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Review article

Mechanism of action of three newly registered drugs for multiple sclerosis treatment

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ABSTRACT

Multiple sclerosis (MS) is a disease of suspected autoimmune origin leading to neurodegeneration. The disease pathomechanism is considered to be primarily based on neuroinflammation directed against myelin antigens caused by autoreactive T cells. MS etiology remains still unknown, which makes it difficult to create an efficient therapy, therefore, MS treatment targets mechanisms involved in disease pathology. In this review, we present the mechanism of action of three newly registered drugs for MS. Dimethyl fumarate (DMF) is an agent presenting a broad spectrum of action. Its main activity is based on activating the nuclear factor E2 dependent pathway leading to antioxidant enzyme synthesis. DMF in general suppresses the pro-inflammatory immune activity and exerts a neuroprotective action. Teriflunomide is a more focused drug, acting as an inhibitor of pyrimidines synthesis, important for rapidly dividing cells such as activated lymphocytes. Similarly, alemtuzumab, an anti-CD52 antibody, causes depletion of mainly lymphocytes. Since in MS pathology, T and B cells are involved, this mode of action is promising.

Contents

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Multiple sclerosis and its treatment

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) of multifactorial etiology, combining genetic and environmental factors [1,2]. The disease usually affects young people, with a peak of onset between 20 and 40 years old and women more often than men. MS is a significant cause of disability, especially in people in their most productive working and childbearing years [3]. MS is characterized by demyelination and axonal loss starting from the dysregulation of the immune system followed by a neurodegenerative process. In the pathogenesis both immune-mediated and neurodegenerative processes play a crucial role, however, the link between these two elements is under debate. Axonal damage is considered critical for permanent deficits in the progressive form of MS but the precise mechanisms of axonal injury remain unclear. Currently available therapies focus on preventing ongoing inflammation in the CNS, which may provide neuroprotection to some extent. None of the currently approved disease-modifying drugs protect neurons and axons from neurodegeneration. There is still a challenge of understanding and measuring neuroprotection at the axonal and neural levels [4–6].

MS is still not a curable disease, however, since interferons (IFNs) were first registered in 1993, some therapeutic progress has been achieved. Many other drugs were introduced to the market, including natalizumab, fingolimod, and a new preparation of old drugs such as pegylated form of IFN-beta-1a (peg-IFN-β-1a). The majority of them are available to treat the most typical form of the disease which is relapsing-remitting multiple sclerosis (RRMS) or alternatively secondary progressive MS with neurological exacerbations (known as relapsing-progressive MS – RPMS). Until now, the progressive form of the disease is a great therapeutic challenge and is still an unmet need of affected patients [7].

In 2013 and 2014, the European Medicine Agency approved three drugs for MS which are dimethyl fumarate, teriflunomide (a metabolite of leflunomide) and alemtuzumab. The first two are used orally and are considered as first-line treatment, the third one, due to its safety profile is considered as second-line or even third-line treatment. It is interesting that all three drugs were originally used for alternative indications including fumarate derivatives for psoriasis, leflunomide for rheumatoid arthritis, and alemtuzumab for chronic lymphatic leukaemia. The mechanism of action of the new drugs in MS is of great interest and still not fully described.

In Poland, a national MS therapeutic programme is available. It gives the opportunity to use first-line drugs which are: interferons, glatiramer acetate and recently dimethyl fumarate available from 1 July 2016. The second-line therapy includes natalizumab and fingolimod.

There is much interest in new MS therapeutic agents, however, the majority of published data focuses on the results of clinical trials [8]. Although during the past few decades there was a better understanding of the basic pathophysiology of the disease, the exact mechanism of action of even well-established therapy is not fully elucidated and little is known about the newly registered drugs [9]. The aim of the current publication is to review the published data on preclinical studies on the possible mode of action of these three recently accepted drugs.

Dimethyl fumarate

Dimethyl fumarate (DMF), the orally administered drug is the ester of fumaric acid metabolized in the gastrointestinal tract by esterases. After gastric digestion, DMF metabolites (methylhydrogen fumarate – MHF and others), but not DMF, are detected in the blood and are probably responsible for the clinical effect of DMF [10–12]. DMF was first used in psoriasis treatment, as the immunomodulating agent [13,14]. Since psoriasis is an autoimmune disease based on similar pathomechanisms as MS, the idea arose to use DMF for MS treatment.

