

Molecular pharmacodynamics of new oral drugs used in the treatment of multiple sclerosis

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Abstract: New oral drugs have considerably enriched the therapeutic armamentarium for the treatment of multiple sclerosis. This review focuses on the molecular pharmacodynamics of fingolimod, dimethyl fumarate (BG-12), laquinimod, and teriflunomide. We specifically comment on the action of these drugs at three levels: 1) the regulation of the immune system; 2) the permeability of the blood–brain barrier; and 3) the central nervous system. Fingolimod phosphate (the active metabolite of fingolimod) has a unique mechanism of action and represents the first ligand of G-protein-coupled receptors (sphingosine-1-phosphate receptors) active in the treatment of multiple sclerosis. Dimethyl fumarate activates the nuclear factor (erythroid-derived 2)-related factor 2 pathway of cell defense as a result of an initial depletion of reduced glutathione. We discuss how this mechanism lies on the border between cell protection and toxicity. Laquinimod has multiple (but less defined) mechanisms of action, which make the drug slightly more effective on disability progression than on annualized relapse rate in clinical studies. Teriflunomide acts as a specific inhibitor of the de novo pyrimidine biosynthesis. We also discuss new unexpected mechanisms of these drugs, such as the induction of brain-derived neurotrophic factor by fingolimod and the possibility that laquinimod and teriflunomide regulate the kynurenine pathway of tryptophan metabolism.

Keywords: demyelinating diseases, pharmacotherapy, fingolimod, dimethyl fumarate, laquinimod, teriflunomide

Introduction

The advent of new oral drugs opens a new era in the treatment of multiple sclerosis (MS). These drugs, some of which are already marketed or in the final stages of clinical development, are highly heterogeneous in terms of mechanism of action and clinical efficacy. Here, we discuss the pharmacodynamics of fingolimod, dimethyl fumarate, laquinimod, and teriflunomide in an attempt to offer a head-to-head comparison of their mechanisms of action at three levels: the immune system, the blood–brain barrier, and the central nervous system (CNS). We searched PubMed-MEDLINE using the headings fingolimod, sphingosine-1-phosphate, dimethyl fumarate, fumarate, nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2), laquinimod, and teriflunomide linked to MS, experimental autoimmune encephalomyelitis (EAE), immune system, immune function, vascular permeability, blood–brain barrier, neurons, astrocytes, oligodendrocytes, and microglia. Primary objectives of the review are to 1) provide a molecular correlate of the beneficial effect of the four drugs in MS and EAE; 2) comment on some ambiguous aspects inherent to their mechanism of action; and 3) to lay the groundwork for the choice of one of these drugs in the treatment of MS.

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Other issues that may critically influence the choice of one of the four drugs, such as pharmacokinetics, safety and tolerability, and costs, are not discussed here.

General mechanisms of action Fingolimod

Fingolimod (Gilenya™, Novartis, Basel, Switzerland) was the first oral drug to receive US Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval for the treatment of relapsing-remitting MS (RRMS). In two phase III clinical studies (TRANSFORMS [Trial assessing Injectable Interferon Versus FTY720 Oral in relapsing-Remitting Multiple Sclerosis] versus interferon [IFN]- β and FREEDOMS [FTY720 Research Evaluating Effects of Daily Oral therapy in Multiple Sclerosis] versus placebo) fingolimod (0.5 mg/day) demonstrated high efficacy in reducing the annual relapse rate (by about 55%) and progression of neurological disability.¹⁻³ Fingolimod also had a remarkable effect on brain atrophy associated with MS, reducing the rate of atrophy to the same levels as those observed in healthy subjects.

Fingolimod has a unique mechanism of action among the drugs that are currently used in the treatment of MS.⁴ Fingolimod is an analog of sphingosine, which is a component of glycosphingolipids, and is more lipophilic than sphingosine as a result of the presence of an aromatic ring in its structure (Figure 1). Sphingosine is phosphorylated inside the cells into sphingosine-1-phosphate (S1P) by two protein kinases, named type-1 and type-2 sphingosine kinase (SphK1 and -2). S1P is then transported outside the cells.⁵⁻⁷

S1P is present in relatively high concentrations in the blood and lymph, but in lower concentrations inside the lymph nodes and other organs.⁸ The concentration gradient between circulatory fluids and tissue interstitium is critical for the regulation of cell migration by S1P. S1P acts as an

'inside-out' signaling molecule by activating five types of G-protein-coupled receptors (S1P1R to S1P5R), which are linked to multiple transduction pathways. Fingolimod shares the same metabolic fate of sphingosine, and is phosphorylated by SphK2 into fingolimod phosphate. Because of the high distribution volume of fingolimod, high levels of fingolimod phosphate are found in the interstitium of lymph nodes and other organs.⁹ Fingolimod phosphate activates S1P1Rs with high potency ($K_D=0.3$ nM) and higher efficacy than S1P. Overactivation of S1P1Rs results in rapid receptor desensitization and internalization.¹⁰ Thus, fingolimod behaves as a functional antagonist of S1P1Rs, although binding of arrestins to S1P1Rs may redirect signaling to alternative G-protein-independent pathways. Fingolimod phosphate can also activate S1P3Rs, S1P4Rs, and S1P5Rs, but not S1P2Rs (Figure 1). The following aspects must be taken into account: 1) functional antagonism is preceded by a transient activation of S1P1Rs, a mechanism that underlies the first-dose bradycardia associated with fingolimod in MS patients; and 2) only receptors that are actively coupled to G proteins can be desensitized in response to agonist activation. Thus, the balance between S1P1R activation and functional antagonism by fingolimod critically depends on the receptor reserve (ie, the proportion of S1P1Rs that are not functionally recruited to the biological response in a particular context and cell type).

