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The sphingosine 1-phosphate receptor modulator fingolimod as a therapeutic agent: recent findings and new perspectives.

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Abstract

The immunomodulatory drug fingolimod (FTY720, Gilenya®) was approved for oral treatment of relapsing-remitting multiple sclerosis, due to its impressive efficacy and good tolerability. Pharmacologically, it acts as an unselective agonist of sphingosine 1-phosphate receptors (S1PR) and as a selective functional antagonist of the S1P₁ subtype by induction of receptor downregulation. Since S1P₁ is crucial for the regulation of lymphocyte trafficking, its downregulation causes redistribution of the immune cells to secondary lymphoid tissues, resulting in the depletion from the circulation and hence immunosuppression. Numerous preclinical studies have since been performed with the aim to increase the spectrum of potential indications for fingolimod with emphasis on other autoimmune disorders and diseases associated with inflammation and uncontrolled cell proliferation, including cancer. As an alternative to fingolimod, novel S1PR modulators with a more selective receptor activation profile and improved pharmacokinetic performance and tolerability have also been developed. Preclinical and clinical studies are ongoing to investigate their therapeutic potential for various indications. This review discusses the most relevant preclinical and clinical findings from S1PR-targeting and from less-well defined off-target effects reported in the literature, and reveals perspectives for using fingolimod and functionally-related derivatives and new formulations in the management of an increasing number of diseases.

Keywords: Fingolimod, S1P receptor modulators, preclinical developments, pharmacology, clinical indications, adverse events
List of abbreviations:

ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotropic factor; CerS, ceramide synthase; CNS, central nervous system; CPL, cecal ligation and puncture; cPLA₂, cytosolic phospholipase A₂; CSA, cyclosporine; CTL, cytotoxic T lymphocytes; CYP450, cytochrome P450; DAMPs, damage-associated molecular patterns; EAE, experimental autoimmune encephalomyelitis; ERK, extracellular signal-regulated protein kinase; FTY720, fingolimod; HDAC, histone deacetylase; HDL, high density lipoprotein; ISP-1, myriocin; KO, knockout; LPA, lysophosphatidic acid; LPP3, lysophospholipid phosphatase 3; MECP2, methyl-CpG-binding protein 2; MMF, mycofenolate mofetil; MS, multiple sclerosis; NK, natural killer cells; PECAM, platelet endothelial cell adhesion molecule; PP2A, protein phosphatase 2A; PPMS, primary progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; Sgpl1, S1P lyase; SIRS, systemic inflammatory response syndrome; SLE, systemic lupus erythematous; SPP1, S1P phosphatase 1; T1D, type 1 diabetes mellitus; T2D, type 2 diabetes mellitus; Th, T helper cell; TLR, Toll-like receptor; TNBS, trinitrobenzene sulfonic acid; TRPM7, transient receptor potential cation channel M member 7.
1. Historic development of fingolimod

Fingolimod (FTY720, Gilenya®, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol) was originally synthesized by the Japanese chemist Tetsuro Fujita from Yoshitomi Pharmaceutical Industries Ltd. (present Mitsubishi Tanabe Pharma Corporation, Japan) (Adachi et al., 1995) using the natural compound myriocin (ISP-1) as a lead. Myriocin was previously isolated by the same group from the culture broth of Isaia sinclairii, the imperfect or asexual stage of the genus Cordyceps sinclairii (cordycipitaceae), a subfamily of parasitic fungi. It was described as an immunosuppressant 10-100 times more potent than cyclosporin A (Chiba et al., 1998; Fujita et al., 1996). Remarkably, extract and powder from the near relative Cordyceps sinensis has been widely used in Traditional Chinese Medicine due to its energy boosting effect and to grant eternal youth. Based on the chemical structure of myriocin, a series of derivatives were synthesized from which FTY720 turned out to be an even more potent immunosuppressant when tested in vitro in a mouse allogenic mixed lymphocyte reaction assay, and in vivo in rat and dog transplantation models where allograft survival was prolonged for several weeks (Chiba et al., 1998; Fujita et al., 1996; Suzuki et al., 1996b).

2. Mechanism of action of fingolimod

The main immunomodulatory mechanism of action of fingolimod is based on its effect on lymphocyte homing. It reversibly redistributes T and B cells from the circulation to secondary lymphoid organs like peripheral and mesenteric lymph nodes and Peyer's patches, thereby causing a state of peripheral lymphopenia (Chiba et al., 1998). Since fingolimod resembles in chemical structure the sphingolipid molecule sphingosine, it can serve as a substrate for sphingosine kinase (SphK) to become phosphorylated to the active metabolite fingolimod-P (Brinkmann et al., 2002). Thus, fingolimod is a pro-drug which requires SphK to become active. Two subtypes of SphK, SphK1 and SphK2, exist and in principle both enzymes can phosphorylate fingolimod in vitro (Billich et al., 2003). However, SphK2 is 30-fold more efficient due to a lower Km value of fingolimod for SphK2 compared to SphK1 (Billich et al., 2003). Furthermore, SphK2 is the only enzyme which activates fingolimod in vivo, since only Sphk2 knockout mice are resistant to fingolimod-induced lymphopenia (Zemann et al., 2006) and lack fingolimod-mediated protection from disease symptoms in experimental autoimmune encephalomyelitis (EAE), a widely used animal model for multiple sclerosis (Imeri et al., 2016).

Fingolimod-P mimics sphingosine 1-phosphate (S1P) in structure and thus, not surprisingly, also binds to S1P receptors (Brinkmann et al., 2002) (Mandala et al., 2002). S1P receptors
were originally described as endothelial differentiation genes (edg) and build a subclass of G protein-coupled lipid receptors, which are most homologous to the lysophosphatidic acid (LPA) receptors. So far, 5 subtypes of S1P receptors have been identified, denoted S1P$_1$-S1P$_5$. They have all been determined to bind S1P with high affinity (Kihara et al., 2014) with binding constants ranging from 1 to 10 nM, except for S1P$_4$, which has a ten-fold lower affinity (Mandala et al., 2002). Fingolimod-P binds with similar affinity as S1P to S1P$_1$, S1P$_3$, and S1P$_5$, but shows much better binding to S1P$_4$ than S1P (6 nM versus 90 nM) (Mandala et al., 2002). Fingolimod is not a ligand for S1P$_2$. Due to the diversity of S1P receptor subtypes with their distinct function and ubiquitous expression in the body (Kihara et al., 2014; O'Sullivan et al., 2017), in theory multiple effects can be expected from using fingolimod, which may either have therapeutic benefit or cause adverse events.

3. Specific Fingolimod targets

3.1. The S1P receptor family

The S1P$_1$ receptor

As outlined above and because of its binding to and activation of the various S1P receptor subtypes, except for S1P$_2$, “active” fingolimod is pharmacologically considered an unselective S1P receptor agonist (Brinkmann, 2007; Brinkmann et al., 2002; Mandala et al., 2002). Moreover, it causes sustained desensitization of the S1P$_1$-mediated signaling pathway by inducing receptor internalization and degradation, which on the cellular level results in functional antagonism. This effect of fingolimod on S1P$_1$ is unique and not seen with the endogenous ligand S1P, which also internalizes S1P$_1$ upon binding but then dissociates in endosomes and the receptor recycles back to the plasma membrane. Similarly, S1P$_3$, S1P$_4$, and S1P$_5$ are also internalized upon fingolimod-P binding and then redistribute back to the cell surface. Particularly, downregulation of S1P$_1$ on T cells is supposed to account for the immunosuppressive effect of fingolimod, whereas downregulation of S1P$_1$ in other cell types, notably in endothelial cells, is likely responsible for the adverse events observed under long-term fingolimod treatment (Brinkmann, 2007). The agonistic effect of fingolimod on S1P$_3$, S1P$_4$, and S1P$_5$ may account for additional biological effects with unknown consequences as outlined below.

These early pioneering studies have fueled research on S1P$_1$ receptor biology to better understand its role in human diseases. Since S1P$_1$ is ubiquitously expressed in almost every cell type, its downregulation by prolonged fingolimod treatment is expected to have multiple consequences on cellular responses and tissue homeostasis. The generation of systemic...


*S1pr1* knockout mice corroborated the vital biological function of \( S1P_1 \), since embryos die in the developmental stage of day E12.5-E14.5, due to impaired vessel maturation and hemorrhagic bleedings.