General anti-inflammatory activity

The first experiments on the DMF mode of action were made in the topic of psoriasis treatment. Important data were obtained by de Jong et al. [15], who observed that the DMF main metabolite, monomethyl fumarate (MMF), influences the peripheral blood mononuclear cells (PBMC) and T cells, enhancing their IL-4 and IL-5 production, even after stimulation. This is the evidence of switching the Th1-dependent immune response towards Th2-dependent that could also be beneficial in MS treatment, since it is a Th1-mediated disease. Similarly, in the experiments involving psoriasis patients, there is evidence that DMF causes a decrease in the number of circulating lymphocytes, especially CD8+ [16], and induces the apoptosis of activated T cells *in vitro* and monocyte-derived dendritic cells [17,18].

Studies performed on animals with experimental allergic encephalomyelitis (EAE), an animal model of MS, revealed that oral administration of DMF or its metabolites ameliorates the disease course. When combined with the widely used interferon therapy, a further improvement was observed [19]. DMF administered to animals with EAE increases the levels of anti-inflammatory plasma cytokines (IL-5, IL-10) [20]. Experiments conducted both on mice with EAE, and PBMCs isolated from psoriasis patients, confirmed the hypothesis about influence of DMF on dendritic cells (DCs) diffrentiation. Results showed that DMF treatment results in generation of type II DCs, that produce IL-10, but not IL-12 or IL-23. Furthermore, IL-10 promotes Th0 cells polarization into Th2 subtype, while IL-12 and IL-23 would promote polarization into Th1 and Th17 respectively. Induction of Th2 cells eventually results in anti-inflammatory cytokines production and attenuation of ongoing EAE/MS [21].

There is strong evidence that DMF inhibits the activity of nuclear factor κB (NF-κB) – transcription factor regulating the inflammatory response. It was shown that MMF in lipopolysaccharide (LPS)-induced cell culture of monocyte-derived DCs reduced the activation of NF-κB [22]. Activity inhibition is due to blockage of nuclear translocation, not the DNA binding. In normal human dermal fibroblasts stimulated with tumor necrosis factor (TNF-α), DMF inhibited the nuclear translocation of NF-κB p50 subunit [23], while in human endothelial cells no effect of DMF on NF-κB binding to DNA was noted [24].

Antioxidant action

The best known and widely described effect of DMF is its antioxidant activity. For diseases based on autoimmune-derived inflammation, anti-oxidative agents are very desirable. A number of studies showed that DMF and its metabolites activate the Nrf2-dependent (nuclear factor E2) intracellular pathway leading to an increase in antioxidant enzymes expression [25,26]. Nrf2 is a cytoplasmatic protein, in basal state coupled with Kelch-like ECH-associated protein 1 (Keap1) protein. Keap1 acts as a suppressor of Nrf2 and as an intracellular sensor of the redox state. Nrf2 bound to Keap1 undergoes ubiquitination and degradation. Oxidative stress modifies the thiols in cysteine residues in Keap1 resulting in the release of Nrf2 and its translocation into the nucleus. In the nucleus, Nrf2 makes heterodimers with MAF proteins, whose accumulation eventually increases the transcription of ARE (antioxidant response elements) regulated genes in the promoter region for genes encoding the second phase antioxidant enzymes. Those enzymes are, among others glutathione-S-transferase (GST),

superoxide dismutase (SOD), and heme oxygenase-1 (HO-1) [27–29].

Linker et al. [25] presented that DMF increased the Nrf2 intracellular levels in astrocyte cultures (human, rodent), and also the activity of Nrf2-dependent antioxidant enzyme in the liver and cerebellum from rats with EAE. Similar results were observed by Scannevin et al. [26]. They also noted the elevated levels of another antioxidant agent, glutathione (GSH) in cells treated with MMF. Modulation of the intracellular GSH levels in astrocytes in a time-dependent manner was presented. Acute DMF exposition decreased the intracellular GSH levels [30], but after prolonged incubation the GSH levels were increased [31]. It was proven elsewhere [32] that DMF (but not MHF) forms intracellular conjugates with GSH, resulting in GSH depletion, which is responsible for type II DCs generation [21].

