

REVIEW

Neurological S1P Signaling as an Emerging Mechanism of Action of Oral FTY720 (Fingolimod) in Multiple Sclerosis

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FTY720 (fingolimod, Novartis) is a promising investigational drug for relapsing forms of multiple sclerosis (MS), an autoimmune and neurodegenerative disorder of the central nervous system. It is currently under FDA review in the United States, and could represent the first approved oral treatment for MS. Extensive, ongoing clinical trials in Phase II/III have supported both the efficacy and safety of FTY720. FTY720 itself is not bioactive, but when phosphorylated (FTY720-P) by sphingosine kinase 2, it becomes active through modulation of 4 of the 5 known G protein-coupled sphingosine 1-phosphate (S1P) receptors. The mechanism of action (MOA) is thought to be immunological, where FTY720 alters lymphocyte trafficking via S1P₁. However, MOA for FTY720 in MS may also involve a direct, neurological action within the central nervous system in view of documented S1P receptor-mediated signaling influences in the brain, and this review considers observations that support an emerging neurological MOA.

Key words: FTY720, Fingolimod, Sphingosine 1-phosphate receptors, Multiple sclerosis, Central nervous system

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disorder that is best known for its effects on the white matter of the central nervous system (CNS). This autoimmune disorder represents one of the most common causes of disease-related demyelination (Steinman, 1996; Zamvil and Steinman, 2003; Frohman et al., 2006). MS affects up to 2.5 million people worldwide, with a 2:1 preponderance of females to males and with 70-80% of cases occurring in young adults (Bashir and Whitaker, 2002). The most common form of MS is characterized by “relapsing-remitting” neurological symptoms that can persist for many years, and which may be associated with severe neurodegenerative impairments, such as axonal damage and neuronal loss (Steinman, 1996; Chabas et al., 2001; Bashir and Whitaker, 2002; Frohman et al., 2006). The primary

factors that initiate MS are still unknown. However, activation of the immune system is regarded as the key disease element in MS, resulting in the production of a variety of cytokines/other factors that have been associated with MS pathological features (Chabas et al., 2001; Filippini et al., 2003; Steinman, 2008). Recently, gene and protein array studies have provided molecular candidates for the potential development of MS therapies (Steinman, 2001; Lock et al., 2002; Steinman and Zamvil, 2003; Han et al., 2008). In addition to molecular targets, many cell types in different systems appear to be involved in MS: immune cells (T-cells, B-cells, natural killer cells, dendritic cells, and macrophages), CNS cells (neurons, astrocytes, and oligodendrocytes), and brain-resident cells (microglia and endothelial cells) (Zamvil and Steinman, 2003; Brinkmann, 2009; Chun and Hartung, 2010).

Currently available, disease-modifying therapies (DMTs) for MS aim to reduce immune response by targeting immunological pathways, but all are only partially effective: interferon beta-1 α (Avonex, Rebif) and -1 β (Betaseron), the synthetic peptide glatiramer

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acetate (Copaxone), the antineoplastic agent mitoxantrone (Novantrone), and the VLA4 blocker natalizumab (Tysabri) (Frohman et al., 2006; Gold et al., 2006; Chun and Hartung, 2010; Rammohan and Shoemaker, 2010). In addition, all DMTs are delivered by injection, requiring MS patients to tolerate the challenges of continual parenteral drug delivery, which is why many MS patients would prefer an oral treatment (Chun and Hartung, 2010; Rammohan and Shoemaker, 2010).

A new class of drug targets are the receptors for the signaling phospholipid known as sphingosine 1-phosphate (S1P) that interact with at least 5 cognate G protein-coupled receptors (GPCRs: S1P₁₋₅) (Chun et al., 2002; Ishii et al., 2004). S1P, one of the most studied signaling phospholipids, was originally known as a metabolite that originated from cell membranes through metabolic pathways: sphingomyelin is cleaved to ceramide and sphingosine and then S1P is produced by phosphorylation of sphingosine via sphingosine

kinases 1/2 (Sphk1/2) (Liu et al., 2002; Hla, 2004) (Fig. 1). Today, S1P is believed to be a potent signaling molecule with its 5 known receptors (S1P₁₋₅) connecting to various downstream signaling pathways (Fig. 1), and which exert a variety of biological effects that have been extensively reviewed (Spiegel and Milstien, 2003; Ishii et al., 2004; Brinkmann, 2007; Herr and Chun, 2007; Choi et al., 2008b).