Besides its role in immune cell trafficking and vascular development, \( S1P_1 \) is implicated in the regulation of vascular integrity. Increased vascular permeability is a typical feature of inflammation and allergy, and therefore a possible barrier protective effect of \( S1P \) signaling is of therapeutic interest. Various *in vitro* and *in vivo* studies have investigated the effect of \( S1P \) and fingolimod on the endothelial barrier function. It was shown that endotoxin-induced microvascular permeability and inflammation leading to acute lung and kidney injury in mice can be reduced by direct application of either \( S1P \) or fingolimod (Peng *et al.*, 2004). On the contrary, application of a \( S1P \), receptor antagonist (W146), induced loss of capillary integrity in mouse skin and lung (Sanna *et al.*, 2006), and the most recently developed potent \( S1P \), selective antagonist NIBR-0213 induced transient lung and heart permeability defects in rats, which promoted chronic inflammatory remodeling (Bigaud *et al.*, 2016). Furthermore, a conditional gene deletion approach was used to demonstrate that plasma \( S1P \) is crucial for vascular integrity. Since \( Sphk1/Sphk2 \) double knockout mice are embryonically lethal, a conditional double knockout (KO) mouse was generated, which contains one conditional \( Sphk1 \) allele and one null \( Sphk1 \) allele in a \( Sphk2 \) null background (\( Sphk^{+/−} \) \( Sphk2^{−/−} \)) and carries a Cre transgene. By inducing Cre, plasma \( S1P \) levels became undetectable. These „pS1Pless“ mice showed a several-fold increased vascular leakage and reduced survival compared to wildtype mice upon administration of platelet activating factor (PAF), and the symptoms could be relieved by injection of a selective \( S1P_1 \) agonist (AUY954) (Camerer *et al.*, 2009).

Improved endothelial barrier function resulting from \( S1P_1 \) activation by \( S1P \) and fingolimod was also demonstrated in cultures of human endothelial cells *in vitro* (Dudek *et al.*, 2007). The molecular mechanisms underlying the barrier enhancing effect are not yet fully understood, but the role of adherens junction molecules, such as VE-cadherin and platelet-endothelial cell adhesion molecule (PECAM-1), appears to be crucial. In this regard, it was shown that \( S1P \) and fingolimod-P can stimulate the translocation of VE-cadherin to cell-cell contact sites of endothelial cells in cultures (Sanchez *et al.*, 2003), and *in vivo* in \( S1pr1 \) knockout mice the retinal vasculature lost VE-cadherin staining (Gaengel *et al.*, 2012). For PECAM-1, upregulation was detected in endothelial cells overexpressing SK-1, which is supposed to increase \( S1P \) levels (Limaye *et al.*, 2005). Furthermore, \( S1P_1 \) silencing was shown to reduce expression of PECAM-1 and VE-cadherin (Krump-Konvalinkova *et al.*, 2005), and deregulation of these adherens junction molecules by fingolimod was also seen in a mouse model of multiple sclerosis, i.e. EAE (Imeri *et al.*, 2016). On the other hand, the barrier enhancing effect of fingolimod seems to be dose-dependent, since higher
concentrations in the range of 10 to 100 µM in cultures of human umbilical vein endothelial cells rather compromised the barrier function, and in vivo in mechanically ventilated mice even aggravated lung injury (Muller et al., 2011). Similarly, in a bleomycin-induced lung injury model in mice, prolonged exposure to fingolimod resulted in vascular leak, fibrosis and increased mortality (Shea et al., 2010).

Altogether, these studies suggest that S1P₁ activation improves the endothelial barrier function, whereas S1P₁ antagonism, notably also by prolonged fingolimod treatment, disrupts it, thereby increasing permeability and vascular leakage.

The S1P₃ receptor

A more detailed characterization of S1P₃ activation by fingolimod-P showed that it has an EC₅₀ value of 7 to 10 nM for S1P₃, which is comparable to the endogenous ligand S1P. However, its efficacy reached only 50% of S1P, suggesting a partial agonistic effect (Riddy et al., 2012). By definition, a partial agonist in the presence of a full agonist produces an antagonistic output. This could mean that in vivo, fingolimod can also antagonize S1P₃ signaling rather than stimulate it, depending on the local S1P concentration. This idea is appealing and warrants further investigation, particularly in view of the potential anti-tumor activity of fingolimod discussed in chapter 5.3. In this context it is noteworthy that S1P₃ was recently shown to have prometastatic properties in breast and lung carcinoma cells (Filipenko et al., 2016; Zhang et al., 2013), and high expression is frequently found in tumors from breast cancer patients where it correlates with poor prognosis (Watson et al., 2010).

Since plasma S1P levels are often elevated in cancer patients (Alberg et al., 2013; Sutphen et al., 2004; Zhang et al., 2015), the requirements for fingolimod to act as a partial S1P₃ agonist and thus impede metastatic growth might be fulfilled.

The S1P₄ receptor

The role of S1P₄ in physiological processes is still poorly understood and therefore effects of fingolimod mediated by S1P₄ are unclear. S1P and fingolimod-P associate with S1P₄ with binding constants of 95 nM and 6 nM, respectively, which means that fingolimod is a much better ligand for this receptor than the endogenous ligand (Mandala et al., 2002). S1P₄ expression is restricted in the body and mainly found in lymphocytes and tissues of the immune and hematopoietic system (Graler et al., 1998).

Knockout of S1pr4 in mice and zebrafish consistently revealed a reduction of the number of circulating neutrophils, suggesting a role for S1P₄ in immunity and infection (Allende et al., 2011; Pankratz, 2016). S1pr4 deficient mice also show differential reactions to inflammation
with exacerbated T helper (Th)2 cell responses and decreased responses of Th1 cells (Allende et al., 2011). Moreover, megakaryocytes generated from S1pr4 deficient mice showed atypical and reduced formation of proplatelets in vitro, and the recovery of platelet numbers after experimental thrombocytopenia was significantly delayed, suggesting a role for S1P4 in thrombopoiesis (Golfier et al., 2010). Of note, in humans, a rare missense variant of S1P4 (Arg365Leu) was reported, which generates a loss-of-function receptor and is associated with reduced white blood cells and neutrophil counts (Pankratz, 2016), thus corroborating the preclinical data from mice (Allende et al., 2011).

The S1P5 receptor

The S1P5 receptor was originally cloned as rat nerve growth factor-regulated G protein-coupled receptor Nrg-1 (Glickman et al., 1999) and later found to be identical to S1P5/edg8. It is predominantly expressed in the brain and spleen (Im et al., 2000; Malek et al., 2001), and in these tissues, it is further concentrated in oligodendrocytes and natural killer (NK) cells (O'Sullivan et al., 2017).

Fingolimod-P and S1P bind to S1P5 with equally high affinity. In oligodendrocytes this triggers two distinct functional responses depending on the developmental stage of the cells. It leads to retraction in pre-oligodendrocytes, whereas it increases the survival of mature cells (Jaillard et al., 2005). In addition, migration of oligodendrocyte precursor cells (OPCs), which normally migrate over considerable distances during brain development, is inhibited by S1P5 activation (Novgorodov et al., 2007). Fingolimod was also shown to protect human oligodendrocytes from apoptosis induced by serum and glucose deprivation, suggesting a neuroprotective effect by activating S1P5 (Miron et al., 2008). Moreover, S1P5 is also expressed on brain microcapillary endothelial cells where it contributes to the blood-brain barrier function and maintains the immunooquiescent state of brain endothelial cells (van Doorn et al., 2012).

In S1pr5 knockout mice the number of circulating NK cells is decreased (Walzer et al., 2007) proposing a function of S1P5 is to promoting NK cell egress from bone marrow and lymph nodes into the blood, and recruiting them to sites of inflammation (Jenne et al., 2009; Walzer et al., 2007). S1pr5 knockout mice also lack circulating Ly6C-negative peripheral monocytes, but maintain normal levels in the bone marrow (Debien et al., 2013).

3.2. Other targets of Fingolimod

Besides S1P receptors, which are activated by fingolimod at low nM concentrations, other intracellular targets of either fingolimod or fingolimod-P have been described. These include
SphK1, S1P lyase (Sgpl1), ceramide synthase (CerS) 2, histone deacetylases (HDACs), cytosolic phospholipase A2, protein phosphatase A2 (PP2A), and the cation channel TRPM7. However, these off-targets respond only at much higher concentrations in the μM range. It was recently reported that fingolimod can in principle accumulate to high μM concentrations in cells in vitro to achieve concentrations required for such “off-target” effects (Schroder et al., 2015). However, it is unlikely and evidence has never been provided that passive tissue targeting of fingolimod occurs in vivo. Moreover, the low target selectivity of fingolimod at such high concentrations is not appropriate for therapeutic use.

SphK1
As determined in vitro, fingolimod is a substrate of SphK-2 and is also phosphorylated by SphK-1, but with much lower efficiency (Billich et al., 2003). In vivo, however, SphK2 is the only kinase which activates fingolimod as demonstrated in Sphk1- and Sphk2-deficient mice. Fingolimod, but not fingolimod-P, acts also as a direct inhibitor of SphK1, but very high concentrations of 50 μM are needed to achieve 50% inhibition (Tonelli et al., 2010; Vessey et al., 2007). As a pharmacological target with oncogenic potential, SphK1 is of interest for cancer therapy (see chapter 5.3). Considering the toxicity profile of fingolimod at 0.5 mg per day in patients (Table 2), however, it is unrealistic to believe that such high doses can be tolerated. Nevertheless, numerous preclinical studies have shown pro-apoptotic and anti-tumor effects of fingolimod with tumor cell lines in vitro and in tumor models in mice, which could be partly attributed to inhibition of SphK1 (Pchejetski et al., 2010).