Interesting observations confirming the antioxidative action were made when neural cells obtained from rat pups were cultured with H_2O_2. 10 μM hydrogen peroxide caused the loss of 80% of cultured cells, but, when MMF was added, there was an increase in cell viability (about 50%) [26].

DMF mechanisms of Nrf2 pathway induction

Anti-oxidative agents, such as DMF activate the Nrf2-dependent pathway due to conformational changes in the Keap1 protein. There is evidence that DMF, except for a direct modification of Keap1, indirectly modifies the Nrf2 protein. It was established that Nrf2 acetylation enhances the specific binding of Nrf2 to its promoter site [33]. Thus, an alternative Keap1-independent mechanism of Nrf2 activation was tested. It was shown that incubation of cultured astrocytes with DMF resulted in a decreased level and activity of histone deacetylases (HDACs) and also HDACs mRNA levels. When the inhibitor of HDACs was added to a cell culture, increased Nrf2 activity was observed. This indicates that the activity of HDACs, reduced by DMF, causes increased activity of Nrf2, most likely by enhanced acetylation. The most probable mechanism of DMF and HDACs interaction is modification of cysteine residues of HDACs by DMF, which decreases HDACs activity [34]. It was previously shown that inhibition of HDAC also reduced the expression of Keap1, stimulated dissociation of Nrf2 from Keap1, and enhanced Nrf2 translocation into the nucleus and binding to ARE [35].

Recently, evidence of involvement of hydroxycarboxylic acid receptor 2 (HCAR2) activation in the DMF mechanism of action has appeared. Activation of microglia through the HCAR2 changes the cell phenotype from pro- to anti-inflammatory. As MMF is known to be an HCAR2 agonist, its effect on cultured mice microglia was tested. Treatment of microglia with HCAR2 antagonist reversed the MMF-induced change of phenotype [36]. The hypothesis about HCAR2 involvement in the DMF mechanism of action is supported by *in vivo* experiments, showing that DMF reverses clinical improvement [37].

Effect on blood-brain barrier

The next postulated mechanism of action of DMF is the possible interference with the blood-brain barrier (BBB). During inflammation, the BBB is more permeable, leading to infiltration of immune cells into the CNS. In MS, among those cells are also autoreactive T cells [38]. Schilling et al. [20] conducted an experiment feeding EAE mice twice daily with DMF or MHF. The animals were sacrificed during the chronic phase of EAE. Histopathological analysis revealed significantly reduced T cell and macrophage infiltration in the spinal cord in mice fed with MHF. DMF also reduced significantly infiltration of macrophages, and slightly reduced T cell infiltration. This led to the idea that DMF influences the BBB, but this failed to be proven. Bénardais et al. [39] tested the influence of DMF on the human brain micro-vascular endothelial cell line (hCMEC). They did not observe an effect of DMF on the expression of either of the tight-junction proteins mRNA: zona occludens (ZO-1) and claudin-5, and neither the proteins itself in hCMEC. Also no effect of DMF oral administration to mice with EAE on claudin-5 expression in CNS blood vessels was noted. However, downregulation of tight-junction proteins is observed during inflammation. Addition of TNF-α to cell culture caused down-regulation of occluding and ZO-1, which was reversed by MMF addition, which indicates an indirect influence of DMF on the BBB.

Findings from the work of Lim et al. indicate the influence of MMF on monocyte migration through the BBB. Incubation of TNF treated hCMEC, with MMF, resulted in a reduction of monocyte migration [40]. Also, incubation of hCMEC with MMF reduced both mRNA and protein expression for vascular cell adhesion molecule 1 (VCAM-1) and the mRNA for ICAM-1, but not the Intercellular Adhesion Molecule 1 (ICAM-1) protein. VCAM-1 expression in endothelial cells is induced by ROS, whose levels are reduced by activation of the Nrf2-dependent pathway [41]. Therefore, MMF by activating the Nrf2-dependent pathway leads to a reduction of VCAM-1 expression [40]. It was also shown that DMF reduced another adhesion molecule – α4-integrin expression on both circulating T-cells and B-cells in mice with EAE. A similar effect was demonstrated in human Jurkat T cells *in vitro*, but only when the cells were activated [42].