Dimethyl fumarate

Dimethyl fumarate (also known as BG-12) is an oral prodrug that is converted into monomethyl fumarate and fumarate inside cells. The extent to which dimethyl fumarate, monomethyl fumarate, and fumarate contribute to the immune suppressant and cytoprotective activity of dimethyl fumarate is unknown. Fumaric acid esters, under the trade name of Fumaderm® (Fumapharm AG, Lucerne, Switzerland), have been used in Germany since 1994 in the treatment of psoriasis.^{11,12} The efficacy of oral dimethyl fumarate in the treatment of MS has been proven in two phase III studies named DEFINE (Placebo-controlled Phase 3 Study of Oral BG-12 for Relapsing Multiple Sclerosis) and CONFIRM (Placebo-controlled Phase 3 Study of Oral BG-12 or Glatiramer in Multiple Sclerosis).^{13,14} The latter study included an arm with glatiramer acetate as an active comparator. In both studies, dimethyl fumarate reduced relapse rates by about 50% compared with placebo. Reduction of disability progression was significant in the DEFINE study,¹³ but not in the CONFIRM study.¹⁴ Dimethyl fumarate is administered at a dose of 240 mg, two or three times per day.

Dimethyl fumarate enhances the activity of Nrf2, a basic leucine zipper transcription factor that controls the

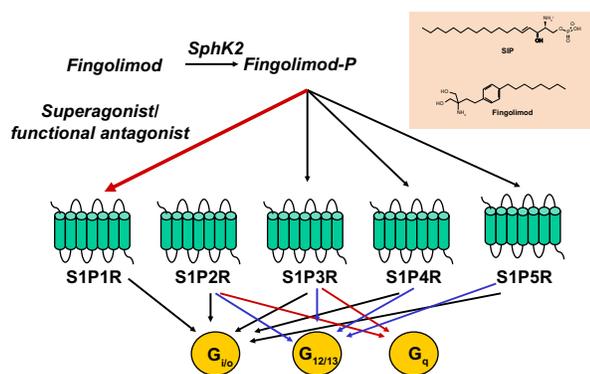


Figure 1 Fingolimod phosphate activates four sphingosine-1-phosphate receptors: S1P1R, S1P3R, S1P4R, and S1P5R. Superactivation of S1P1R results in functional antagonism. Coupling of S1PRs to G proteins is also shown.

Abbreviations: SphK2, type-2 sphingosine kinase; S1P, sphingosine-1-phosphate.

expression of a wide range of antioxidant proteins and detoxification enzymes.^{15,16} Under physiological conditions, Nrf2 is retained in the cytoplasm by a dimer of the Kelch-like ECH-associated protein (Keap1), which serves as an adapter for the ring box protein (Rbx1)-bound Cullin3 (Cul3)-based E3 ligase. Cul3 primes Nrf2 to degradation by the ubiquitin-proteasome system. Keap1, which is a Zn-finger protein, acts as the redox sensor of the system. Its highly reactive cysteine residues can be oxidized to sulfenic acid, form disulfides, or be alkylated by electrophiles. This leads to destabilization of the complex and release of Nrf2, which is no longer degraded and translocates into the nucleus. Nuclear import of Nrf2 is facilitated by protein kinase C- δ /t-mediated Nrf2 phosphorylation. Nrf2 binds in a complex with a small Maf protein or a member of the activator protein-1 (AP-1) family to the antioxidant response element sequence in the promoter of its target genes.^{17,18} Activation of Nrf2 results in the induction of genes encoding enzymes that detoxify reactive oxygen species (ROS) or enhance glutathione (GSH) levels, eg, GSH-synthesizing enzymes, NAD(P)H: quinone oxidoreductase, thioredoxin, and heme oxygenase-1 (HO-1).¹⁹ Some of the proteins encoded by these genes are vital for cells' defense against oxidative damage. Nuclear export of Nrf2 is promoted by phosphorylation of Tyr568 mediated by the tyrosine kinase, Fyn. Activation of glycogen

synthase kinase-3 β promotes nuclear translocation of Fyn, thus limiting the nuclear availability of glycogen-synthase kinase-3 β (Figure 2).^{20,21}

Activators of Nrf2 include ROS, electrophiles, and other molecules that are potentially hazardous to cells and can be neutralized by Nrf2-regulated genes. Subtoxic concentrations of oxidants or electrophiles can afford cell protection against toxic concentrations of the same or other molecules via the activation of the Keap1-Nrf2 pathway. This toxicological phenomenon, called "hormesis", is reminiscent of the phenomenon of ischemic preconditioning, in which a sub-lethal ischemic insult protects cells against a subsequent ischemic challenge. Activation of Nrf2 by dimethyl fumarate fits into this scenario. Dimethyl fumarate forms conjugates with GSH, thereby causing depletion of intracellular free GSH.^{12,19-21} This potentially harmful event may, in principle, activate the Nrf2 pathway in response to dimethyl fumarate, although other mechanisms of activation are also possible. There are some caveats: 1) one cannot exclude that the initial GSH depletion may reach the threshold for cell toxicity if combined with additional toxic insults; 2) GSH conjugation is considered to be one of the major defensive mechanisms against oxidative damage, but can paradoxically generate harmful species (eg, episulfonium ions) under particular circumstances; 3) activation of the Nrf2 system may inhibit tumor initiation but may also facilitate

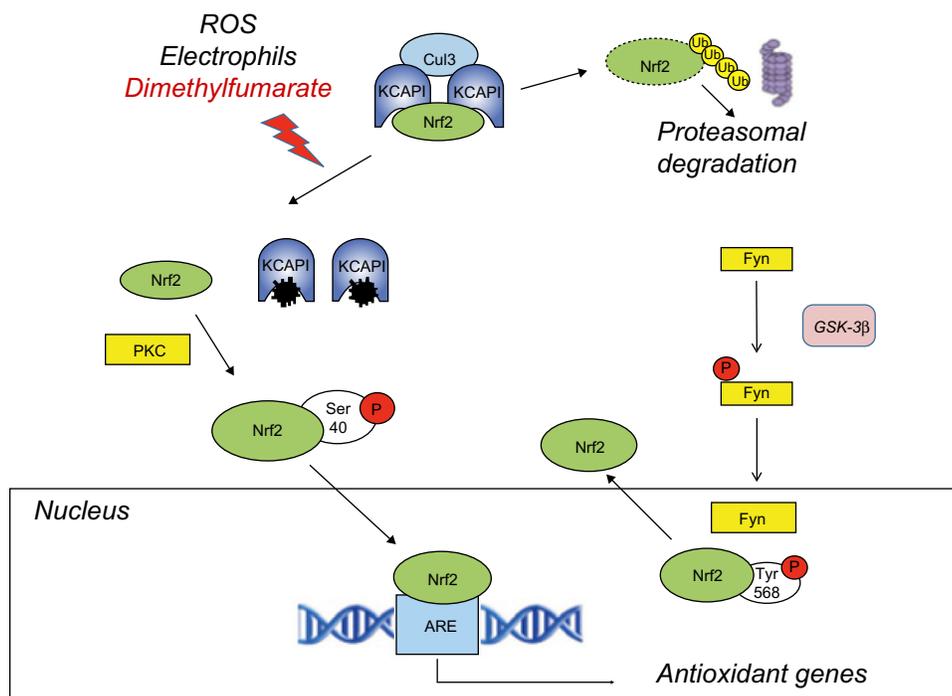


Figure 2 Activation of the Nrf2 pathway by dimethyl fumarate.