S1P lyase
S1P lyase (Sgpl1) irreversibly degrades S1P to hexadecenal and phosphoethanolamine as the final step for eliminating sphingolipids in the cell in addition to secretion. In contrast to S1P, fingolimod is not a substrate for Sgpl1 and was even shown to inhibit it (Bandhuvula et al., 2005). Moreover, treatment of mice with fingolimod inhibits tissue Sgpl1 within 12 h, resulting in stable or even increased S1P levels. It was therefore proposed that disruption of S1P metabolism by inhibiting Sgpl1 accounts for some of the effects of fingolimod on immune cells (Bandhuvula et al., 2005). In support of this, inhibition of Sgpl1 using novel selective inhibitors can mimic the effect of fingolimod on lymphocyte homing (Bagdanoff et al., 2010; Weiler et al., 2014).

Ceramide synthase 2
Ceramide synthases (CerS) comprise a family of enzymes that acylate dihydrosphingosine to dihydroceramides and therefore are crucial for de-novo synthesis of ceramides (Park et al., 2014; Wegner et al., 2016) and consequently, also the downstream metabolite S1P. Six subtypes of CerS have been identified which all have certain substrate preferences and utilize a restricted subset of fatty acid-CoAs for dihydroceramide synthesis, thereby generating ceramides of different chain lengths (Wegner et al., 2016). In vitro activity studies revealed non-competitive inhibition of CerS2 by S1P with an IC\textsubscript{50} value of approx. 20 \mu M (Laviad et al., 2008). This inhibition may represent a negative feedback regulation ensuring that the overproduction of cellular S1P turns off de-novo synthesis of the precursor ceramide and thus ensures a balanced sphingolipid homeostasis. Fingolimod, but not the phosphorylated form, was shown to inhibit CerS only at high concentrations in a cell-free assay with an IC\textsubscript{50} value of 30 to 50 \mu M (Lahiri et al., 2009), and also in experiments with human pulmonary artery endothelial cells (Berdyshev et al., 2009).

**Protein phosphatase 2A**

The protein phosphatase 2A (PP2A) is an important cellular Ser/Thr phosphatase that regulates multiple signal transduction pathways by dephosphorylating protein kinases and other enzymes (Millward et al., 1999). Its role as a tumor suppressor became evident for the first time when it was found to be strongly inhibited by the tumor promoter ocadaic acid (Bialojan et al., 1988). PP2A is a heterotrimeric complex built by three subunits, including a scaffolding subunit, a regulatory subunit and a catalytic subunit. Each subunit again exists in several isoforms and additional splice variants which all together allow the existence of 80 different subforms of the PP2A holoenzyme. This finally directs its subcellular localization and allows for such a huge diversification of substrates.

The first observation that PP2A is affected by sphingolipids was reported in 1993 showing that short-chain C2-ceramide could increase PP2A activity in vitro, whereas other sphingolipids such as dihydro-C2-ceramide or sphingosine had no activating effect (Dobrowsky et al., 1993). Later on, activity studies using the purified PP2A holoenzyme revealed a weak effect of fingolimod on PP2A activation in the range of a 60% increase at 10 \mu M, which was sufficient to inhibit Akt and induce apoptosis in leukemia cells (Matsuoka et al., 2003). The relevance of PP2A as a target of fingolimod in cancer therapy is discussed in chapter 5.3.

**Histone deacetylases (HDAC)**
Histone acetylation is implicated in the epigenetic regulation of gene expression. The acetylation reaction is catalyzed by histone acetyltransferases (HAT) and associates with active gene transcription because acetylation of histones loosens the contact between core nucleosome proteins and DNA and facilitates the binding of transcription factors to their respective binding sites (Eckschlager et al., 2017; Kelly et al., 2013). On the contrary, histone deacetylases (HDAC) remove acetyl groups from histones, thereby tightening the chromatin structure, which hinders binding of transcription factors and hence suppress gene transcription. Numerous studies demonstrated that HDAC inhibitors have therapeutic potential to treat diseases such as cancer, inflammation, fibrosis and even cognitive disorders (Eckschlager et al., 2017; Fischer et al., 2010; Hull et al., 2016; Liu et al., 2015). Notably, certain HDAC inhibitors are already FDA approved and used for the treatment of relapsed and refractory cutaneous T-cell lymphoma (vorinostat), peripheral T cell lymphoma (belinostat) and multiple myeloma (panobinostat) (Eckschlager et al., 2017). Recently, it was reported that S1P generated by Sphk2 in the nucleus binds directly to HDAC and inhibits its activity. This results in hyperacetylation of histones and regulates expression of certain genes such as the cyclin-dependent kinase inhibitor p21 and c-fos (Hait et al., 2009). A similar effect was also exerted by fingolimod-P (Hait et al., 2014) suggesting that SphK2 is a driver of epigenetic gene regulation.

The phospholipase A₂

The phospholipase A₂ (PLA₂) hydrolyses phospholipids at the sn2 position and thereby liberates fatty acids and lysophospholipids. Particularly, the liberation of arachidonic acid, which serves as a precursor for all eicosanoids including prostaglandins, leukotrienes and thromboxane, is a crucial and rate limiting step in inflammatory reactions (Dennis et al., 2011). PLA₂ exists as a family of several subtypes subclassified as secreted low molecular weight enzymes (sPLA₂s), cytosolic Ca²⁺-dependent enzymes (cPLA₂s) and Ca²⁺-independent enzymes (iPLA₂s). Inhibition of sPLA₂s and cPLA₂ was shown to reduce inflammatory reactions in vitro and in vivo (Dennis et al., 2011; Kokotou et al., 2017) and the development of subtype specific PLA₂ inhibitors as anti-inflammatory drugs and as alternatives to cyclooxygenase inhibitors is of great interest. In mast cells, a central cell type involved in inflammatory allergic disorders, fingolimod, but not fingolimod-P, inhibited antigen-stimulated arachidonic acid release (by 30% at 1 μM) and even more potently prostaglandin D₂ synthesis (EC₅₀ approx. 100 nM) (Payne et al., 2007). Furthermore, in renal mesangial cells, which are implicated in chronic inflammatory kidney diseases like glomerulonephritis, fingolimod downregulated cytokine-stimulated sPLA₂ production and secretion (Xin et al., 2007), which, however, was not due to direct inhibition but rather to
inhibition of a transcriptional mechanism. Thus, in addition to its immunomodulatory role, fingolimod has direct anti-inflammatory effects and thus potential for treating inflammatory and allergic diseases.

The cation channel TRPM7

Transient receptor potential cation channel subfamily M member 7 (TRPM7) is a plasma membrane ion channel that contains a cytosolic protein kinase domain. It has a variety of physiological functions, including the regulation of Ca\textsuperscript{2+} signalling, Mg\textsuperscript{2+} homeostasis, cell migration, proliferation and differentiation and immune responses. Inhibition or depletion of TRPM7 causes anoxic neuronal death, cardiac fibrosis, tumour progression and macrothrombocytopenia (Chubanov et al., 2017). Recently, it was shown that in HEK293 cells overexpressing TRPM7 or in cardiac fibroblasts, TRPM7 is dose-dependently inhibited by fingolimod and the endogenous analog sphingosine with IC\textsubscript{50} values of 720 nM and 300 nM, respectively (Qin et al., 2013). No inhibition was seen by the phosphorylated species S1P and fingolimod-P. It was suggested that TRPM7 inhibition by fingolimod contributes to the drug’s anti-proliferative and anti-migratory effects (Qin et al., 2013).

4. Pharmacokinetic characteristics of Fingolimod

The pharmacokinetic characteristics of fingolimod were summarized from the report NDA 22-527 submitted to the FDA for drug approval. A major advantage of fingolimod as a therapeutic agent is the possibility of its oral application. Absorption is food-independent and slow (maximal plasma concentration after 12-16 h), but extensive, and its bioavailability is high (93%). It reaches steady-state concentrations after 1-2 months during daily intake (Clinical Pharmacology and Biopharmaceutics Review- Application number : NDA 22-527; https://www.accessdata.fda.gov/drugsatfda_docs/nda/2009/022115s0).

Fingolimod shows high plasma protein binding (>99.7%), mainly to albumin, and in contrast to S1P, there is no evidence for fingolimod binding to ApoM/HDL. It has a large volume of distribution of approx. 20 L/kg and shows slow blood clearance (6.3±2.3 L/h), resulting in a half-life of 6-9 days.

In the blood, there is a stable equilibrium between fingolimod and fingolimod-P and both plasma levels decrease in parallel following similar elimination kinetics. The enzymes involved in regulating the equilibrium between the inactive and active compound are Sphk2 and ecto-phosphatase lysophospholipid phosphatase 3 (LPP3). It is noteworthy, however, that fingolimod-P can also be dephosphorylated intracellularly by the S1P phosphatase 1 (SPP1) (Mechtcheriakova et al., 2007), and then reenters the fingolimod blood pool. In
addition to phosphorylation, two further pathways of fingolimod biotransformation exist which are depicted in Fig. 2.