Effect in the CNS

Many studies show that DMF exerts an anti-inflammatory effect in the CNS. In cultured astrocytes, DMF reduced the activity and expression of NOS2 (nitric oxide synthase 2), increased the Nrf2 binding to DNA, and decreased the NFκB activity [30,31].

In vivo studies, conducted in mice with Myelin Oligodendrocyte Glycoprotein (MOG)-induced EAE, showed that oral administration of DMF caused alleviation of the disease course, a reduction of demyelination by 60%, lesser axon loss in lesion sites, reduction of astrocyte activation in both white and gray matter of the spinal cord, and also cerebellar, brainstem and spinal neuron preservation. Immunostaining for Nrf2 revealed increased Nrf2 expression in neurons, oligodendrocytes and astrocytes, but not in microglia and T cells after DMF treatment. Cultured motor neurons presented a reduced apoptotic marker presence. This effect was not observed in Nrf2-knockout mice [25].

Wilms et al. [43] performed experiments with cultured microglia. The results indicated that DMF reduced the levels of iNOS (nitric oxide synthase), nitric oxide synthesis, and the expression of proinflammatory cytokines in LPS-activated cells. The observed effect was Nrf2-dependent, while the nuclear levels of Nrf2 in microglia were elevated after DMF treatment. DMF also has a protective effect on neural stem/progenitor cells and neurons surviving the oxidative stress. In cultured neural stem/progenitor cells, isolated from rat embryonic pups, DMF reduced the H_2O_2-induced apoptosis, as well as H_2O_2-induced reactive oxygen species production [44]. The DMF neuroprotective effect was also measured by neurite outgrowth in motor neurons. No outgrowth was observed in neurons under oxidative-stress condition, while DMF reversed this effect. Furthermore, the antioxidative effect of DMF in neural stem/progenitor cells is also Nrf2-dependent, as there was an increase in both mRNA and Nrf2 protein levels after DMF treatment and upregulation of a second phase antioxidant gene expression.

Recent studies show that DMF may exert a neuroprotective action by influencing the glutamatergic excitotoxicity. The DMF metabolite, MMF, was shown to delay the onset and reduce the severity of glutamatergic excitotoxicity induced morphological

changes observed in the brainstem in mice. Also levels of Glu-induced Ca^{2+} was decreased after MMF treatment [45].

Teriflunomide

Teriflunomide, the active metabolite of leflunomide is, similarly to DMF, an orally used drug newly registered for treatment of relapsing-remitting multiple sclerosis. Leflunomide, first used for the treatment of another autoimmune disease, rheumatoid arthritis, converts into teriflunomide in a non-enzymatic manner in the plasma or gut mucosa [46]. It was decided to examine teriflunomide, as the main leflunomide active metabolite, in multiple sclerosis due to its anti-inflammatory properties, and also efficiency in the treatment of autoimmune based rheumatoid arthritis [47].

General antiproliferative agent

Teriflunomide is an inhibitor of the mitochondrial enzyme, dihydroorotate dehydrogenase (DHODH), which is the key enzyme for the de novo pyrimidine synthesis. DHODH is necessary for rapidly dividing cells, such as activated lymphocytes, where pyrimidines are not only used for DNA and RNA synthesis, but also for glycosylation of proteins or membrane lipid biosynthesis [48]. Except for the DHODH-dependent pathway, cells may use the salvage pathway for pyrimidine synthesis, which are then recycled from the intracellular pool, which is not effective in highly proliferating cells. Therefore, teriflunomide does not affect the resting cells, inhibiting only those rapidly dividing [46,49]. As MS is an autoimmune-based disease, where the autoreactive T and B cells are, among many others, involved in the pathomechanism, a reduction in the number of circulating T cells and B cells producing autoantibodies would be beneficial for disease course attenuation [50]. In animal studies, teriflunomide delayed EAE onset and reduced the clinical score in Dark Agouti [51] and Lewis [52] rats. Moreover, significantly reduced inflammation, demyelination and axonal loss in CNS were observed, which may be the result of a decreased number of both B and T autoreactive lymphocytes [51]. Another study showed that treatment with teriflunomide resulted in a decreased number of infiltrating immune cells (T cells, NK cells, macrophages, neutrophils) into the spinal cord, and a decrease in spleen weight in EAE Dark Agouti rats [53].

