumin-bound S1P results in $S1P_2$-mediated disruption of cellular junctions, increased vascular leakage and reduced endothelial integrity (Figure 2).[49]

Relationship between S1P and VEGF signaling

VEGF affects S1P signaling in different ways. It increases S1P production by upregulating SphK expression in endothelial cells,[72] and also increases SphK activity in retinal endothelial cells (RECs).[80] While both actions raise intracellular S1P concentration, most of the intracellular S1P is transported to the extracellular space to act on $S1P_{1-5}$ receptors in autocrine and paracrine manners. VEGF specifically upregulates $S1P_1$ receptor expression in aortic endothelial cells, potentiating nitric oxide/Akt signaling, but has no effect on $S1P_2$ or $S1P_3$ receptor expression, suggesting that under hypoxic conditions, these endothelial cells might be more sensitive to $S1P_1$ signaling.[81] Yet, $S1P_1$ activation with SEW2871 blocks VEGF-induced sprouting in HUVECs and mouse microvascular endothelial cells, while the $S1P_1$ antagonist W146 increases VEGF-induced angiogenesis.[74] This suggests that upregulation of $S1P_1$ receptors in response to VEGF can lessen the overall angiogenic response to VEGF under pathological conditions. S1P results in increased VEGF expression in RPE cells, a response that is diminished after $S1P_2$ blockade with JTE013.[49] Additionally, S1P can transiently activate VEGFR2 receptors in bovine aortic endothelial cells in a tyrosine kinase inhibitor-sensitive manner, resulting in eNOS phosphorylation and activation.[82] SphK inhibition decreases VEGF-mediated RECs proliferation, migration and vascular leakage, indicating that S1P release is involved in VEGF-mediated angiogenesis.[80]

S1P ROLE IN OCULAR INFLAMMATION AND RELEASE OF INFLAMMATORY MEDIATORS

Inflammatory processes also play a role in AMD, DR and RVO pathophysiology. Indeed, the lower incidence of retinopathy among diabetic patients on salicylate therapy for rheumatoid arthritis, and the significant effect of corticosteroid therapy on reducing macular edema and neovascularization in DR highlight the therapeutic relevance of anti-inflammatory drugs in

progression of DR.[83] Retinal ischemia is known to induce the expression of potent inflammatory cytokines including monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α),[84] resulting in leukocyte infiltration and macrophage recruitment. Activated macrophage and microglia secrete inflammatory molecules such as TNF-α and interleukins,[85] which subsequently trigger a complex chain of cellular and vascular responses, the details of which are outside the scope of this review. Levels of MCP-1 are markedly increased in vitreous of patients with DR [86,87] and RVO.[87] Furthermore, markedly elevated IL-8 levels are detected in the vitreous fluid of DR and RVO patients,[87,88] higher IL-6 levels are also detected in the vitreous of DR patients,[86] and complement system activation is reported in AMD.[89] Which highlight the significant role of inflammatory response in neovascular ocular diseases.

In addition to its role in vascular integrity, S1P signaling can modulate inflammation. For instance, S1P reduces vascular leakage, neutrophils infiltration and lung edema after intratracheal administration of lipopolysaccharide.[90] But S1P also increases the production of inflammatory cytokines such as IL-8 and IL-6 among others.[43] S1P increases cyclooxygenase-2 (COX-2) expression and prostaglandin production via $S1P_2$ receptors in renal mesangial cells.[91] Fingolimod suppresses inflammation in an uveoretinitis model,[92] and inhibits leukocyte infiltration when administered as a single dose before induction of ocular inflammation.[93] Fingolimod-treated MS patients show a lower incidence of ocular inflammation compared to other MS patients.[94] It is unclear whether the ocular anti-inflammatory effects of fingolimod are due to its agonist or functional antagonist activity.[61,95] S1P increases IL-8, but not IL-6, production by RPE cells in a Pertussis toxin sensitive manner, suggesting $S1P_1$ receptor involvement.[43] However, another study suggests a role for $S1P_2$ receptors, as S1P-induced production of IL-8 and

CCL2 in RPE cells is decreased by JTE013, but not by $S1P_1$ or $S1P_3$ antagonists.[49] These apparently contradictory reports suggest that further work is needed to assess the role of different S1P receptors in retinal inflammation.