(1) Cytochrome P450 (CYP450)-mediated ω-hydroxylation/oxidation followed by fatty acid-like-β-oxidation steps in the liver. Fingolimod blood clearance is slow with 6.3 ± 2.3 L/h. The main CYP450 isoform identified is CYP4F2 with only minor contributions of other CYP450 isoforms. Inactive metabolites are then eliminated by the renal system (81%).

(2) Fingolimod acylation by a still unknown acyltransferase to form atypical ceramides. Ceramides have attracted attention due to their pro-apoptotic effect on various cell types, and it is possible that such atypical ceramides are involved.

The main metabolites detectable in the blood are M3 and M4 from the CYP4F2 route and M29 and M30 from the acylation route (Fig. 2). Quantification of the metabolites in blood analysis after a single oral dose of radiolabeled 14C-Fingolimod revealed that 23.3% of the radioactivity was bound to fingolimod, 10.3% to fingolimod-P, 8.3% to M3, 8.9% to M29 and 7.3% to M30.

The finding that CYP4F2 is a key enzyme in the biotransformation of fingolimod has prompted studies investigating the role of CYP4F2 inhibitors and possible drug interactions. Indeed, co-administration of the CYP4F2 inhibitor and antimycotic drug Ketoconazole was shown to increase the concentrations of fingolimod and fingolimod-P in the blood. Other drug interactions which may affect bioavailability have not been reported so far. Since fingolimod is metabolized mainly in the liver, dosing must be adjusted in case of liver insufficiency.

5. Fingolimod as a therapeutic agent

From its main effect to deplete peripheral lymphocytes, it is obvious that fingolimod has potential for the treatment of diseases associated with inappropriate immune responses, such as in allograft rejection and autoimmune. First preclinical studies in rats, mice and dogs revealed a delay in allograft rejection with fingolimod alone and an even synergistic effect when used together with cyclosporine A (CSA) (Chiba et al., 1996; Suzuki et al., 1996a). It was then tested in several phase 1 to phase 3 clinical trials (Tedesco-Silva et al., 2004) (Mulgaonkar et al., 2006) (NCT00239811) (Tedesco-Silva et al., 2006) (Salvadori et al., 2006) for kidney transplantation. In the phase 3 trials, de-novo renal transplant recipients were randomized to receive either 5 mg fingolimod plus a reduced dose CSA, 2.5 mg fingolimod plus a full dose CSA or mycophenolate mofetil (MMF) plus a full dose CSA over 1 year. Primary endpoints were incidence of acute rejection, graft loss, death or discontinuation of treatment. Unfortunately, these trials could not prove a superior effect of fingolimod/CSA treatment compared to the standard therapy with MMF/CSA, nor did fingolimod allow
reduction of the CSA dose. In addition, more complications were reported in the fingolimod/CSA groups, including decreased renal function and macula edema, leading to discontinuation of the studies.

On the other hand, fingolimod could prove successful for treating multiple sclerosis, and under the name Gilenya® has been approved for oral treatment of RRMS. RRMS currently remains as the only approved indication for fingolimod, all other indications of interest are still under preclinical or phase 1-2 clinical investigation.

5.1. Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune and chronic neurodegenerative disease that disrupts the normal functioning of the brain, optic nerves and spinal cord through inflammation-mediated white matter demyelination and axonal loss (Dutta et al., 2014; Milo et al., 2010). This ultimately leads to a progressive decay of both physical and cognitive functions. There are three main types of MS: the relapsing-remitting MS (RRMS), the primary progressive MS (PPMS) and the secondary progressive MS (SPMS). The transition from RRMS to SPMS is thought to occur when axonal loss outweighs the compensatory capacity of the central nervous system (CNS) such as neuroprotection and remyelination (Dutta et al., 2014; Milo et al., 2010).

Early on, it was recognized that fingolimod can reduce disease symptoms in various animal models of multiple sclerosis. These promising results prompted testing in clinical trials of RRMS. It is of note that for this indication fingolimod can be applied as monotherapy and at a ten-fold lower dose than used in the renal transplantation studies, thereby minimizing adverse events. Several clinical trials were performed from phase 1 to 3, where fingolimod proved to be effective in reducing disease symptoms and the number of relapses. In 2010, the FDA and EMA granted approval for this indication.

In addition, fingolimod was tested in clinical studies for PPMS. However, the primary endpoint delay of MS disability progression compared to placebo, was not met. Altogether, fingolimod represents a clear improvement in the therapy of RRMS, mainly due to its oral applicability and good tolerability at clinically effective doses (Table 2). New S1PR modulators with higher selectivity for S1P₁ or dual activity against S1P₁,5 are currently under investigation for various other indications for which S1PR modulation represents a reasonable therapeutic concept (Table 3).

5.2. Inflammatory bowel diseases
Chronic inflammatory bowel disease (IBD), which mainly includes Crohn's disease and ulcerative colitis, is an idiopathic disease caused by multiple factors of genetic, inflammatory or environmental origin (Peyrin-Biroulet et al., 2017). The detailed mechanisms of IBD pathogenesis are not completely understood. It is characterized by intestinal inflammation and a dysregulated immune response to host intestinal microflora (Baumgart et al., 2007).

Since T cell infiltration into the mucosa is a common feature, it is expected that fingolimod by redistributing immune cells to secondary lymphoid organs, can relieve disease symptoms and progression (Peyrin-Biroulet et al., 2017).

First promising data with fingolimod came from a mouse model of chronic colitis. In this model, the disease was caused by depletion of interleukin 10 using gene knockout. Treatment of mice with fingolimod for 4 weeks efficiently reduced the number of CD4+ T cells in the colonic lamina propria and decreased the production of IFN-γ production in colonic lymphocytes thereby mitigating the severity of the disease (Mizushima et al., 2004). In other experimental models of colitis, such as colitis induced by dextran sulfate sodium (DSS), trinitrobenzene sulfonic acid (TNBS) or by colitogenic T cell transfer, similar protective effects of fingolimod were reported (Daniel et al., 2007; Deguchi et al., 2006; Mizushima et al., 2004). However, in other preclinical models of colitis the therapeutic effect of fingolimod could not be confirmed, suggesting this indication to be more critically investigated to clarify this inconsistency (Montrose et al., 2013; Radi et al., 2011).

Recent data from using the new S1P1,5-selective immunomodulator ozanimod support the idea of targeting S1P1,5 in the treatment of IBD. In this regard, ozanimod was shown to decrease inflammation and disease parameters in TNBS-induced colitis and colitis induced by adoptive T cell transfer in mice (Scott et al., 2016). It was then further tested in a phase 2 clinical study (NCT02435992) in patients with moderately or severe active ulcerative colitis. Daily oral treatment with 1 mg ozanimod achieved higher clinical response rates and remissions, and improved mucosal healing resulting in lower Mayo Clinic scores compared to placebo. This effect was already seen 8 weeks after the start of treatment and was maintained up to week 32 (Sandborn et al., 2016). Similarly, the new S1P1-selective agonist KRP-203 (2-amino-2-[2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl]-1,3-propanediol hydrochloride) (Fujishiro et al., 2006; Shimizu et al., 2005) also proved to be effective in mitigating the symptoms of colitis (Song et al., 2008). A clinical phase 2 study (NCT01375179) to assess its potential in the treatment of ulcerative colitis was conducted but terminated early due to non-convincing results at a first interim analyses after 18 months (according to the company’s clinical trial results database https://www.novctrd.com/).
More recently, the next generation S1P$_{1,4,5}$-selective modulator etrasimod (APD334) (Buzard et al., 2014) also entered a phase 2 study (NCT03139032) in IBD patients and it remains to be seen whether this compound holds more promise to reduce disease symptoms.

5.3. Psoriasis

Psoriasis is a chronic inflammatory skin disease characterized by infiltration of T cells into skin lesions and by focal formation of inflamed raised plaques which constantly shed scales derived from hyperproliferating skin epithelial cells. First-line and still the most effective therapy is the use of glucocorticoids, which classically act by immune suppression and by reducing keratinocyte proliferation (Das et al., 2009).

In principle, fingolimod could be useful in the treatment of psoriasis by redistribution of the T cells away from the skin and by blocking migration of skin dendritic cells to draining lymph nodes where antigen presentation to naïve lymphocytes occurs (Han et al., 2015). Unfortunately, since animal models of psoriasis do not exist, efficacy of fingolimod has not been demonstrated. Nevertheless, second-generation S1PR modulators with higher selectivity have been developed. One is ponesimod which was tested in a phase 2 study (NCT01208090) in patients with moderate to severe chronic plaque psoriasis. The primary endpoint was defined as 75% reduction in psoriatic area after 16 weeks. The conclusion from the study was that treatment with ponesimod resulted in clinical responses after 16 weeks and symptoms improved further when it was continued (Vaclavkova et al., 2014).

5.4. Rheumatoid arthritis

Rheumatoid arthritis is another autoimmune disease that mainly affects the joints and leads to a progressive disability (Mclnnes et al., 2011). It is characterized by synovial inflammation and hyperplasia, autoantibody production, cartilage and bone destruction, but is also associated with systemic complications such as cardiovascular, pulmonary and skeletal disorders (Mclnnes et al., 2011). Reactive T cells are implicated in the pathogenesis of the disease and it is plausible to assume that fingolimod has therapeutic benefit. Indeed, in various rat and mouse models of arthritis fingolimod was shown to reduce the incidence of arthritis, and to reduce hindpaw swelling and bone destruction (Matsuura et al., 2000; Tsunemi et al., 2010; Wang et al., 2007; Yoshida et al., 2013). Clinical studies with fingolimod or novel S1PR modulators in patients with arthritis have not been performed yet.