It is noteworthy that teriflunomide is not cytotoxic. Arresting cell cycle at S phase is not followed by cell apoptosis. A number of experiments using both leflunomide and teriflunomide were conducted, showing inhibition of cell proliferation, which was reversed by uridine addition in cell cultures of human PBMC [54], rat spleen mononuclear cells [55] and human B cells [56] with no cytotoxicity observed.

Interestingly, there was stronger inhibition of proliferation observed for T cells with higher than with lower antigen avidity [57].

Effect on antigen presentation/transmembrane migration

Presentation of antigens by professional antigen presenting cells (APCs) to T cells is necessary for antigen-specific T cell activation. The site of contact between those two types of cells is called immunological synapse and contains many adhesion and signaling molecules. Contact between those molecules, on both T cells and APCs, is vital for proper immune response [58]. *In vitro* teriflunomide altered the integrin function and disrupted immunological synapse formation. Also, the effect was not reversed by uridine addition indicating that it is not dependent on pyrimidine synthesis [59].

Adhesion molecules are also important for transendothelial cell migration. In PMBC obtained both from healthy and rheumatoid arthritis patients, cultured *in vitro* leflunomide inhibited transendothelial migration. Except for interaction with adhesion molecules, especially CD44, essential for diapedesis, the effect is DHODH dependent because of reversing the effects by uridine addition. As was mentioned above, DHODH also contributes to membrane lipid synthesis, so improper membrane structure may impair its plasticity [60].

Anti-inflammatory agent

Except for the antiproliferative mode of action, teriflunomide exerts a number of anti-inflammatory effects.

Modulation of cytokine production was observed by Korn T et al. [52]. The study showed a decrease in IFN-γ and a slight increase in IL-10 production by T cells isolated from EAE Lewis rats after teriflunomide addition. In human activated PBMC, teriflunomide decreased levels of pro-inflammatory cytokines [54]. In isolated human peripheral blood lymphocytes, leflunomide inhibited the TNF and IL-17 production [61].

Teriflunomide also promotes stimulated, naïve T cells differentiation into Th2 cells rather than Th1, which further causes the promotion of anti-inflammatory cytokine production (Th2-dependent) while decreasing the production of pro-inflammatory ones (Th1-dependent). The observed effect was reversed by uridine, therefore, indicating a relationship of pyrimidine synthesis inhibition to T cell differentiation [62].

Another action of teriflunomide is inhibiting the protein tyrosine kinases, which are responsible for protein phosphorylation. This affects a number of mechanisms resulting in the immune activity of the cells. As binding of cytokine to its receptor activates the intracellular pathway requiring protein phosphorylation, inhibition of kinases disrupts such pathways. Inhibition of Jak1 and Jak3 kinases by teriflunomide affects IL-2R signaling, responsible for cell proliferation [63]. Jak1 and Jak3 proteins, both with STAT6, are involved in IL-4R signal transduction, responsible for Ig secretion from LPS induced B cells [64]. Protein phosphorylation is also essential for TCR/CD3 dependent activity, and may be inhibited by teriflunomide [65]. The abovementioned inhibition of TNF and IL-17 production are also dependent on the JAK/STAT pathway [61], which may be affected by leflunomide. In animal studies, leflunomide decreased clinical symptoms of autoimmune disease, and also reduced tyrosine phosphorylated proteins in mice lymph nodes [66].

Teriflunomide is also known for inhibition of NfκB activation. In cell lines activated with TNF, teriflunomide inhibits NfκB activity. The effect was reversed by uridine, which implicates the role of pyrimidines in TNF-dependent cellular activity [67,68].

Alemtuzumab

Alemtuzumab, similar to DMF and teriflunomide, is a newly registered drug for RRMS treatment, which was first used for chronic lymphocytic leukaemia. In contrast to the previously described agents, it is administered intravenously (*iv*) [50,69,70].