Abbreviations: ARE, antioxidant responsive elements; Cul3, Cullin3; GSK3 β , glycogen synthase kinase-3 β ; KEAP, Kelch-like ECH-associated protein; Nrf2, nuclear factor (erythroid-derived 2)-related factor 2; PKC, protein kinase C; ROS, reactive oxygen species.

tumor growth by inhibiting apoptosis and autophagy and by enhancing the resistance to chemotherapeutic drugs via the multidrug-resistant proteins;^{20,22} and 4) agents that activate the Nrf2 pathway may cause toxic effects due to their interaction with nucleophiles such as GSH.²³ This may explain the high frequency of gastrointestinal adverse effects found in patients treated with dimethyl fumarate in the DEFINE and CONFIRM studies.^{13,14}

Laquinimod

Laquinimod is structurally similar to roquinimex (linomide), a quinoline-3-carboxamide analog. The efficacy of laquinimod in MS was evaluated in a placebo-controlled phase III study (ALLEGRO [Placebo-controlled Trial of Oral Laquinimod for Multiple Sclerosis]), in which the drug reduced disability progression and showed a modest effect on annualized relapse rate.²⁴

The mechanism of action of laquinimod is only partially known. Interestingly, laquinimod is also structurally similar to kynurenic acid and xanthurenic acid, which are two metabolites of the kynurenine pathway of tryptophan metabolism (Figure 3). Kynurenic acid acts as an antagonist at the glycine site of N-methyl-D-aspartate (NMDA) receptors, quinolinic acid is an orthosteric NMDA receptor agonist, and xanthurenic acid is a putative type-2/3 metabotropic glutamate receptor agonist.²⁵ Interestingly, indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the kynurenine pathway is induced by pro-inflammatory cytokines, and its expression in antigen-presenting cells (APC) supports T-regulatory (T_{Reg}) cell generation and immune tolerance.^{26,27} Some of the metabolites of the kynurenine pathway have been considered as potential effectors of IDO in the regulation of immune tolerance.^{28,29} Thus, the kynurenine pathway is

highly relevant to the pathophysiology of neuroinflammation and EAE, being at the interface between immune regulation and mechanisms of neurodegeneration/neuroprotection. In one report, laquinimod was indicated as an inhibitor of the kynurenine pathway.³⁰ However, no information was provided on the enzymatic reaction of the kynurenine pathway that is targeted by laquinimod.

Teriflunomide

Teriflunomide is the active metabolite of leflunomide, an immunosuppressive drug approved as a disease-modifying agent in the treatment of rheumatoid arthritis.³¹ The efficacy of teriflunomide in MS was shown in a placebo-controlled phase III study (Teriflunomide Multiple Sclerosis Oral [TEMSO]), in which the drug reduced both the annualized relapse rate (by about 30% versus placebo) and the disability progression.³² In contrast with laquinimod, the mechanism of action of teriflunomide is well established.

Teriflunomide behaves as a high-affinity inhibitor of dihydroorotate dehydrogenase (DHODH), the enzyme that converts dihydroorotate into orotate in the de novo synthesis of pyrimidines.^{33,34} De novo synthesis of pyrimidines is pivotal for the metabolism of highly proliferative cells such as activated lymphocytes, and, therefore, inhibition of DHODH accounts for the immunosuppressive effect of teriflunomide (Figure 4). Teriflunomide inhibits DHODH activity in a competitive manner, with a half maximal inhibitory concentration (IC₅₀) of 1.25 μM.³⁵ All DHODH-related effects of teriflunomide can be overcome by increases in intracellular levels of pyrimidines.

Pyrimidine nucleotides originate either from the de novo synthesis described above or from a 'salvage pathway' that is independent of DHODH. Thus, cells might become resistant to teriflunomide by enhancing the activity of the salvage pathway. However, this mechanism is effective in resting cells, but not in highly proliferating cells, such as blasting lymphocytes, which strictly depend on de novo synthesis of pyrimidines. Teriflunomide can be considered as an 'atypical' antimetabolite because it inhibits proliferation of blasting lymphocytes without affecting resting and homeostatically expanding lymphocytes.³⁶

Interestingly, DHODH-independent effects of teriflunomide have been described in vitro. For example, teriflunomide inhibits Janus kinases 1 and 3 (JAK1/3), two particular tyrosine kinases involved in cytokine signaling.^{37,38} Src family tyrosine kinases and receptor tyrosine kinases can also be targeted by teriflunomide.³⁹ Two of the most important intracellular signaling pathways, the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B

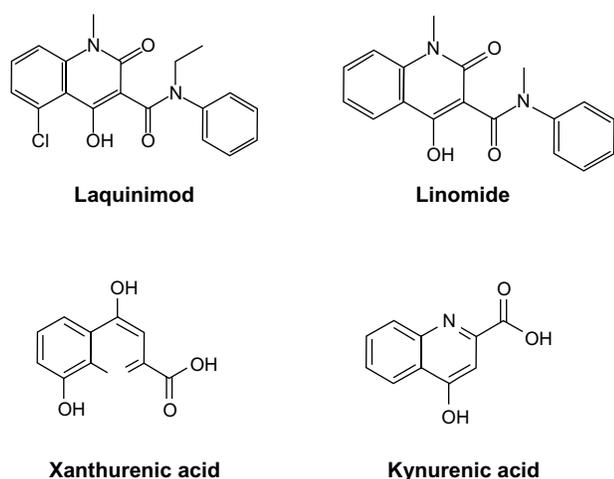


Figure 3 Molecular structures of laquinimod, linomide, xanthurenic acid, and kynurenic acid.