5.5. Cancer
5.5.1. **S1PR-dependent effects**

The immune suppressive effect of fingolimod results from impeding egress of naïve and central memory T cells from lymph nodes upon S1P₁ binding of its phosphorylated analog fingolimod-P generated by SphK2 (Brinkmann et al., 2010; Kharel et al., 2005; Mehling et al., 2008; Zemann et al., 2006). Whereas a decrease of circulating lymphocytes and reduced cell trafficking to sites of inflammation like in the CNS accounts for efficacy in MS, immune suppression is counterproductive in cancer patients. Even if it may affect also regulatory T cells implicated in downregulating anti-tumor immune responses (Wolf et al., 2009), the decline of circulating cytotoxic T cells (CTLs) and certain NK cells (Johnson et al., 2011) potentially hampers tumor rejection and immune surveillance. To overcome the immunosuppressive effect of fingolimod, derivatives were generated which cannot be phosphorylated and bind to S1P₁, but still maintain biological activity in cells (Omar et al., 2011; Segura-Ulate et al., 2017). In addition to inducing a decline in circulating immune cells by S1P₁ binding, data from preclinical mouse models suggest that fingolimod also stimulates the accumulation of myeloid-derived suppressor cells in tumors via S1P₃-dependent stimulation of GM-CSF release, which would favor immune escape and tumor growth (Li et al., 2017). In this context, it is noteworthy that S1P₃ signaling was recently shown to have prometastatic properties in breast and lung carcinoma cells (Filipenko et al., 2016; Zhang et al., 2013). Since fingolimod-P was found to act only as a partial agonist of S1P₃ with about 50% of the activity of S1P (Riddy et al., 2012), it would be interesting to see if it has anti-metastatic activity by antagonizing S1P₃ in the presence of high levels of S1P.

5.5.2. **S1PR-independent effects (off-target)**

Fingolimod was shown to inhibit cancer-relevant signal transduction pathways independent of S1PR binding, in part due to its structural similarity to sphingosine. The heterogeneity of responses to fingolimod includes increased cell death, decreased cell proliferation, angiogenesis, migration, invasion, metastasis and inflammation (Patmanathan et al., 2015; White et al., 2016). Thus, by targeting multiple oncogenic signaling pathways and compensatory pathways implicated in drug resistance, fingolimod in principle holds promise for drug repurposing as an anticancer agent for various oncology indications characterized by different molecular abnormalities.

The mechanisms underlying the S1PR-independent anti-tumor activity of fingolimod are less well defined and all were measured at relatively high µM concentrations. One of the better characterized effects is inhibition and proteasomal degradation of the oncoprotein SphK1 (Tonelli et al., 2010) and hence downmodulation of various well-characterized pro-
tumorigenic processes associated with this pathway (Lim et al., 2011; Pchejetski et al., 2010; Vessey et al., 2007).

Another target of fingolimod in cancer is PP2A, a tumor suppressor with decreased activity in various human tumors (Cristobal et al., 2016; Grech et al., 2016; Perrotti et al., 2013). Inhibition of PP2A occurs by mutations, hyperphosphorylation of the catalytic subunit or deregulation of the endogenous inhibitors SET and CIP2A (Chen et al., 1992; Cristobal et al., 2016; Saddoughi et al., 2013), leading to constitutive activation of survival signaling pathways (Pitman et al., 2012). Surface plasmon resonance (SPR) analysis indeed demonstrated that fingolimod binds to I2PP2A/SET with a $K_d$ of 11 nM (Saddoughi et al., 2013) but biological activity was again only measured at µM concentrations. Further effects of fingolimod on PP2A function seem to include CIP2A downregulation and PP2A hypophosphorylation (Rincon et al., 2015), but these mechanisms need to be explored in more detail. In BCL/ABL-transformed leukemia cells, 2.5 µM fingolimod was shown to increase cellular PP2A activity by 400 % and consequently dephosphorylate and degrade BCR/ABL with an EC$_{50}$ value of 80 nM (Neviani et al., 2007). It was suggested that this mechanism accounts for the anti-tumor effect in Philadelphia chromosome (Ph1)-positive leukemia. Fingolimod was also shown to inhibit activation of the chemokine receptor CXCR4 in multiple myeloma. CXCR4 and its ligand CXCL12 are crucial for multiple myeloma cell proliferation and regulate the migration and homing to the bone marrow (Azab et al., 2009). The anti-leukemic effect of fingolimod occurred in cooperation with bortezomib and the molecular data suggest a cross-talk between the S1P and the CXCR4 pathways (Beider et al., 2017). However, inhibition of CXCR4 determined by analysis of intracellular survival signaling via extracellular signal-regulated protein kinase (ERK)1/2 and S6 proteins was measured at a concentration of 20 µM fingolimod without showing data from lower doses at which cell viability was already decreased. Anti-leukemic activity of fingolimod was also measured in mice, but only after daily injections of very high doses of 10 mg/kg, which makes it difficult to predict the clinical potential of the treatment.

Current preclinical data suggest that a downside of fingolimod for cancer therapy is its low efficacy and potency. In vitro, biological responses in cancer cells were reported at µM concentrations, and in tumor xenograft models in mice, anti-tumor activity was always measured after daily injections of aberrantly high doses, ranging from 2.5-10 mg/kg (Beider et al., 2017; Cristobal et al., 2014; Gstalder et al., 2016; Pchejetski et al., 2010; Rincon et al., 2015). This is up to 1400-fold more than used in patients to treat MS, and it is clear that at such high doses numerous targets are affected which are crucial for normal tissue function. The safety data for fingolimod shown for the 0.5 mg daily schedule (Table 1) make it
unrealistic to believe that such high doses can be applied to patients without escalation of
toxicity.

To improve the efficacy and tolerability of fingolimod, second-generation derivatives and
complex pharmaceutical formulations were engineered and tested in various preclinical
tumor models. These include the analog OSU-2S, which shows reduced S1PR modulation
and increased inhibition of tumor cell proliferation (Omar et al., 2011), lipid-encapsulated
ingolimod for oral delivery (Estella-Heramoso de Mendoza et al., 2015), nanoparticles
combining fingolimod with docetaxel (Alshaker et al., 2017; Wang et al., 2017), and
fingolimod-loaded nanocarriers targeting CD19, CD20 or CD37 (Mao et al., 2014). However,
again, these new developments were tested at clinically unrealistic mg/kg doses and
therefore conclusions for anti-tumor activity in patients cannot be drawn.

Altogether, these preclinical studies reveal that very high concentration of fingolimod are
needed to reduce cancer cell viability in vitro and affect tumor growth in xenograft models in vivo. At such high doses, effects of low specificity are common and indeed multiple low-
affinity targets of fingolimod have been described, increasing the risk of unpredictable
complications. We therefore conclude that fingolimod will likely fail to achieve clinical
responses in cancer patients at tolerable doses.

5.6. Stroke

Ischemic stroke occurs as a result of occlusion of a cerebral artery by a blood clot, which
causes oxygen and glucose deprivation and results in neuronal death, either by apoptosis or
necrosis, and in metabolic and functional deficits. Dying cells then release factors such as
damage-associated molecular patterns (DAMPs) that stimulate the pathogen recognition
receptors toll-like receptor (TLR)-2 and -4, activate NF-κB and the synthesis of various pro-
inflammatory mediators, including IL-1β, TNF-α, and chemokines. These in turn recruit
immune cells to the affected brain area to clear cell debris and promote healing. Immune
cells infiltrating during the first phase consist of macrophages and neutrophils, and in a
second delayed phase of T- and B cells (Santos Samary et al., 2016). However,
ischemia/reperfusion injury is not restricted to the brain but occurs also in other organs, and it
was first shown that fingolimod is capable of reducing tissue injury in kidney and liver upon
ischemia/reperfusion induction (Anselmo et al., 2002; Troncoso et al., 2001). In view of this
finding, animal models of stroke were performed where it was demonstrated that fingolimod
reduces lesion size and improves neurological outcome after experimental stroke (Czech et
al., 2009). In the meantime, several more preclinical studies using fingolimod in animal
models of stroke were reported, and a systematic review and a meta-analysis of the data
was conducted to prove the drug’s efficiency in such animal models. Results indicated an
overall beneficial efficacy of fingolimod in stroke models with one negative study that used a permanent middle cerebral artery occlusion model (Liu et al., 2013). However, it should be kept in mind that animal experiments are routinely not performed in aged mice which would resemble the situation in humans with comorbidities like diabetes and hypertension (Liu et al., 2013).