S1P ROLE IN PHOTORECEPTORS APOPTOSIS AND NEURODEGENERATION

Retina, being a part of the CNS, comprises full neuronal circuits to acquire, convert and transfer electrical activity of photoreceptors to the brain, which is known as neural retina. Neural retina is a multilayered interconnected structure composed of five cell types, these are photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells.[96] Neurodegeneration in retinal diseases usually refer to apoptosis of retinal ganglionic cells and photoreceptors which leads to significant and progressive loss in visual function [24]. Retinal neurodegeneration is evident in diabetic retinopathy,[15] and neovascular AMD,[21] although the exact mechanism of neurodegeneration is not fully elucidated. Nevertheless, hypoxia-associated distorted retinal blood flow in these diseases is suggested to be the main reason to trigger neuronal death.[24] The role of S1P signaling in normal development of CNS is thoroughly reported, as sphingosine kinase null mice embryos suffered from neuronal tube defects with massive apoptosis in neuroepithelium.[97] Additionally, S1P signaling is involved in nerve growth factor mediated neurite extension,[98] and neuronal excitability.[99] S1P is required for development, differentiation and proliferation of photoreceptors in rat retinas.[51]

Under stress-induced photoreceptors and retinal ganglionic cells apoptosis, S1P can elicit different responses.[100,101] On one hand, S1P promotes cellular proliferation and reduces photoreceptor apoptosis.[102] Decosahexanoic acid (DHA, a mediator of photoreceptor survival and differentiation) increases intracellular S1P levels by upregulating SphK, and the protective

effects of DHA are blocked after SphK inhibition.[51] Similarly, action of S1P on $S1P_1$ receptors increases the survival of and mitigates the damage to retinal ganglionic cells following optic nerve injury,[103] and the selective $S1P_1$ receptor agonist CYM-5442 reduces retinal ganglionic cell damage after endothelin-1 induced vasoconstriction.[104] On the other hand, S1P acts as a pro-apoptotic mediator that can intensify the degenerative response in photoreceptors.[10] an action that is suggested to be mainly mediated by $S1P_2$ receptor activation.[35]

Under pathological conditions in the retina as hypoxic and oxidative stress, or optic nerve injury, $S1P_2$ receptors are upregulated while $S1P_1$ receptors are down-regulated.[33,103] This makes the role of $S1P_1$ receptors in ganglionic cell and photoreceptor survival is less obvious under pathological conditions.[103] At variance with the trophic effect of $S1P_1$ receptors, $S1P/S1P_2$ signaling elicits a detrimental effect on neuronal cells.[103]

S1P ROLE IN FIBROSIS, GLIOSIS AND EPITHELIAL TO MESENCHYMAL TRANSITION (EMT)

Fibrosis is a reparative process that occurs in response to tissue injury, where the injured tissue is replaced by non-functional, collagen rich fibrous matrix. Outside the CNS, fibroblasts are the main players in fibrosis, as they migrate to the injured location, proliferate, synthesize and deposit extracellular matrix proteins.[105] As retina is considered a part of the CNS, the fibrotic response in the posterior chamber utilizes different mechanisms and cellular incorporation compared to that seen in non-CNS tissues.[106] Retina has a scarce fibroblast population; instead the fibrotic response is mainly mediated by RPE and Müller glial cells. RPE and glial cells are quiescent and non migratory under normal condition. Under inflammatory conditions or tissue injury, these cells undergo specific trans-differentiation to acquire fibroblast-like phenotype in processes known as EMT or gliosis. During EMT, RPE cells lose their epithelial traits and acquire

mesenchymal/fibroblast like phenotype, becoming invasive, migratory, lacking tight junction proteins and expressing mesenchymal markers.[107]. The fibrocontractile nature of transformed RPE can result in retinal detachment and severe vision impairment ending in further disease exacerbation. Similar transitional events occur in Müller cell gliosis, where Müller glial cells transdifferentiate to a fibroblast-like phenotype, release trophic mediators, and acquire proliferative and migratory properties.[108,109] Again, this transition to fibrocontractile structure results in gliotic scar tissue formation, which further exacerbates retinal damage.[110] While multiple cytokines and several proinflammatory signals can trigger a fibrotic response, there is growing evidence that TGF-β is one of the most important cytokines that contribute to EMT [111,112] and gliosis.[113], as it is detected at higher levels in the vitreous of patients with DR.[114]