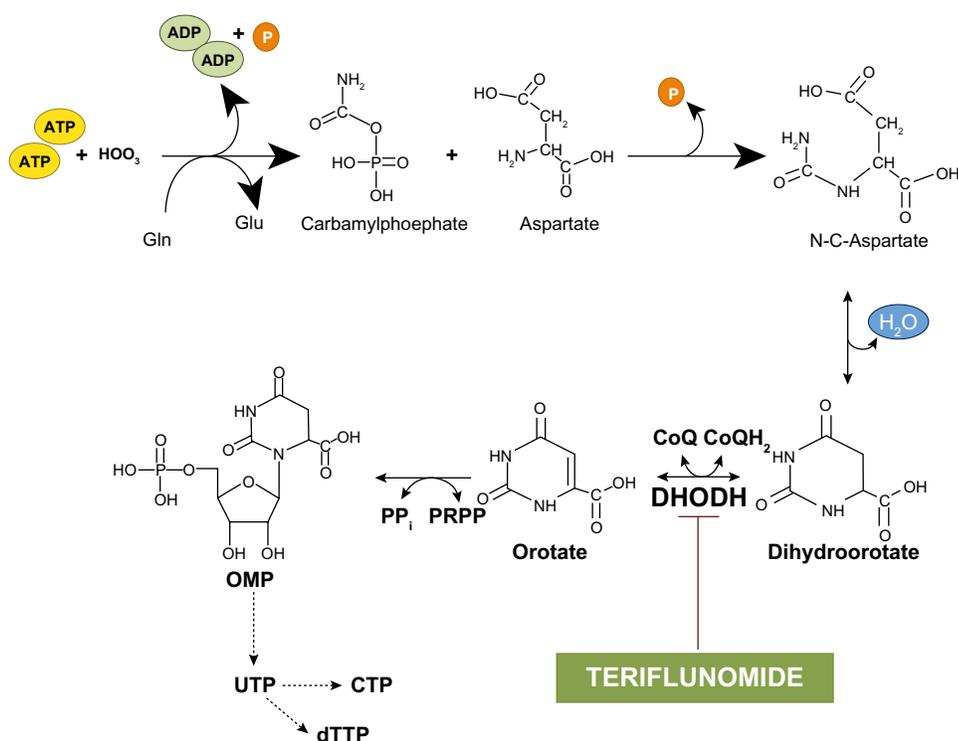


Figure 4 De novo synthesis of pyrimidines: inhibition of dihydroorotate dehydrogenase by teriflunomide.

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHODH, dihydroorotate dehydrogenase; OMP, orotidine-5'-phosphate; UTP, uridine-5'-triphosphate; CTP, cytosine-5'-triphosphate; dTTP, deoxythymine-5'-triphosphate; PRPP, phosphoribosyl pyrophosphate; PPI, pyrophosphate.

(NF- κ B) pathways, are inhibited by teriflunomide in cultured cells. Finally, teriflunomide inhibits type-2 cyclooxygenase (COX-2) activity, with an IC₅₀ of 0.5–20 μ M,⁴⁰ and it has also been suggested that teriflunomide acts as an inhibitor of the kynurenine pathway.³⁰

Effects on the immune system

Fingolimod

The main action of fingolimod in the immune system is to regulate the trafficking of lymphocytes between primary and secondary lymphoid organs and circulating fluids (blood or lymph).⁴¹ Fingolimod causes transient and reversible lymphopenia without affecting lymphocyte proliferation or survival. Lymphopenia results from the retention of T-naïve and central memory T-cells (T_n and T_{CM}) in secondary lymphoid organs. The retention of T_{CM} cells strongly contributes to the therapeutic efficacy of fingolimod because T_{CM} cells account for the vast majority of T-cells invading the CNS in MS.^{42,43} Fingolimod has no effect on circulating granulocytes, monocytes, eosinophils, erythrocytes, and platelets, and does not affect T-cell activation, proliferation, differentiation, or cytokine and antibody production.

The S1P1R, which is the main target of fingolimod, is one of the G-protein-coupled receptors that regulate migration of immune cells from primary and secondary lymphoid organs

to the blood or lymph and vice versa. This particular function is also shared by S1P3Rs, S1P5Rs, the CXCR4, CXCR5, and CCR7 chemokine receptors, and the CB2 cannabinoid receptors.⁴⁴ What is critical for the chemoattractive or chemorepulsive action of S1PRs is the concentration gradient of the ligand, S1P, between the blood or lymph and the interstitial fluid. S1P is present in relatively high concentrations in the blood or lymph, but only in low amounts in the interstitial fluid of lymphoid organs. Thus, S1P1Rs are maintained in a desensitized state by the high amount of S1P in the blood and lymph, whereas they are fully active in the interstitium of lymph nodes or other lymphoid organs. Circulating lymphocytes enter the lymph nodes across high endothelial venules, and T lymphocytes are attracted to the T zone in response to CCR7 activation by their cognate chemokine ligands (CCL19/CCL21). In contrast, B lymphocytes are attracted to the follicles by the CXCR5 ligand, CXCL13. Activation of S1P1Rs by endogenous S1P allows the egress of CCR7⁺ cluster of differentiation (CD)-45RA⁺ T_n cells and CCR7⁺CD45RA⁻ T_{CM} cells from the lymph nodes by over-riding the retention signal of CCR7. It is curious that the two 'antagonistic' receptors, S1P1R and CCR7, are both coupled to G_i proteins. Because of its high lipophilicity, fingolimod penetrates in large amounts inside the lymph nodes, and over-stimulates S1P1Rs after its transformation into fingolimod phosphate. This results in

rapid desensitization and functional antagonism of S1P₁R in spite of the low levels of S1P. Thus, fingolimod tips the balance between CCR7 and S1P₁R in favor of CCR7 by simulating an environment rich in extracellular S1P. Cells lacking the chemokine retention signal, ie, CCR7⁺CD45RA⁺ and CCR7⁻CD45RA⁻ effector memory T-cells (T_{EM}) exit the lymph nodes independently of S1P₁Rs and are therefore insensitive to fingolimod. T_{EM} cells play an important role in mechanisms of immune surveillance at the site of entry of pathogens (eg, the intestinal mucosa), and represent a major defense against viral infections. Thus, fingolimod acts as an immune suppressant in MS with minimal or no effect on peripheral mechanisms of immune surveillance (Figure 5). Using a metaphor, fingolimod retains the army in the barracks, leaving the sentinels outside. S1P₁Rs also regulate T-cell egress from the thymus as well as the cyclical shuttling of B-cells between the marginal and follicular zones of the spleen.⁴⁵⁻⁴⁹