A pilot trial and a clinical phase 2 study (NCT02002390, NCT02956200) were performed to provide proof-of-concept for the efficacy of fingolimod, also in combination with alteplase and thrombectomy, to reduce brain inflammation and improve clinical outcomes of acute ischemic stroke in patients. The data revealed that patients treated with alteplase plus fingolimod 0.5 mg daily for 3 consecutive days within 4.5 hours after the onset of ischemic stroke showed a significant reduction of neurologic impairment compared to patients in the standard therapy receiving group. Neurologic functions improved in these patients during the first week in coincidence with a reduction of circulating lymphocyte counts. After 3 months, more patients in the fingolimod group achieved full recovery of their neurologic capability (Fu et al., 2014; Zhu et al., 2015).

5.7. Systemic inflammatory response syndrome /sepsis

Systemic inflammatory response syndrome (SIRS), sepsis and septic shock are typically characterized by a strong inflammatory reaction and dysregulated vascular permeability, leading to hypovolaemia and severe tissue edema finally culminating in multiorgan failure. Numerous options to block the inflammatory response, i.e. by neutralizing or antagonizing TNFα or IL-1β, have been explored over years, but all failed due to the multifactorial nature of the disease. Moreover, hypovolaemia is normally treated by volume substitution, but due to the increased vascular permeability the added fluid can even aggravate edema formation. Therefore, likely more promising is to directly target the vascular endothelial cells and increase their barrier function. Since S1P1 activation is well described as a barrier enhancing mechanism, the activation of this receptor signaling system is desirable. Interestingly, in sepsis, serum levels of S1P (Coldewey et al., 2016; Frej et al., 2016; Winkler et al., 2015) and its carrier ApoM/HDL (Christoffersen et al., 2012; Wu et al., 2004) are downregulated, which further suggests the usefulness of increasing S1P/S1P1 signaling to reduce symptoms of sepsis. In this context it was previously reported that application of reconstituted HDL attenuates organ injury in a rat model of endotoxic shock (McDonald et al., 2003). Although not explicitly investigated, it is likely that S1P, which is coupled to HDL in the blood, accounts for the protective effect of HDL. Furthermore, fingolimod could prove protective also in preclinical models of sepsis. For instance, in an acute LPS-induced lung injury model in mice, symptoms were significantly relieved by applied S1P (at 1 μM) or fingolimod (at 0.1
mg/kg) (Peng et al., 2004), and, in a cecal ligation and puncture (CLP)-induced sepsis model in rats, fingolimod, at 0.2 mg/kg decreased volume loss by 30% (Lundblad et al., 2013). Moreover, it improved LPS- and (CLP)-induced cardiac impairment in mice (Coldewey et al., 2016), and reduced vascular and immune defects (Hemdan et al., 2016). On the other hand, the S1P1 agonist SEW2871, which was claimed to be selective for S1P1, but manifold less potent than S1P and lacking a functional antagonistic effect (Jo et al., 2005), could not substitute for fingolimod in a rat model of sepsis, but instead caused severe cardiac complications and no improvement of the vascular barrier function (Flemming et al., 2017). This is probably due to low potency and off-target effects caused by the use of higher doses. Collectively, more data are needed to rate the therapeutic potential of fingolimod in the treatment of SIRS and septic shock in patients.

5.8. Chronic inflammatory kidney diseases

Systemic lupus erythematosus:

Systemic lupus erythematosus (SLE) is a systemic and poly-etiological autoimmune disease that is associated with a broad spectrum of clinical and immunologic manifestations. Early symptoms most frequently affect the skin and joints, whereas morbidity and mortality is mainly due to kidney damage (Bai et al., 2017; Yu et al., 2017). The development of nephritis in patients with SLE involves multiple pathways including aberrant apoptosis, autoantibody production, immune complex deposition, complement activation and inflammatory cytokines and interferon-γ production (Bai et al., 2017; Yu et al., 2017). Additional typical renal features are glomerular crescents, podocyte injury, tubulointerstitial lesions and vascular injury. Although outcomes for patients with lupus nephritis have improved over the past 30 years, curative treatment of this disease still remains a challenge (Bai et al., 2017; Yu et al., 2017).

Several animal models exist where the autoimmune disease spontaneously develops. Most commonly used are NZB/W F1, MRL-lpr/lpr and BXBS mice (Perry et al., 2011). In all three models, fingolimod was tested and found effective in reducing disease pathology and improving renal function (Alperovich et al., 2007; Ando et al., 2010; Okazaki et al., 2002). Notably, the novel S1P1-selective agonist KRP-203 was also tested in the MRL-lpr/lpr mice. When these mice received KRP-203 for 8-12 weeks, a significant reduction of kidney injury was observed, which was explained by its ability to block T-cell infiltration into the kidney (Wenderfer et al., 2008). The novel S1P1-selective cenerimod (ACT-334441) is currently under investigation in a phase 1 study (NCT02914223), and it will be interesting to see whether this compound holds the promise to mitigate disease symptoms and the progression of SLE.
Other forms of inflammatory kidney diseases:

Fingolimod was also found effective in other inflammatory kidney diseases such as in mesangioproliferative glomerulonephritis or tubulointerstitial fibrosis, which have no autoimmune character. In rats in which chronic progressive glomerulosclerosis was induced, the treatment with oral fingolimod, at 0.3mg/kg daily, resulted in an effective reduction of blood lymphocyte counts and renal lymphocyte infiltration and proteinuria and matrix expansion were reduced and renal function improved (Martini et al., 2007; Peters et al., 2004). In another study in mice, fingolimod administered orally at 1mg/kg for 5 days relieved tubulointerstitial fibrosis induced by unilateral ureter obstruction (Thangada et al., 2014).

5.9. Atherosclerosis

Atherosclerosis is a multifactorial disease of inflammatory nature and associated with increased vascular plaque formation that at some point causes rupture and thrombus formation. Patients therefore have a strongly increased risk for cardiovascular complications including coronary artery disease, myocardial infarcts and stroke (Ross, 1999). Risk factors for the pathogenesis of atherosclerosis include high serum levels of cholesterol/LDL, smoking, hypertension and diabetes mellitus (Ross, 1999). On the cellular level, atherosclerosis is mainly characterized by endothelial dysfunction, but other cell types are also involved such as macrophages, T cells and smooth muscle cells. The activated endothelial cells show increased permeability and adhesion of immune cells, and change their normally anticoagulant state into a pro-coagulant state (Dahal et al., 2017; Ross, 1999). In addition, the inflammatory environment stimulates proliferation and migration of vascular smooth-muscle cells leading to thickening and remodeling of the vascular wall (Ross, 1999). To date, the standard of care for this disease still aims at reducing plasma cholesterol levels with statins and fibrates. However, new strategies are underway to more specifically reduce vascular inflammation, for instance by inhibiting or neutralizing IL-1β (Ridker et al., 2017). Since S1P modulators like fingolimod have demonstrated anti-inflammatory potential in the clinical setting, their use in the treatment of atherosclerosis is suggested.

Mice are resistant to atherosclerosis by nature and hence require certain genetic manipulations to establish suitable preclinical models. Particularly, ApoE knockout (KO) mice, LDL receptor KO mice, hepatic lipase KO mice or human cholesterol-ester transfer protein transgenic mice were shown to develop atherosclerosis with high incidence (Kapourchali et al., 2014). Unfortunately, however, when fingolimod was tested in ApoE KO and LDL receptor KO mice under various diet conditions, outcomes were controversial and
difficult to explain. On the one hand, in mice kept on a cholesterol-rich diet, fingolimod attenuated the development of atherosclerosis, which was attributed to its effect on the distribution and activation of T cells and macrophages in plaques, and to reduce plasma cytokine levels (Keul et al., 2007; Nofer et al., 2007). On the other hand, in ApoE KO mice kept under standard diet (Klingenberg et al., 2007), and in LDL receptor KO mice fed with a low cholesterol Western-like diet (0.25%) (Poti et al., 2012), two procedures causing milder hypercholesterolaemia, smaller lesions and no increase of plasma cytokines, fingolimod could not prevent atherosclerotic lesions, although lymphocyte counts were reduced. The conflicting data of the studies were discussed (Poti et al., 2012), and it was proposed that fingolimod may be protective only under conditions of massively increased cholesterol burden and exacerbated inflammation. In another study, the S1P₁ agonist KRP-203 also improved atherosclerosis lesions in LDL-R-deficient mice fed a cholesterol-rich diet (Poti et al., 2013), but because of these controversial findings it remains unclear whether clinical studies with S1PR modulators in atherosclerosis are warranted.

5.10. Diabetes mellitus

Two types of diabetes mellitus exist of which type 1 (T1D) has a juvenile onset and accounts for 10% of all cases of diabetes. It is characterized as an autoimmune destruction of pancreatic beta-cells in the islets of Langerhans. Consequently, there is a loss of insulin secretion and the main therapeutic option is to substitute insulin by administration. In contrast, type 2 diabetes (T2D) has a adult onset and covers 90% of diabetics. It is mainly characterized by the development of insulin resistance in peripheral tissues, and obesity and associated inflammation are the prevalent risk factors (Ng et al., 2017). In T2D, there is no evidence for autoimmune-mediated destruction of beta-cells, although amyloid deposition in the islets frequently occurs which may interfere with beta-cell function and insulin secretion.