S1P signaling generally triggers fibrotic events in neovascular ocular disease. Administration of an anti-S1P antibody reduces collagen precipitation in sub-retinal structures after rupture of Bruch's membrane in a mouse model of CNV.[44] Likewise, locally injected anti-S1P monoclonal antibodies can alleviate conjunctival scarring following glaucoma filtering surgery.[115] S1P increases the production of contractile actin fibers and facilitates collagen deposition by RPE in vitro, one of the mesenchymal characteristics of EMT,[42] but the exact mechanism and S1P receptor subtype involved has not been reported. Migration of Müller glial cells in vitro is significantly increased by exogenously added S1P. Additionally, inhibition of SphK1, the main isoform in Müller glial cells, abolishes filopodia formation and cellular migration, suggesting that both endogenous and exogenous S1P amplify glial cell migration. S1P mediated actions are reduced by pre-treatment of Müller glial cells with the $S1P_3$ antagonist BML-241, suggesting that the effects are primarily mediated by $S1P_3$ receptors.[109]. Likewise, SphK1-

null mice show diminished gliosis and slower progression of Sandhoff disease, a central neurodegenerative disease. Similar results are obtained by $S1P_3$ receptor gene deletion.[116]

Although the relationship between S1P and TGF-β signaling in neovascular ocular disease is not yet reported, there is established evidence of crosstalk between S1P and TGF-β in renal mesangial cells.[117]. TGF-β increases SphK1 in endometriotic stromal cells [118], human fibroblasts,[119] and in human kidney podocytes.[120] SphK1 upregulation in kidney is associated with protective rather than detrimental effects, as SphK1 deficient mice develop more drastic streptozocin induced nephropathy.[120]

CONCLUSION

S1P is a promising therapeutic target that modulates angiogenesis, inflammation, apoptosis and fibrosis associated with neovascular ocular diseases. S1P/$S1P_1$ signaling can induce formation of competent blood vessel sprouts, increase retinal perfusion and reduce cell apoptosis and neurodegeneration. Blocking $S1P_2$ receptors achieves similar beneficial outcomes, while $S1P_3$ receptor antagonism or $S1P_1$ activation can inhibit gliosis. Under hypoxic conditions, SphK1 and $S1P_2$ are upregulated; this can be accompanied by $S1P_1$ downregulation, resulting in increased S1P production and predominant signaling through $S1P_2$ receptors. Therefore, inhibition of $SphK_1$, $S1P_1$ activation and $S1P_2$/$S1P_3$ antagonism might be used to attenuate retinal damage in ocular neovascular disease. Recent clinically approved S1P receptor modulators include siponimod and ozanimod; both can selectively activate $S1P_1$ receptors (along with $S1P_5$ receptors, which are less prevalent in ocular tissue). Evidence to date suggests that S1P receptor modulation plays important roles in the pathogenesis and treatment of neovascular ocular diseases. However, the role of these agents in the progression of neovascular ocular disease should be elucidated in preclinical models

to inform future clinical trials involving S1P receptor modulators already approved for other conditions.

ACKNOWLEDGMENTS

Figure 1 was created in part using with BioRender. Elements of Figure 2 are from the Servier medical arts database.

COMPETING INTERESTS:

NA

FUNDING

This work was funded by the Irish research council (IRC).

CONTRIBUTORSHIP STATEMENT

RAA, KBR, and CW contributed to the drafting of manuscript. RAA and CW contributed to the interpretation of the data in the literature. All authors contributed to the critical appraisal and final approval of the manuscript. CW provided the overall supervision of this work.

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FIGURE LEGEND

Figure 1.

Schematic representation of synthesis and metabolism of sphingolipids, with special emphasis on S1P and the 5 receptor subtypes it can activate ($S1P_{1-5}$ receptors) as well as the G proteins they are coupled to. Specific S1P receptor modulators discussed in this review are listed at the bottom of the figure (blue and red circles indicate agonist and antagonist activities, respectively). This figure was created by the authors, using the software Biorender.

Figure 2.