Differentiation of CD4⁺ T_n cells into diverse effectors and regulatory lineages is a central event in the regulation of immune function, with T-helper (T_H)-1 and T_H 17 cells supporting autoimmunity in EAE and MS, and T_{Reg} cells mediating mechanisms of immune tolerance. Liu et al⁵⁰ found that activation of S1P₁Rs restrains the thymic generation, peripheral maintenance, and suppressive activity of T_{Reg} cells. S1P₁Rs signal via the phosphatidylinositol-3-kinase/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) kinase pathway to over-ride the T_{Reg} -promoting function of transforming growth factor- β (TGF- β). More specifically, S1P₁Rs restrain the action of the TGF- β -related signaling molecule, Smad-3^{50,51} (Figure 6). One expects that

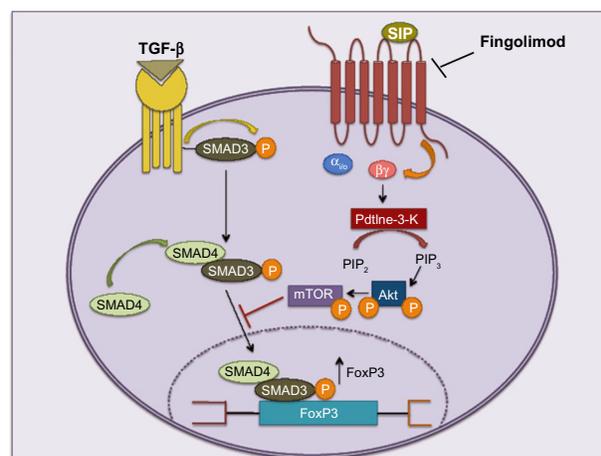


Figure 6 Fingolimod enhances the production of T_{Reg} cells by acting as a functional antagonist of S1P₁Rs.

Abbreviations: Akt, protein kinase B; mTOR, mammalian target of rapamycin; S1P, sphingosine-1-phosphate; TGF, transforming growth factor; T_{Reg} , T-regulatory cell.

fingolimod, acting as a functional antagonist of S1P₁Rs, enhances T_{Reg} differentiation and activity at the expense of T_H 1 and T_H 17. Accordingly, fingolimod enhances the conversion of Foxp3⁻CD4⁺ T-cells into Foxp3⁺ T_{Reg} cells in lymphoid organs of non-obese diabetic mice,⁵² and improves T_H 1-mediated colitis and acute kidney injury induced by ischemia/reperfusion by enhancing the formation and activity of T_{Reg} cells.^{53,54} There are no published studies on the effect of fingolimod on T_{Reg} cells in MS patients. However, it is known that fingolimod reduces blood levels of T_H 17 cells by >90% in MS patients, perhaps because these cells are found primarily within the pool of T_{CM} cells that are retained in the lymph nodes.⁵⁵

Dimethyl fumarate

Treumer et al⁵⁶ showed that dimethyl fumarate causes apoptotic death of purified human T-lymphocytes stimulated with anti-CD3 antibodies and/or cytokines, a mechanism that might explain the beneficial effect of Fumaderm[®] in the treatment of psoriasis.^{11,12,57,58} How does dimethyl fumarate cause T-cell death when the Nrf2 system represents one of the cell's main defenses against oxidative damage? One possible explanation is that T-cell death results from the initial trigger of Nrf2 activation, represented by the GSH depletion caused by dimethyl fumarate. If dimethyl fumarate concentrations at the target site are high, then the resulting GSH depletion may be sufficient to reach the threshold for the induction of cell death. Accordingly, dimethyl fumarate was found to cause death of activated splenocytes only at high micromolar concentrations, but did not affect cell viability at concentrations of 1 or 10 μ M. However, low micromolar

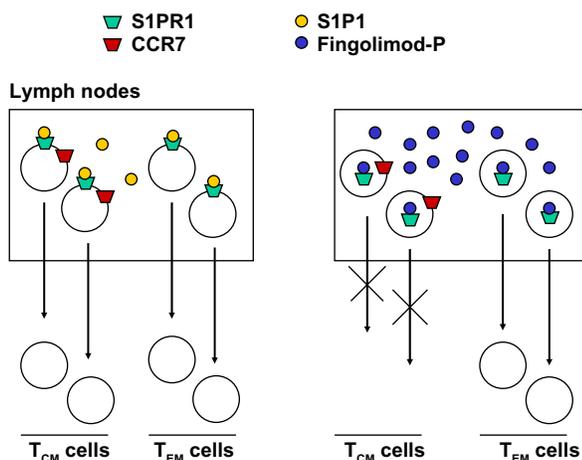


Figure 5 Fingolimod inhibits the egress of T_{CM} cells from lymph nodes without affecting the egress of T_{EM} cells. T_{EM} cells do not express CCR7 receptors.

Abbreviations: CCR, chemokine receptor; T_{CM} , central memory T-cells; T_{EM} , effector memory T-cells.

concentrations of dimethyl fumarate were sufficient to inhibit cytokine production from splenocytes,⁵⁹ suggesting that the real mechanism responsible for the immune-suppressive action of dimethyl fumarate involves changes in the cytokine network rather than a direct effect on T-cell viability.