**Beta-cell apoptosis:** Since apoptosis of beta-cells is crucial in the development of T1D, understanding the regulation of apoptosis in this cell type is mandatory. The trigger for beta-cell death comes from auto-reactive T cells that are directed against multiple islet antigens, and therefore, therapeutic interference with the activated immune cells seems promising. Several immunomodulatory drugs, including anti-CD3, anti-CD20, and CTLA4-Ig, were tested in clinical trials of new-onset T1D patients (Orban et al., 2011; Pescovitz et al., 2009; Sherry et al., 2011). Although these treatments resulted in short-term improvement of beta-cell function for several months, durable improvement was not observed. This was explained by the low regenerative capacity of human beta-cells (Maganti et al., 2014). Fingolimod was so far only tested in animal models of autoimmune- and drug-induced diabetes, and in many of these models, it was found effective to normalize blood glucose levels and to protect islet
graft destruction (Fu et al., 2002; Maki et al., 2002; Suzuki et al., 1998; Tsuji et al., 2012; Yang et al., 2003). Interestingly, fingolimod also increased beta-cell survival and improved their regeneration in vivo in hyperglycemic db/db mice (Moon et al., 2013; Zhao et al., 2012). Whether fingolimod deserves further investigation in clinical trials for T1D, particularly also in view of disappointing long-term results with other immunomodulators, is disputable. 

Alternatively, beta-cell destruction could be impeded by targeting the molecular pathways involved in the death response, possibly in combination with immunomodulators like fingolimod. In this context, the cellular sphingolipid rheostat has attracted attention as the balance between pro-apoptotic ceramide and pro-survival S1P was shown to affect the cell’s life/death decision. In numerous studies with beta-cells, inhibition of ceramide formation or stimulation of SphK1/S1P signaling could protect cells from apoptosis (Jessup et al., 2011) (Ng et al., 2017) (Meikle et al., 2017).

**Insulin resistance:** Insulin resistance is the main risk factor for T2D. There is ample evidence from cell culture and animal experiments that the deregulation of ceramides, glucosylceramide and gangliosides is involved in insulin resistance (Chaurasia et al., 2015; Chavez et al., 2012). Procedures to block or deplete enzymes in the de-novo synthesis of ceramide, glucosylceramide synthase, or GM3 synthase, all improve glucose tolerance and prevent insulin resistance and diabetes (Chavez et al., 2012). Furthermore, it is was recently reported that adiponectin, a peptide hormone secreted by adipocytes and acting via adiponectin receptors (AdipoR)/AMPK to stimulate glucose uptake and fatty acid oxidation, has insulin-sensitizing, anti-inflammatory and anti-atherogenic activity by modulating ceramides. Interestingly, AdipoR has an intrinsic ceramidase activity (Holland et al., 2011), and thereby, reduces cellular ceramide levels by converting it to sphingosine and further to the prosurvival S1P. The prosurvival effect of S1P on beta-cells was further supported by data from SphK1 knockout mice. When fed with high-fat diet, SphK1-deficient islets were more susceptible to lipotoxic stress factors (Qi et al., 2013). Fingolimod also reversed insulin resistance in animal models (Bruce et al., 2013; Kendall et al., 2008) and it was proposed that this effect was due to reduced ceramide formation in muscles (Bruce et al., 2013).

Altogether, based on available data, fingolimod seems to have some short-term benefit in T1D by downregulating the immune response against beta-cell, but long-term responses are missing as it fails to halt disease progression. Furthermore, it must be kept in mind that in diabetic patients the risk for developing macula edema under fingolimod is increased (Moss, 2017). This risk must be weighed against its rather limited benefit as an insulin sensitizer in diabetes.
5.11. Rett’s syndrome

The Rett’s syndrome is a progressive neurodevelopmental disorder that predominantly affects young females and manifests with autistic-like behavior and severe mental retardation. Typical symptoms include motor impairment, such as ataxia, apraxia and tremor, stereotypic hand movements, seizures, hyperventilation, apnea and even cardiac dysrhythmias (Cronk et al., 2016; Liyanage et al., 2014; Pecorelli et al., 2016). The disorder is mainly characterized as a genetic disease which in most cases is caused by a mutation in the X-linked gene coding for the methyl-CpG-binding protein 2 (MECP2). In rare cases, other mutations, such as in the genes coding for the cyclin-dependent kinase-like 5 protein (CDKL5) and forkhead box G1 (FOXG1), were also described. The MECP2 protein is known to regulate the transcriptional activity of various genes and modulate the chromatin structure (Liyanage et al., 2014). A more global function as genome-wide epigenetic modulator has also been proposed (Liyanage et al., 2014). A well described target gene of MECP2 is the brain-derived neurotropic factor (BDNF) (Liyanage et al., 2014). Notably, MECP2 knockout mice, which represent a model for Rett syndrome and consequently develop neuronal and motor deficits comparable to Rett patients, showed reduced BDNF expression in various brain regions including hippocampus, striatum and cerebellum (Deogracias et al., 2012). In these mice, application of fingolimod not only increased BDNF protein expression in the brain, but also improved neuronal and motor deficits, and even prolonged the life-expectancy of the animals (Deogracias et al., 2012). Furthermore, in a rat model of valproic acid-induced autism, fingolimod mitigated learning and memory impairments and social deficits (Wu et al., 2017). A phase 1 study (NCT02061137) currently investigates the safety and efficacy of fingolimod in children older than 6 years with Rett syndrome, and it will be interesting to see if fingolimod can mitigate the regression of motor and language skills.

5.12. Fear extinction memory

Since fingolimod-P was previously shown to directly inhibit HDAC activity in cell nuclei in vitro as well as in vivo in mouse brain (Hait et al., 2014), and in view of the finding that HDAC inhibitors can improve cognitive disorders (Fischer et al., 2010), it may be hypothesized that fingolimod can have beneficial cognitive and memory enhancing effects. In a mouse model of fear extinction memory testing, fingolimod application over 3 days followed by electric foot shock leads to a continuous reduction of the freezing time (a measure of fear towards the electric shock). When an extinction session was introduced after day 2 in which mice were reexposed to the conditioned stimulus but without footshock, they returned to a higher freezing time upon footshock on day 3. In contrast, fingolimod treated mice remained at the
low freezing time, suggesting that the extinction of aversive memories was improved (Hait et al., 2014). Since these experiments were carried out in immunocompromised SCID mice, which are deficient in both B and T cell responses, the involvement of immune-mediated mechanisms in the fingolimod effect was excluded, and particularly involvement of HADC inhibition in the hippocampus was suggested as a possible mechanism. More cognitive tests are needed to better rate the potential of fingolimod as enhancer of cognitive ability and memory.

5.13. Other potential indications for fingolimod
The above mentioned indications for fingolimod have been extensively investigated in preclinical studies or have already moved forward to clinical trials. In addition, fingolimod is under consideration for various other disorders as well, most notable are disorders associated with neuroinflammatory processes, such as Amyotrophic Lateral Sclerosis (ALS) (Potenza et al., 2016), Alzheimer (Takasugi et al., 2013), Parkinson (Zhao et al., 2017) and cerebral malaria (Finney et al., 2011). Currently, a clinical phase 2 study (NCT01786174) is ongoing to determine safety and tolerability of fingolimod in patients with ALS. It will be interesting to see whether it can mitigate the clinical symptoms of this still incurable neurodegenerative disease.

6. Adverse effects of fingolimod
Table 2 summarizes some of the adverse events reported from patients with relapsing-remitting MS treated with low doses of 0.5 mg fingolimod daily for 24 months (FREEDOMS II). Those seen more frequently with fingolimod than with placebo included lymphopenia, increased liver transaminases, herpes viral infections, hypertension, and initial bradycardia and first degree AV block both as first-dose effects. Of note, analysis of pooled safety data from various phase 2 and phase 3 studies revealed that the risk for macular edema, which in FREEDOMS II occurred in 1% of the patients in both the fingolimod and the placebo group, was seven times more common in individuals with coexisting diabetes or history of uveitis when treated with 1.25 mg fingolimod (Moss, 2017; Zarbin et al., 2013).

Also, data derived from the phase II trials on renal transplant recipients, which received fingolimod at 10 times higher doses and also in combination with other immunosuppressive drugs like CSA and MMF, additional adverse effects to the ones summarized in table 2, were reported including reduced lung function and renal function (Budde et al., 2006; Salvadori et al., 2006).
Here, we want to point out that based on published preclinical data much higher doses of fingolimod would be required to achieve anti-tumor effects in patients. To illustrate this, in tumor-xenografted mice doses ranging from 2.5 to 10 mg/kg daily were used, which would correspond to doses of 175 to 700 mg per day in cancer patients. Even though this simplified calculation does not fully respect differences in pharmacokinetics and drug metabolism between mouse and man, and between i.p. and oral delivery, considering the safety data shown in Table 2 for 0.5 mg fingolimod, it goes without saying that such aberrantly high doses will result in many more complications.