Summary of S1P-mediated effects on angiogenesis. Top: $S1P_1$ receptor activation by S1P reduces proangiogenic factors release in response to hypoxia (most importantly VEGF) leading to fewer tip cell formation and fewer branching points per unit area. This leads to formation of fewer vessel branches. $S1P_1$ receptors also increase intercellular junction proteins expression and perivascular cells coverage of newly formed sprouts. This leads to formation of competent blood vessels with normal blood flow that restore tissue perfusion (red areas) and downregulate the angiogenic signal. Bottom: Hypoxia results in over-expression of SphK1 and $S1P_2$ receptors. This receptor subtype increases VEGF release and tip cells number resulting in increased vessel branching per unit area. The formed branches have defective expression of intercellular junction proteins and irregular perivascular cells coverage. This leads to formation of leaky endothelium with diminished blood flow which further exacerbate tissue hypoxia (blue areas). Due to sustained angiogenesis, further branching of the new sprouts occurs leading to further leaking and hemorrhage. The net effect of these two opposite signals depends on relative receptors densities and specific receptor upregulation in response to hypoxia. Although their abundance in the eye is not known, the balance

of S1P carrier proteins may also play a role, as ApoM-bound S1P and Albumin-bound S1P preferentially activate $S1P_1$ and $S1P_2$ mediated cascades, respectively. This figure was created by the authors using elements from the Servier medical arts database.

Figure 1:

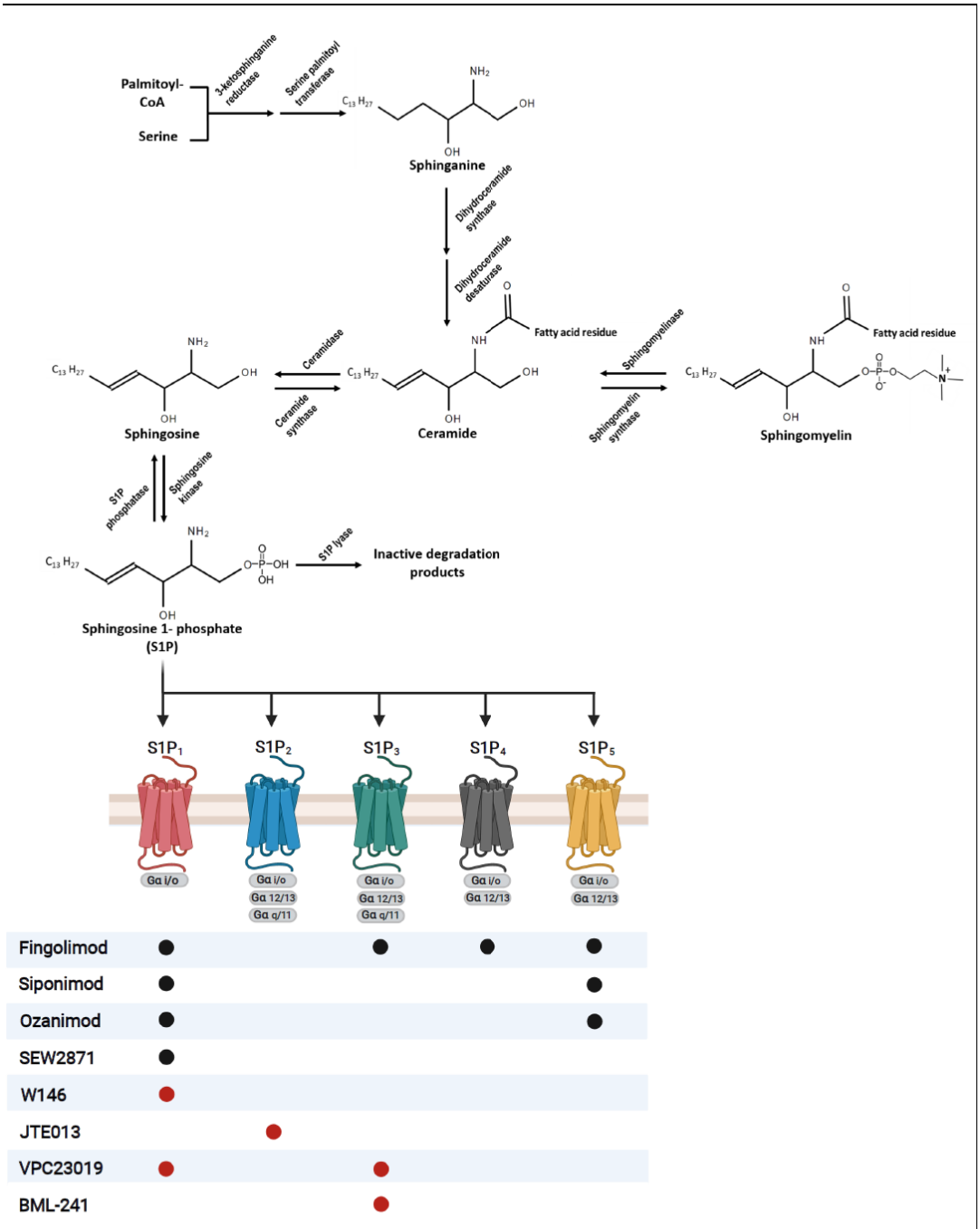
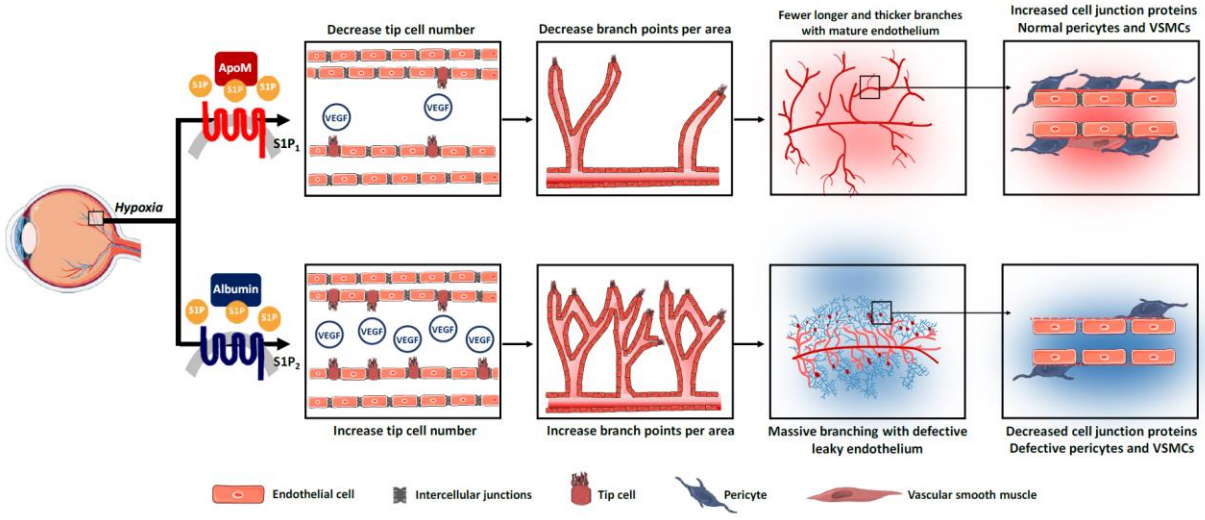

Palmitoyl-CoA
Serine
3-ketosphinganine reductase
Serine palmitoyl transferase
Sphinganine
Dihydroceramide synthase
Dihydroceramide desaturase
Fatty acid residue
Sphingosine
Ceramidase
Ceramide synthase
Ceramide
Sphingomyelinase
Sphingomyelin synthase
Sphingomyelin
S1P phosphatase
Sphingosine kinase
Sphingosine 1- phosphate (S1P)
S1P lyase
Inactive degradation products
S1P1
S1P2
S1P3
S1P4
S1P5
Gα i/o
Gα 12/13
Gα q/11
Fingolimod
Siponimod
Ozanimod
SEW2871
W146
JTE013
VPC23019
BML-241

Figure 2


Hypoxia
ApoM
S1P
S1P1
Albumin
S1P2
VEGF
Decrease tip cell number
Decrease branch points per area
Fewer longer and thicker branches with mature endothelium
Increased cell junction proteins Normal pericytes and VSMCs
Increase tip cell number
Increase branch points per area
Massive branching with defective leaky endothelium
Decreased cell junction proteins Defective pericytes and VSMCs
Endothelial cell
Intercellular junctions
Tip cell
Pericyte
Vascular smooth muscle