Recent evidence indicates that dimethyl fumarate acts at the core of the immunological synapse by inhibiting dendritic cell maturation and differentiation of T_n cells into autoreactive T_H1 and T_H17 cells. Dimethyl fumarate suppresses NF- κ B signaling in dendritic cells by reducing p65 nuclear translocation and phosphorylation by the ERK (extracellular signal-regulated kinase)1/2 kinase target, mitogen stress-activated kinase 1.⁶⁰ Dimethyl fumarate treatment in humans generates type-II dendritic cells that produce interleukin (IL)-10 instead of IL-12 and IL-23, and drive the differentiation of T_n cells into IL-4-producing T_H2 cells instead of T_H1 and T_H17 . The underlying mechanism has been clarified in mice, where dimethyl fumarate produced an initial GSH depletion followed by Nrf2 activation, induction of HO-1, and impaired signal transducers and activators of transcription 1 (STAT1) phosphorylation. HO-1 translocates to the nucleus, where it prevents transcription of the p19 subunit of IL-23, whereas STAT1 inactivation inhibits transcription of the p35 subunit of IL-12.⁶¹ This epigenetic mechanism may underlie the ability of dimethyl fumarate to inhibit the T_H1 and T_H17 branches of the adaptive immune response, which are critically involved in the pathogenesis of MS and psoriasis. No data are available on the effect of dimethyl fumarate on differentiation and activity of T_{Reg} cells. Thus, it is not known whether dimethyl fumarate affects mechanisms of immune tolerance in autoimmune disorders.

Laquinimod

Laquinimod reduces the expression of pro-inflammatory cytokines and enhances the expression of regulatory cytokines, thus hindering over-reactive immune responses.^{62,63} Central to the anti-inflammatory action of laquinimod is the inhibition of NF- κ B, a signaling pathway that is activated by pro-inflammatory cytokines and, in turn, drives the expression of pro-inflammatory cytokines. Gurevich et al⁶⁴ found that laquinimod inhibits the NF- κ B pathway by suppressing the beta-transducin repeat containing encoding gene that increases ubiquitination and degradation of the NF- κ B inhibitor protein, I κ B. This was followed by the suppression of NF- κ B downstream signaling genes.⁶⁴ In vitro studies have shown that laquinimod does not affect viability or proliferation of immune cells and does not influence humoral immune responses.⁶²

Laquinimod acts at the core of the immunological synapse by reducing the expression of genes related to the antigen presentation pathway in APCs.⁶⁴ In addition, laquinimod down-regulates the expression of genes that are involved in the activation of APCs and T-lymphocyte chemotaxis.⁶⁴ As a result of a primary action on APCs, laquinimod polarizes the immune response towards T_H2 and T_{Reg} cells at the expense of T_H1 and T_H17 cells, thus protecting mice against EAE or experimental autoimmune neuritis (animal model of the Guillain-Barré syndrome).⁶⁵⁻⁶⁸ This effect on antigen presentation is similar to that produced by glatiramer acetate, a first-line drug in the treatment of MS. The precise molecular mechanism by which laquinimod influences antigen presentation in dendritic cells and other APCs is unknown. Laquinimod might influence the kynurenine pathway, thus driving kynurenine metabolism towards the formation of molecules that promote immune tolerance at the immunological synapse. This interesting hypothesis warrants in-depth investigation.

Laquinimod can also modulate B-cell phenotypes by increasing the percentage of CD25⁺ cells in B lymphocytes isolated from healthy donors. However, this effect was not seen when lymphocytes were cultured from MS patients.⁶³

Finally, laquinimod affects innate immunity by targeting monocytes and APC subpopulations. In macrophages, laquinimod down-regulates genes related to cell proliferation, phagocytosis, and T-cell stimulation; in addition, laquinimod treatment reduces monocyte infiltration and promotes the proliferation of anti-inflammatory subsets of monocytes and dendritic cells in mouse models of EAE and in MS patients.^{64,68}

Teriflunomide

Teriflunomide restrains T-cell activation by inhibiting the formation of the immunological synapse, and by altering intracellular signaling.^{69,70} Activated T lymphocytes expand the intracellular pyrimidine pool by up to 8-fold, which not only serves as a substrate for DNA synthesis but is also involved in lipid biosynthesis, protein glycosylation, and other metabolic processes.⁷¹ Thus, teriflunomide behaves as a cytostatic agent. T-cells exposed to teriflunomide are still able to proliferate when activated ex vivo by mitogens,³⁶ and teriflunomide administration does not reduce CD4⁺ and CD8⁺ blood counts in patients.

In addition to its activity on cell proliferation, teriflunomide directly affects lymphocyte function, affects B-cell proliferation and activation,³⁶ and polarizes T-cell differentiation

towards the T_H2 phenotype at the expense of T_H1 cells.^{36,72} The inhibition of JAK/STAT signaling, COX-2 activity, and NF-κB may contribute to the activity of teriflunomide against autoreactive immune responses.

Effects on the permeability of the blood–brain barrier

Fingolimod

S1P regulates the permeability of the endothelial barrier by interacting with different S1PRs expressed by endothelial cells. Activation of S1P1Rs reinforces the endothelial barrier by a Rac-dependent mechanism, which leads to membrane translocation of claudin (CLN)-5, occludins, or zonula occludens proteins (ZO-1 and -2).⁷³ In contrast, activation of S1P2Rs and S1P3Rs enhances the permeability of the endothelial barrier by stimulating Rho activity, with ensuing activation of Rho-associated protein kinase (ROCK) and phosphate and tensin homologue (PTEN).^{74,75} Fingolimod reinforces the endothelial barrier in models of neuroinflammation,^{76,77} as well as in other models of vascular leakage.⁴³ The endothelium of human brain microvessels expresses type-2 SphK, which phosphorylates fingolimod into fingolimod phosphate.⁷⁸ It is possible that fingolimod phosphate acts as an agonist (and not as a functional antagonist) of endothelial S1P1Rs, which might be resistant to receptor desensitization because of a high receptor reserve or other mechanisms. However, this remains to be demonstrated. An alternative hypothesis is that fingolimod acts on reactive astrocytes associated with MS lesions by reducing the activity of acidic sphingomyelinase and the resulting formation of ceramide. Ceramide acts as a pro-inflammatory lipid molecule in EAE and MS, enhancing the permeability of the blood–brain barrier.⁷⁹ The two hypotheses on the mechanism of action of fingolimod are not mutually exclusive. Interestingly, both fingolimod and S1P reduce the activity of P-glycoprotein at the blood–brain barrier.^{80,81} P-glycoprotein is the main drug efflux pump, mediating mechanisms of drug resistance in the CNS and other organs. By inhibiting P-glycoprotein activity, fingolimod might enhance drug delivery in the CNS and boost the activity of multiple classes of drugs acting inside the brain parenchyma.