**Rebound syndrome upon drug discontinuation**

Fingolimod discontinuation may be necessary due to different reasons, the most common are severe adverse effects, breakthrough disease activity or pregnancy. In a recent study involving 50 patients, fingolimod treatment was discontinued in 5 patients who developed a severe disease recurrence known as rebound phenomenon (Hatcher et al., 2016). Such a frequency is indeed clinically relevant and deserves more detailed investigation. Symptoms of recurrence usually start 4-12 weeks after drug withdrawal when lymphocytes reenter the circulation and the CNS. The mechanism underlying the rebound phenomenon is still unclear, but it has been speculated that different T cell subsets repopulate the circulating pool of immune cells with different kinetics. This is supported by the finding that in two patients with severe rebound after fingolimod withdrawal CD8+ T cells recovered more rapidly than the CD4+ and the CD19+ subsets (Hatcher et al., 2016).

In an approach to investigate possible changes that occur in the immune system upon fingolimod withdrawal, the experimental autoimmune encephalomyelitis (EAE) model was performed in mice. Interestingly, T cells of mice under fingolimod withdrawal showed (1) a several-fold increase of S1P1 mRNA and (2) increased phospho-Akt signaling. Furthermore, compared to mice under continuous drug exposure, (3) an increased TH17/TH1 cell ratio, as analyzed in the spinal cord, and (4) reduced Treg cell function in lymph nodes was reported (Cavone et al., 2015). Functional rebound is often associated with treatment discontinuation and has also been described for various other immunosuppressive agents after abrupt dose reduction or withdrawal (Sueki et al., 2017). Clearly, further studies are warranted to pinpoint the type of immune cells responsible for the clinical symptoms after fingolimod withdrawal.

**7. Perspectives**

Fingolimod was the first S1PR modulator reported to have immunosuppressive activity and it is so far the only one that was approved for clinical use. However, fingolimod is a nonspecific
S1PR agonist and therefore new functionally related compounds have been developed, which better discriminate between the various S1PR subtypes and hence are expected to have improved efficacy and tolerability. These include the S1P₁ selective compounds KRP-203, ponesimod and cenerimod, the S1P₁+5 selective siponimod and ozanimod, and the S1P₁+4+5 selective amiselimod and etrosimod. Due to their higher selectivity, these new drug developments hold great promise for clinical benefit in a broad range of inflammatory and autoimmune diseases.

On the other hand, for cancer therapy, the S1P₁-dependent immunosuppressive effect of fingolimod is supposed to be counterproductive and therefore numerous preclinical investigations have focused on S1PR-independent (off-target) effects. In addition to fingolimod, several new formulations with improved tumor-targeting properties have been tested, all of them achieved measurable cytotoxic effects only at high concentrations in the µM range. Remarkably, to achieve anti-tumor effects in vivo doses of 10 mg/kg fingolimod per day were used, which is far beyond the dose of 0.5-1.25 mg per day at which adverse events already occur in patients treated for multiple sclerosis. We therefore conclude that fingolimod and the currently available new formulations will fail to achieve clinical responses in cancer patients at tolerable doses.

Acknowledgements
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Conflict of interest
none
References


Bagdanoff JT, Donoviel MS, Nouraldeen A, Carlsen M, Jessop TC, Tarver J, et al. (2010). Inhibition of sphingosine 1-phosphate lyase for the treatment of rheumatoid arthritis: discovery of (E)-1-{4-[(1R,2S,3R)-1,2,3,4-tetrahydroxybutyl]-1H-imidazol-2-yl]ethanone oxime (LX2931) and (1R,2S,3R)-1-(2-(isoxazol-3-yl)-1H-imidazol-4-yl)butane-1,2,3,4-tetraol (LX2932). *J Med Chem* 53(24): 8650-8662.


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**Table 1:** Pharmacokinetic parameters of fingolimod

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosing</td>
<td>0.5 mg daily p.o.</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>93%</td>
</tr>
<tr>
<td>Absorption</td>
<td>Slow, high, food independent</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Liver: by CYP4F2</td>
</tr>
<tr>
<td>Elimination</td>
<td>Renal, 81%</td>
</tr>
<tr>
<td>Bound in plasma</td>
<td>99.7% (mainly to albumin)</td>
</tr>
<tr>
<td>Blood clearance</td>
<td>Low, (6.3 L/h)</td>
</tr>
<tr>
<td>Distribution volume</td>
<td>20 L/kg</td>
</tr>
<tr>
<td>Half-live</td>
<td>6 - 9 days</td>
</tr>
<tr>
<td>Max. plasma conc.</td>
<td>12-16 hours</td>
</tr>
<tr>
<td>Steady state after</td>
<td>1-2 months</td>
</tr>
<tr>
<td>Drug interactions</td>
<td>Ketoconazole</td>
</tr>
</tbody>
</table>
Table 2: Selected adverse events reported from patients with relapsing-remitting MS treated with 0.5 mg fingolimod daily for 24 months

<table>
<thead>
<tr>
<th>Event*</th>
<th>No. of patients fingolimod (N = 358) vs. placebo (N=355)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphopenia</td>
<td>27 (8%) vs. 0</td>
<td>After one month, peripheral blood lymphocyte counts dropped by an average of 75%. This is an indication for a positive biological response and was not reported as adverse event. Lymphopenia as a complication was defined as less than (0.2 \times 10^9) lymphocytes per liter.</td>
</tr>
<tr>
<td>Increased alanine amino transferase</td>
<td>29 (8%) vs. 6 (2%)</td>
<td></td>
</tr>
<tr>
<td>Increased gamma glutamyltransferase</td>
<td>23 (6%) vs. 2 (14%)</td>
<td></td>
</tr>
<tr>
<td>Herpes viral infections</td>
<td>30 (8%) vs. 19 (5%)</td>
<td>Two fatal cases were reported from a previous study after treatment with 1.25 mg fingolimod daily (Cohen et al., 2010)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>32 (9%) vs. 11 (3%)</td>
<td>Small increases in systolic/diastolic blood pressure (~3/~1 mm Hg) after 2-6 months (Camm et al., 2014)</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>5 (1%) vs. 1 (&lt;0.5%)</td>
<td>Mild to moderate with a mean decrease of 8 beats per minute in the first 6 h after treatment initiation.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Initial S1P₁ agonism activates GIRK channels in atrial myocytes in the sinoatrial</td>
<td></td>
</tr>
<tr>
<td></td>
<td>node, leading to hyperpolarization and reduction in excitability (Camm et al.,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2014)</td>
<td></td>
</tr>
<tr>
<td>First-degree AV block</td>
<td>17 (5%) vs. 7 (2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In the first 6 h after treatment initiation, transient and asymptomatic. Same</td>
<td></td>
</tr>
<tr>
<td></td>
<td>molecular effect on atrial myocytes in the AV node as described for bradycardia in</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the sinoatrial node (Camm et al., 2014).</td>
<td></td>
</tr>
</tbody>
</table>

Data were taken from Calabresi et al (Calabresi et al., 2014) based on the FREEDOMS II study (ClinicalTrials.gov number NCT00355134). Only complications are listed which occurred more frequently than in the placebo group and comprised >1% of the treated patients.
### Table 3: Fingolimod and novel S1PR modulators in clinical studies

<table>
<thead>
<tr>
<th>Compound name</th>
<th>S1PR profile</th>
<th>approved</th>
<th>Phase 3</th>
<th>Phase 2</th>
<th>Phase 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fingolimod (FTY720)</td>
<td>S1P&lt;sub&gt;1,3,4,5&lt;/sub&gt;</td>
<td>RRMS</td>
<td>PPMS</td>
<td>Stroke</td>
<td>Uveitis</td>
</tr>
<tr>
<td>KRP-203</td>
<td>S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>UC (terminated)</td>
<td>SLE (terminated)</td>
</tr>
<tr>
<td>Ponesimod (ACT-128800)</td>
<td>S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>RRMS</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>Cenerimod (ACT-334441)</td>
<td>S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>SLE</td>
<td></td>
</tr>
<tr>
<td>Siponimod (BAF312)</td>
<td>S1P&lt;sub&gt;1,4,5&lt;/sub&gt;</td>
<td>SPMS</td>
<td></td>
<td>RRMS</td>
<td>Dermatomyositis</td>
</tr>
<tr>
<td>Ozanimod (RPC-1063)</td>
<td>S1P&lt;sub&gt;1,4,5&lt;/sub&gt;</td>
<td>RRMS</td>
<td>UC</td>
<td></td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>Amiselimod (MT-1303)</td>
<td>S1P&lt;sub&gt;1,4,5&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>RRMS</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>Etrasimod (APD334)</td>
<td>S1P&lt;sub&gt;1,4,5&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>UC</td>
<td></td>
</tr>
</tbody>
</table>
ALS, amyotrophic lateral sclerosis; PPMS, primary progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; SLE, systemic lupus erythematosus; UC, ulcerative colitis.
Fig. 1
Fig. 2
Fig. 3

- Fingolimod (FTY720)
- Siponimod (BAF312)
- Cenerimod (ACT-33441)
- Amiselimod (MT-1303)
- KRP203
- Ponesimod (ACT-128800)
- Ozanimod (RPC1063)
- Etrasimod