S1P receptors are expressed in a variety of cell types, including immune and CNS cells, and are thought to play a critical role in MS, indicating the possible link between S1P signaling and MS. In fact, S1P signaling via its receptors influences not only immune cells (Brinkmann et al., 2002; Graeler and Goetzl, 2002; Mandala et al., 2002), but also CNS cells (Rao et al., 2003; Gardell et al., 2007; Herr and Chun, 2007; Kimura et al., 2007; Miron et al., 2008c). Remarkably, S1P signaling has gained relevance to MS through FTY720 (known clinically as fingolimod, Novartis), an orally administered immunomodulatory agent current-

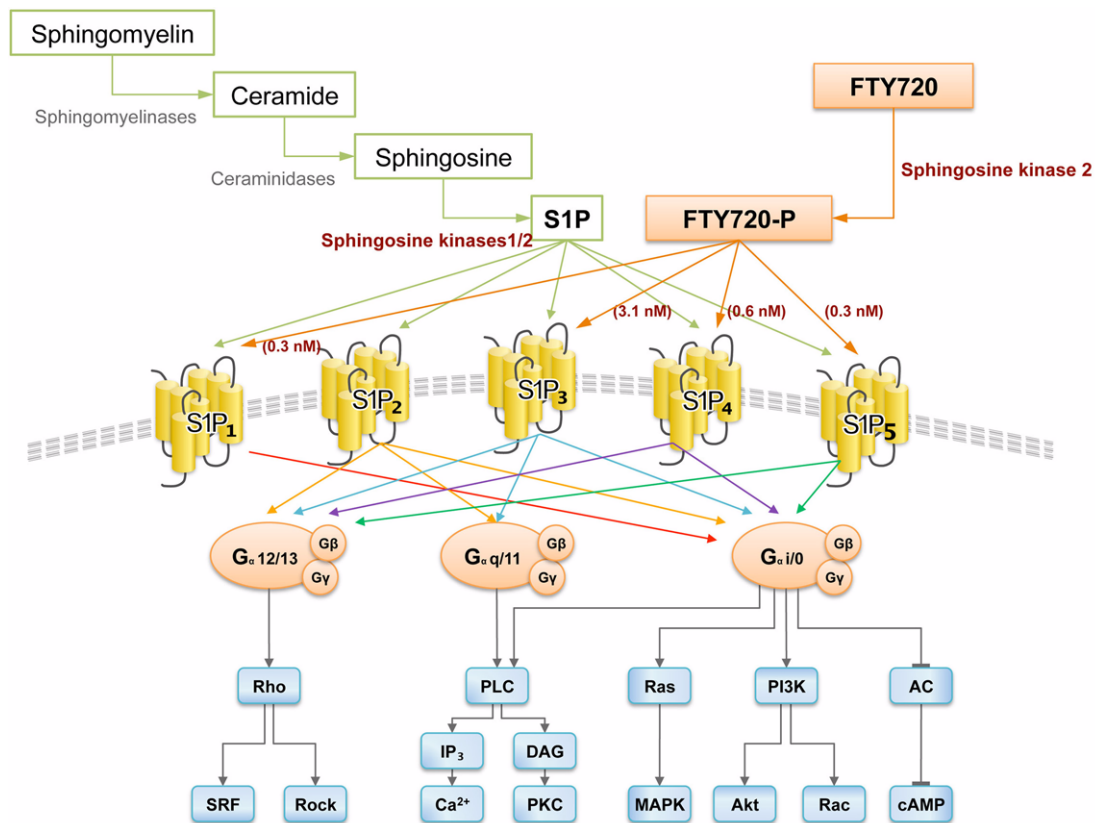


Fig. 1. Phosphorylated FTY720 activates 4 of the 5 known S1P receptors. S1P and FTY720-P that are produced by sphingosine kinases activate S1P receptor-mediated signaling pathways. Activation of S1P signaling plays important biological roles in diverse systems. It is of note that FTY720-P activates 4 of the 5 S1P receptors (S1P_{1/3/4/5}) with high affinity (binding affinity is shown except for S1P₂; FTY720-P does not bind to S1P₂ (> 10,000 nM)). Until now, FTY720 efficacy via S1P signaling was only shown in a few pathological systems, including MS and ischemia. In view of the diverse biological roles of S1P signaling, many more aspects of FTY720 efficacy remain to be evaluated.

ly under phase II/III clinical trials for MS (Kappos et al., 2006, 2010; O'Connor et al., 2009; Cohen et al., 2010).