There are exceptions to the reinforcing action of fingolimod on the endothelial barrier. Of 2,564 MS patients treated with fingolimod in phase III clinical studies, 13 developed reversible macular edema, suggesting that fingolimod may enhance permeability of the blood–retinal barrier. However, it should be highlighted that this adverse effect was dose-dependent, and macular edema was observed only in two

patients treated with the approved dose of fingolimod (0.5 mg/day) for the treatment of RRMS.⁸² The exact mechanism by which fingolimod affects permeability at the blood–retinal barrier is unknown.

Dimethyl fumarate

To our knowledge, studies on the action of dimethyl fumarate on blood–brain barrier permeability have not been published. Dimethyl fumarate inhibits nuclear translocation and activation of NF-κB in human endothelial cells, thereby suppressing the induction of adhesion molecules in response to pro-inflammatory cytokines.⁸³ More recent data demonstrate that dimethyl fumarate suppresses the expression of type-2 vascular endothelial growth factor (VEGF) receptors (VEGFR2) in human endothelial cells by inhibiting the interaction of the Sp1 transcription factor with specific responsive elements at the VEGFR2 gene promoter.⁸⁴ This particular mechanism might restrain the action of VEGF at the blood–brain barrier, and, therefore, be relevant to the treatment of MS. Whether, and in which particular context, dimethyl fumarate inhibits the action of VEGF in the endothelium of brain microvessels remains to be established.

Laquinimod

The effect of laquinimod on the permeability of the blood–brain barrier remains to be investigated. Wegner et al⁶⁶ demonstrated a reduced migratory capacity of immune cells into the CNS in EAE-challenged mice treated with laquinimod. However, when the number of T-cells and macrophages was analyzed within CNS lesions, there was no significant difference between control mice and mice treated with laquinimod.⁶⁶

Teriflunomide

To the best of our knowledge, there are no published studies on the effect of teriflunomide on the blood–brain barrier.

Direct effects on CNS cells

Fingolimod

Fingolimod, which is highly lipophilic, easily crosses the blood–brain barrier. In addition, S1P1Rs, which are the main targets of fingolimod phosphate, are present in neurons, astrocytes, oligodendrocytes, and microglia, and are more abundant in the CNS than in any other organ. S1P3Rs are less abundant in the CNS, but they are found in neurons, astrocytes, and microglia. S1P5Rs are predominantly expressed in oligodendrocytes.^{43,85} S1P1Rs and S1P3Rs are strongly up-regulated in astrocytes present in MS lesions,⁸⁶

suggesting that these receptors have a role in the process of reactive gliosis and contribute to the overall mechanism of inflammation-induced cell death or repair. The specific role of astrocytic S1P1Rs in the action of fingolimod has been examined in an elegant study using conditional null mouse mutants lacking S1P1Rs either in astrocytes or neurons and challenged with EAE. Treatment with fingolimod, administered at the peak of the neurological symptoms, caused the expected peripheral lymphopenia in all mutants, but showed efficacy in relieving neurological signs of EAE only in wild-type mice or in mice lacking S1P1Rs in neurons. Mice lacking S1P1Rs in astrocytes developed less severe EAE, which was completely resistant to fingolimod.⁸⁷ These data suggest that S1P1Rs present in astrocytes support the development of EAE and are specifically targeted by fingolimod. However, the study is not sufficient to exclude the involvement of neuronal S1P1Rs in the overall effect of fingolimod in MS.⁸⁷ We and others have found that fingolimod protects cultured neurons against excitotoxic death. Neuroprotection was mediated by S1P1R-mediated activation of the MAPK and the phosphatidylinositol-3-kinase pathways, which are known to support cell survival.^{88,89} This mechanism might contribute to the protective activity of fingolimod in models of brain ischemia.^{90–92} Interestingly, fingolimod enhances the production of brain-derived neurotrophic factor (BDNF) in cultured neurons and in the mouse brain.⁸⁸ This effect has been shown to mediate the protective effect of fingolimod phosphate against β -amyloid neurotoxicity in cultured neurons.⁹³ Carriers of the low-BDNF expressing Met-66 allele show memory deficits and low hippocampal volume.^{94–97} In addition, BDNF is tightly linked to the pathophysiology of major depression, and serum BDNF levels predict response to antidepressant medication.^{98–100} We expect that fingolimod improves cognitive function and depressive mood in patients with MS by enhancing brain BDNF levels. This interesting hypothesis warrants further investigation.

The presence of S1P1Rs in oligodendrocytes suggests that fingolimod may have direct effects on mechanisms of demyelination and remyelination in MS. *In vitro* studies show that fingolimod phosphate promotes the differentiation of neural stem cells towards the oligodendroglial lineage and supports the proliferation and survival of oligodendrocyte progenitors.^{78,101–104} In non-immunological *in vivo* models of demyelination, fingolimod protects against myelin and axonal damage, but does not promote remyelination.^{54,105} In contrast, fingolimod has been found to enhance remyelination markers in *in vitro* models of demyelination.^{106,107} Thus, the possibility

that fingolimod facilitates mechanisms of myelin repair via a direct action on oligodendrocytes is still open.¹⁰⁸

Dimethyl fumarate

Dimethyl fumarate activates the Nrf2 system, which represents one of the major intracellular protective pathways against oxidative damage. ROS are critically involved in the pathophysiology of MS by promoting transendothelial leukocyte migration and causing myelin and axonal damage.^{109–111} Thus, in principle, dimethyl fumarate has the potential to protect neurons and oligodendrocytes in EAE and MS.