General anti-inflammatory agent

Alemtuzumab is a monoclonal humanized antibody, against CD52 glycoprotein, expressed on the surface of both T and B lymphocytes, and also on other immune cells, such as monocytes, macrophages, eosinophils, basophils and NK cells [70,71]. The function of CD52 remains mostly unknown, but there is evidence that CD52 acts as a costimulatory molecule for T cell activation and contributes to T cell migration [72]. Alemtuzumab action is simply

antibody-dependent cellular cytolysis accompanied by complement-mediated cell lysis and apoptosis [73]. After alemtuzumab administration to MS patients, depletion of CD52+ cells is observed, mostly lymphocytes and very few NK cells or eosinophils, followed by repopulation occurring after about 3 months for monocytes and B cells, after 30 months for CD8+ T cells, and after 61 months for CD4+ T cells [74,75]. The changed proportions in immune cells also include an increased number of Tregs and memory T and B cells [76]. Predominant regulatory T cells also create a "tolerogenic" environment after alemtuzumab application. Long-time CD4+ cell depletion is considered the most important for the therapeutic effect of alemtuzumab.

Long-lasting CD4+ cell depletion may be caused by upregulation of TGF-β+CD4+ cells after alemtuzumab administration, since TGF-β (transforming growth factor) reduces T cell proliferation and antigen presentation [77]. Additionally, it was observed that one year after a single alemtuzumab administration, there was a sustained reduced number of circulating naïve T cells, pointing to the suppressed function of the thymus [78].

For animal studies, creating the transgenic mice expressing human CD52 was needed, while there is no cross reactivity between human and mouse CD52. Human and animal studies showed a similar effect of alemtuzumab. Depleted immune cells were mostly lymphocytes with a small amount of NK cells and neutrophils, which may explain why patients are not completely susceptible to infections, as innate immunity cells remain almost intact after alemtuzumab administration [79]. Moreover, despite depletion of lymphocytes in the periphery, there is a significant amount of these cells remaining in the lymphoid organs. For immune cell repopulation, a similar scheme as in patients was observed, with B cell repopulation taking place earlier than T cell repopulation [80]. Likewise, a transient increase in cytokine levels in serum was observed, with congenial observations in the patients [81], due to "cytokine-release syndrome" which may occur due to simple lymphocyte lysis and also interactions with NK cells [82].

Effect in the CNS

MRI (magnetic resonance imaging) in MS patients shows inhibition of the inflammatory process in CNS after alemtuzumab treatment [69]. In mice with EAE, the beneficial effects of alemtuzumab in CNS were also observed. Histopathological studies showed decreased numbers of infiltrating lymphocytes, with following decreased demyelination and axonal damage. The axonal conductance was preserved in the spinal cord. In periphery, decreased numbers of autoreactive T cells and levels of pro-inflammatory cytokines were observed [83].

Based on information about the clinical improvement in MS patients after alemtuzumab therapy, Jones et al. [84] conducted experiments on PBMC isolated from patients' blood. The study revealed that immune cells, regenerating after alemtuzumab-dependent depletion, produce growth factors such as BDNF (brain-derived neurotrophic factor), PDGF (platelet-derived growth factor) or FGF (fibroblast growth factor), which is further increased by stimulation with myelin antigens. Those growth factors, produced by immune cells are able to promote neuron survival, axon growth, and also oligodendrocyte precursor differentiation and proliferation *in vitro*. Therefore, after alemtuzumab treatment, repopulating immune cells, while entering the CNS, are the source of beneficial neurotrophins [85].

Summary

In this review, we presented data relating to the mechanism of action of three newly registered drugs for MS treatment. To compare, the two first, DMF and teriflunomide, are orally given, while alemtuzumab requires intravenous administration. Clinical trials show high alemtuzumab efficacy, unfortunately connected to the possibility of the appearance of severe adverse effects, which rules it out from being the first-line drug. Interestingly, the presented drugs differ in the range of activity, from multi-modal for dimethyl fumarate, to very specific for alemtuzumab. Also, the drug targets differ. DMF is mostly an antioxidant agent, interfering with the Nrf2-dependent antioxidant pathway, and teriflunomide and alemtuzumab rather work by eliminating lymphocytes due to inhibited proliferation or depletion of those cells, respectively.

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