The mechanism of action (MOA) of FTY720 in MS is believed to be immunological, through the prevention of lymphocyte egress from thymus and peripheral lymphoid organs, and involves the receptor subtype S1P₁. However, emerging evidence provides an important clue that a non-immunological pathway may also be at work in FTY720 efficacy for MS.

FTY720 EFFICACY IN MS AND CLASSICAL ASPECTS of FTY720's MOA

FTY720 (fingolimod) was initially identified as a derivative of myriocin, a metabolite of the ascomycete fungus *Isaria sinclairia*, and has some structural resemblance to S1P (Im, 2003). FTY720 is a novel immunomodulatory agent that is effective in animal models of graft rejection and autoimmune disease through mature T cell depletion (Chiba et al., 1996; Matsuura et al., 2000; Brinkmann et al., 2001; Brinkmann, 2007). An important discovery is that FTY720 is a prodrug, which is phosphorylated (FTY720-P) *in vivo* by Sphk2 and the resultant phosphorylated form has been reported to be a high-affinity, non-selective agonist of 4 of the 5 known G protein-coupled S1P receptors (S1P_{1/3/4/5}) (Brinkmann et al., 2002; Mandala et al., 2002; Albert et al., 2005; Zemann et al., 2006) (Fig. 1).

FTY720 is effective in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Brinkmann et al., 2002; Fujino et al., 2003; Webb et al., 2004; Kataoka et al., 2005; Balatoni et al., 2007; Foster et al., 2007). Extensive Phase II/III clinical trials have supported FTY720 as an orally bioavailable, efficacious agent (Kappos et al., 2006, 2010; O'Connor et al., 2009; Cohen et al., 2010), which may be FDA approved as the first oral drug for relapsing MS patients in 2010. Based on data from ongoing phase III clinical trials, including FREEDOMS (FTY720 *vs* placebo) and TRANSFORMS (FTY720 *vs* IFN- β , currently used drug for MS), FTY720 (daily treatment of 0.5 and 1.25 mg) was shown to be superior to both paired controls (Cohen et al., 2010; Kappos et al., 2010).

The MOA of FTY720 has been thought to be associated with *in vivo* sequestration of circulating lymphocytes in peripheral lymph nodes via S1P₁ (Fujino et al., 2003; Matloubian et al., 2004; Webb et al., 2004; Pappu et al., 2007; Schwab and Cyster, 2007), as seen in cells lacking S1P/S1P₁ signaling pathways (Allende et al., 2004; Matloubian et al., 2004; Pappu et al., 2007), resulting in a marked re-

duction in peripheral blood lymphocytes that generally follows a dose-response to FTY720. One interesting view is that FTY720-P mimics the effect of S1P₁ signaling loss and that the loss of S1P/S1P₁ signaling prevents lymphocyte egress (Allende et al., 2004; Matloubian et al., 2004; Pappu et al., 2007; Liao et al., 2009), as does exposure to FTY720 (FTY720-P) (Mandala et al., 2002). This paradox can be explained by a mechanism through which FTY720 (FTY720-P) acts as a functional antagonist for S1P₁, removing cell surface S1P₁ via irreversible internalization upon receptor activation while internalized S1P₁ is, in sharp contrast, recycled to the cell surface upon S1P-induced receptor activation (Graler and Goetzl, 2004; Matloubian et al., 2004; Oo et al., 2007).

While the currently available body of evidence provides support for FTY720 targeting S1P₁, doing so as a functional antagonist in T lymphocytes in the treatment of MS, there remain other observations that implicate neurological contributions in FTY720's MOA (detailed in the next section).

EMERGING ASPECT OF FINGOLIMOD'S MOA

Even though the immune system is believed to be the main locus for the MOA of FTY720 in MS therapy (see above), other studies suggest the possibility of the CNS as a new locus for FTY720, in addition to the immune system. This possibility is important since all current, major MS therapies target the immune system and can be associated with a range of undesirable side effects including injection-related problems, flu-like symptoms, and/or immunosuppression (Chun and Hartung, 2010; Rammohan and Shoemaker, 2010). Evidence in support of a neurological MOA of FTY720 includes: 1) S1P receptors are highly expressed in CNS cells, with varied expression level depending on cell type, and S1P influences the biology of CNS cells such as astrocytes and oligodendrocytes that are closely related to MS pathologic features, 2) there is a discordance between peripheral lymphocyte levels and FTY720 clinical efficacy in EAE, and 3) FTY720 administered orally is accumulated mainly in the CNS where it is phosphorylated by Sphk2 (see below).