Dimethyl fumarate activates the Nrf2 pathway by causing an initial GSH depletion, which does not reach the threshold for the induction of cell death. This mechanism is reminiscent of ‘ischemic tolerance’, whereby short episodes of ischemia protect the brain or the heart against a subsequent ischemic insult. *In vitro* studies have shown that dimethyl fumarate protects neurons against oxidative damage.^{15,59} However, neuroprotection is critically time- and context-dependent. In one study, cultured neurons were challenged with high concentrations of glutamate, which reduce GSH levels by limiting the intracellular transport of the GSH precursor, cystine.⁵⁹ Under these conditions, pretreatment with dimethyl fumarate was highly neuroprotective. In contrast, dimethyl fumarate became neurotoxic when co-applied with sub-maximal concentrations of glutamate. Once again, the initial GSH depletion caused by dimethyl fumarate might be permissive in causing neuronal death if combined with proinflammatory cytokines, excitotoxins, or other harmful stimuli.⁵⁹ This raises the question of whether the drug exerts neuroprotective or neurotoxic effects under conditions in which neurons are repeatedly exposed to toxic insults, as occurs during neuroinflammatory flare-ups in MS. Studies in models of relapsing-remitting EAE in which the drug is applied at time intervals that coincide with the onset or peak of relapses may help to clarify this issue.

Astrocytes and microglia, which are critically involved in the neuroinflammatory cascade in EAE and MS, are also targeted by dimethyl fumarate via the activation of Nrf2 and the inhibition of NF- κ B. This is associated with induction of HO-1, activation of GSH synthesis, and inhibition of proinflammatory cytokine production and inducible nitric oxide synthase.^{19,112} However, again, dimethyl fumarate applied to cultured astrocytes was shown to acutely deplete GSH levels, which was not associated with cell death.¹¹³ The precise consequences of acute GSH depletion in astrocytes under conditions of neuroinflammation remain to be determined. Only a few studies have examined the effects of dimethyl fumarate

or other fumarate esters on oligodendrocytes. Dimethyl fumarate causes GSH depletion and Mrf2 activation in an OLN-93 oligodendroglial cell line, similar to what is observed in other cell types.¹¹⁴ However, in a non-immune-dependent *in vivo* model of demyelination (the cuprizone model in mice), dimethyl fumarate showed no protective activity on oligodendrocytes.¹¹⁵

Laquinimod

Laquinimod can cross the blood–brain barrier,⁶² and a series of indirect observations suggest that the drug affects the viability of neurons and oligodendrocytes independently of its anti-inflammatory effects. In the ALLEGRO study, laquinimod showed a greater efficacy on disability progression than on annualized relapse rate, in contrast with the majority of drugs used in the treatment of MS. The evidence that laquinimod reduces demyelination, axonal damage, and lesion size in the EAE model^{65–68} is also consistent with direct action of the drug in the CNS. Regulation of BDNF levels may represent a point of convergence between the central actions of fingolimod and laquinimod. Accordingly, laquinimod treatment enhances BDNF expression and reduces CNS injury in EAE mice.⁶⁷ Laquinimod can also modulate inhibitory gamma-aminobutyric acid (GABA)-ergic transmission in the CNS, under both normal and inflammatory conditions.¹¹⁶ However, it should be noted that the enhancing effect of laquinimod on GABA-mediated inhibitory postsynaptic currents requires concentrations of the drug that are several-fold higher than those reached inside the CNS during treatment.¹¹⁶

Laquinimod reduces astrogliosis and impairs astrocyte activation *in vitro* by negatively regulating the NF- κ B pathway. Interestingly, in contrast with dimethyl fumarate, laquinimod protects oligodendrocytes in the cuprizone model of demyelination, an effect that has been ascribed to its action on astrocytes.¹¹⁷

As highlighted above, laquinimod is a quinoline carboxamide derivative, and, as such, has the potential to modulate the activity of the kynurenine pathway.³⁰ This pathway has been implicated in the pathophysiology of multiple disorders of the CNS, including acute and chronic neurodegenerative disorders.²⁵ Two questions must be addressed: 1) which specific enzyme of the kynurenine pathway is inhibited by laquinimod; and 2) whether, and to what extent, activity of laquinimod on this pathway contributes to the central effects of the drug in EAE and MS.

Teriflunomide

There is no evidence suggesting a direct effect of teriflunomide on neurons and other cells resident in the CNS.

However, this is a possibility if the modulation of the kynurenine pathway by teriflunomide³⁰ is confirmed and better characterized.

Conclusion

We have discussed the molecular pharmacodynamics of fingolimod, dimethyl fumarate, laquinimod, and teriflunomide in an attempt to compare their mechanism of action in MS.

In brief, fingolimod has a unique mechanism of action among current MS therapies. It prevents the egress of particular subpopulations of T lymphocytes (ie, T_n and T_{CM} cells) from secondary lymphoid organs, thereby restraining autoimmunity in patients with MS. This particular mechanism results from desensitization and internalization of S1P1Rs on T-cells. Interestingly, fingolimod does not cause peripheral depletion of T_{EM} cells, which mediate immune surveillance at the site of entry of pathogens. Fingolimod also decreases the permeability of the blood–brain barrier and acts inside the CNS parenchyma by supporting neuronal survival. The latter effect may be mediated by an increased BDNF formation.

Dimethyl fumarate is thought to work in MS in two ways: via immune-suppressive activity and via protective effects in the CNS. Dimethyl fumarate alters the balance in the blood of immune reactive and immune tolerant T-cells, thereby causing immune suppression. Dimethyl fumarate activates the Nrf2 pathway of cell defense as a result of an initial depletion of GSH. In principle, this mechanism may result in cell protection; however, the initial GSH depletion may synergize with environmental insults, thereby promoting cell death. Thus, the impact of dimethyl fumarate on mechanisms of cell death and survival may be context-dependent.

Laquinimod has multiple (but less defined) mechanisms of action in MS. It is thought to act by modulating the immune system towards an anti-inflammatory rather than a pro-inflammatory response. Laquinimod may also prevent autoreactive T-cells from entering the CNS. Teriflunomide acts as a specific inhibitor of the *de novo* pyrimidine synthesis, thereby inhibiting lymphocyte proliferation. In addition, teriflunomide polarizes T-cell differentiation towards a T_H2 phenotype at the expense of autoreactive T_H1 cells.

While there are no available data on a direct effect of laquinimod and teriflunomide on CNS cells, it is possible that both drugs influence mechanisms of neuronal death and survival by interfering with the kynurenine pathway of tryptophan metabolism.

The pharmacodynamic profile of new oral drugs at the level of the immune system, blood–brain barrier, and CNS cells should be taken into account when choosing one of these drugs in the treatment of MS.

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Disclosure

The authors report no conflicts of interest in this work.

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