MS-relevant CNS cell types as major loci for S1P signaling

Among the CNS cell types, astrocytes and oligodendrocytes are main components of MS pathologies, such as astrogliosis (Tani et al., 1996; Luo et al., 2007; Nair et al., 2008) and demyelination or remyelination (Steinman, 1996; Miller and Mi, 2007), respectively.

In addition, 4 of the 5 S1P receptors are expressed in both cell types under at least some conditions of analysis: $S1P_3 > S1P_1 > S1P_2 > S1P_5$ in astrocytes and $S1P_5 > S1P_1 > S1P_2 > S1P_3$ in oligodendrocytes (Foster et al., 2007). In this regard, there have been several *in vitro* reports to assess whether FTY720 regulates the biological aspects of those cell types.

Studies have shown that S1P signaling is a critical factor in astrogliosis. It is apparent that S1P induces astrogliosis *in vivo* (Sorensen et al., 2003) via an unspecified receptor subtype, and activation of the S1P/S1P₃ signaling axis is the mechanism responsible for astrogliosis and subsequent development of Sandhoff-like disease (Wu et al., 2008). Recent studies also indicated that FTY720-P acts as an anti-inflammatory agent in neuropathological conditions, such as MS, where S1P_{1/3} are upregulated in reactive astrocytes (Van Doorn et al., 2010). Therefore, a new aspect of FTY720 efficacy may be related to the reduction of astrogliosis in MS. This view can be, in part, supported by several *in vitro* studies where FTY720 regulates astrogliosis-related biological activities, including the activity of astrocytic mitogen-activated protein kinases and migration of astrocytes via S1P₁ and/or S1P₅ (Mullershausen et al., 2007; Osinde et al., 2007), which provide support for a link between FTY720 efficacy and a possible reduction in astrogliosis. However, FTY720 exposure unexpectedly potentiated astrogliosis via S1P₃ and S1P₅ in spinal cord slices, accompanied by a reduction of demyelination produced by lysolecithin exposure (see below) (Miron et al., 2010). This unexpected result may be accounted for through acute demyelination, and the authors of this study suggest that increased astrogliosis produced by FTY720 treatment may limit lesion areas to facilitate remyelination. Astrogliosis has been reported to be beneficial as well as harmful in many neurodegenerative diseases, including MS (Williams et al., 2007). Interestingly, one report showed that astrogliosis precedes immune cell infiltration during EAE (Luo et al., 2007), supporting the notion that the regulation of astrogliosis, perhaps via S1P signaling inside of the CNS may be a distinct process compared to lymphocyte trafficking mechanisms. Preliminary data in MS animal models support FTY720 treatment in reducing astrogliosis (Choi et al., 2008a); future studies will be required to determine the actual functional relationships between FTY720 and astrogliosis in MS models and MS.

Oligodendrocytic biology is another important aspect for MS pathologies and therapies because oligodendrocytes are the main cell type required for CNS myelination. Even though there is no direct *in vivo* evidence

for the role of oligodendrocytic S1P signaling in processes of demyelination and remyelination, several *in vitro* studies have shown that an FTY720-driven rescue of local demyelination in an EAE model increases the possibility of relevance (Jaillard et al., 2005; Kataoka et al., 2005; Papadopoulos et al., 2010). *In vitro* studies also have shown that FTY720 does affect the biology of oligodendrocyte cell lineages (oligodendrocytes and oligodendrocyte precursor cells), including survival, proliferation, migration, and differentiation, despite some contradictory reports that may reflect FTY720 dosage, among other variables (Coelho et al., 2007; Jung et al., 2007; Novgorodov et al., 2007; Dev et al., 2008; Miron et al., 2008a, 2008b, 2008c). Since these biological activities have relevance to remyelination, this may explain how FTY720 reduces demyelination during EAE and/or MS. Importantly, the impact of FTY720 on oligodendrocytes is possibly through S1P_{1/3/5} that are highly expressed in these cell lineages. Recently an organotypic culture system was used to assess effects of FTY720 on the CNS, which demonstrated actions independent of an intact immune system (Miron et al., 2010). FTY720 enhanced remyelination in demyelinated slices by lysolecithin exposure and was accompanied by process extension of oligodendrocyte lineage cells, which might be mediated by S1P₅, as revealed by pharmacological approaches (Miron et al., 2010). In other studies, no defects in myelination were reported in mice with a genetic deletion of S1P₅ (Jaillard et al., 2005), although remyelination after chemical lesion may involve distinct mechanisms. Further studies focused on *in vivo* effects utilizing EAE-induced mice lacking oligodendrocytic S1P₁ or S1P₅ should aid in clarifying these interesting results.

Discordance between lymphocyte levels and clinical severity

While there is a clear relationship between peripheral blood lymphocyte levels and FTY720 dosage-increasing dose reduces lymphocyte levels – this relationship did not track with the clinical score following the termination of FTY720 treatment in a relapsing-remitting EAE model (Webb et al., 2004). Upon FTY720 treatment, clinical severity of EAE-induced mice was reduced along with a reduction in plasma lymphocyte levels. When FTY720 treatment ended, a surprising result occurred: clinical severity returned, but lymphocyte levels were not reversed. If reduced lymphocyte levels by FTY720 treatment is the primary MOA in reducing clinical signs, then a return of lymphocyte levels should accompany a return of clinical signs upon the termination of

FTY720 treatment, which was not observed. One possible explanation for this discrepancy is a non-lymphoid mechanism of FTY720 efficacy. As already noted, a major locus of S1P receptor expression is the CNS (Rao et al., 2003; Foster et al., 2007; Gardell et al., 2007; Herr and Chun, 2007; Kimura et al., 2007; Miron et al., 2008c), while the receptor subtype, S1P₁, plays an important role in modulating lymphoid cells relevant to EAE (Fujino et al., 2003; Allende et al., 2004; Matloubian et al., 2004; Webb et al., 2004; Pappu et al., 2007; Schwab and Cyster, 2007), suggesting that S1P₁ in CNS cells could also be involved. Moreover, FTY720 can be specifically accumulated in the CNS after administration (Foster et al., 2007) (see below). This possibility could be addressed in the future through the use of a conditional mutant where S1P₁ is specifically removed in certain CNS cell types while maintaining expression in non-neural (e.g., lymphoid) cell types.

Preferential localization of FTY720 in the CNS

FTY720 preferentially localizes in the CNS (Foster et al., 2007). Using autoradiography, FTY720 distribution was determined after treatment. FTY720 penetrated the blood brain barrier and localized in the white matter, and likely other areas, of the CNS (Foster et al., 2007); the white matter is the area where damage mainly occurs during EAE and/or MS. Moreover, the accumulation of FTY720 is increased in a dose-dependent manner and FTY720 is phosphorylated to its bioactive form (FTY720-P) by Sphk2 at the accumulation site (Foster et al., 2007). What is interesting is that the levels of FTY720 are much higher in the CNS than in blood and that FTY720 deposition is mainly observed in CNS tissues rather than cerebrospinal fluid (CSF) (Meno-Tetang et al., 2006; Foster et al., 2007). These data implicate the possibility of a novel, neurological MOA for FTY720 efficacy that remains to be functionally assessed.

CONCLUSION

This review focused on data related to FTY720's MOA in MS, particularly the possible biologic activities within the CNS. Even though FTY720's MOA in MS is believed to be immunological, several reports support the possibility that there is a distinct neurological MOA. FTY720 influences on astrogliosis via S1P signaling may be one of the candidates for this MOA, although conflicting data for increasing vs decreasing astrogliosis require further study. Ongoing preliminary studies using combined pharmacological and genetic approaches have suggested that S1P₁

signaling in the CNS is critical for FTY720 efficacy in EAE, along with the regulation of astrogliosis (Choi et al., 2008a). In addition, other cell types that are present in the CNS must be considered. MS pathology is complicated because of the involvement of many cell types from different systems. Even within the CNS, other neurological or non-neurological cell types are involved in MS pathologies and possibly in FTY720 efficacy via S1P signaling, including neurons (Edsall et al., 1997; Toman et al., 2004; Mizugishi et al., 2005; Zhang et al., 2006; Kimura et al., 2007) and microglia (Tham et al., 2003; Nayak et al., 2010) that are also important cell types in MS.

Taken together, S1P signaling represents a new target for the development of MS therapies and its modulator, FTY720 (fingolimod) may soon enter clinical practice as the first oral agent. Further studies are still required to establish the MOA of FTY720 in MS, with a growing body of circumstantial data supporting a possible CNS MOA that could be distinct from those affecting the immune system. If substantiated, this emerging MOA could represent a new paradigm for understanding and therapeutically addressing MS through the development of oral medicines with non-immunosuppressive activities.

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