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Neuroprotection: A New Therapeutic Approach of Relapsing Remitting Multiple Sclerosis

Juan Espinosa-Parrilla, Marco Pugliese,
Nicole Mahy and Manuel J Rodríguez

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Abstract

Neurodegenerative changes occurring early from primary acute immune-mediated inflammation support the hypothesis that multiple sclerosis (MS) is a complex disease. Axonal loss progresses with the disease course and represents the principal driver of disability. In this context, the pursuit of neuroprotective therapies in multiple sclerosis provides new valid alternatives that could significantly impact on disease progression and neurodegenerative changes, including the promotion of restoration of myelin sheaths through the remyelination process. This chapter reviews promising drugs with proposed neuroprotective or neuroregenerative effects that are currently approved or in clinical trials for the treatment of multiple sclerosis. Although the chapter highlights the diazoxide action on neuroinflammation and the results of a clinical trial with this drug, the review also includes other molecules with oral or parenteral administration.

Keywords: relapsing-remitting multiple sclerosis, disease-modifying drugs, neuroprotection, neuroregeneration, neuroinflammation, central nervous system

1. Introduction

Immunosuppressive and immunomodulatory drugs have been designed for years to treat multiple sclerosis (MS), as it was considered the most appropriate approach to balance the effects of the patients' immune reaction. However, the new data recently offered by basic and clinical research increase our understanding of this complex disease, allowing changes in its therapeutic approach. As a consequence, the first disease-modifying drug with a suggested

neuroprotective mechanism of action has been recently approved, broadening and improving the therapeutic landscape against MS.

In this chapter, we review new drugs with proposed neuroprotective or neuroregenerative effects that are currently approved or in clinical trials for MS treatment. Although we highlight the diazoxide action on neuroinflammation and the results of a clinical trial with this drug, the review also includes other molecules with oral or parenteral administration.

The articles analysed for this review were selected using PubMed database filtered by English language. Clinical trials were selected at the ClinicalTrials.gov database. Studies in the database labelled as “unknown status” were excluded. The initial search was done in December 2015.

2. Treating neurodegeneration in multiple sclerosis

MS is an autoimmune disease in which elements of the neuronal myelin sheath are recognized as antigens by the immune system. Focal inflammatory and immune actions against neuronal cover cause a neurodegenerative process that leads to neurological impairment. The difficulty to characterize the neurodegenerative process supports the hypothesis that MS is a complex disease, with high variations among individuals. In any case, as a consequence of the immune aggression, an axonal loss progresses with the disease and represents the principal driver of disability in the chronic course of MS [1]. In this context, multiple mechanisms could contribute to demyelination and axonal injury including energy imbalance, ion accumulation, neuroinflammation and astroglial response to oxidative and metabolic stress.

2.1. Chronic neurodegenerative processes in multiple sclerosis

About 85% of MS patients present the so-called relapsing-remitting (RR) form, characterized by unpredictable relapses related to inflammatory activity, followed by periods of months to years of relative quietness. During the remitting period, patients present no new signs of disease activity and, occasionally, partial remissions of symptoms associated with reparative processes are observed [2]. Progressive forms are less frequent forms of initial MS, but about 55–65% of relapsing-remitting multiple sclerosis (RRMS) patients develop a secondary progressive course [1].

At the pathophysiological level, the neurological impairment develops due to focal inflammatory and immune attacks against the neuronal cover, which cause a chronic neurodegenerative process. The reason why and where these focal demyelinating inflammatory lesions appear within the central nervous system (CNS) remains unknown. Current hypothesis establish that CD4-positive T lymphocytes are mainly responsible for the initial autoimmune attack, but with a secondary role in chronic neuronal damage [3]. Resident microglia and astrocytes, and infiltrated macrophages, B lymphocytes, natural killer (NK)-positive and CD8-positive T cells would be the major players in the neurodegenerative process [4, 5].

At the subcellular level, a plethora of inflammatory effectors mediating myelin and neuronal damage have been described. These effectors include molecules such as nitric oxide (NO), reactive oxygen species (ROS), tumour necrosis factor α (TNF- α), interferon γ (IFN γ), granzymes, perforin and matrix metalloproteinases, and also molecular processes such as activation of the complement, antibody secretion and phagocytosis [4, 6, 7]. Furthermore, as a concomitant event, glutamate release from damaged axons will trigger excitotoxicity, which leads to ion imbalance and mitochondrial metabolic stress, feeding neuronal damage and neuroinflammation [8, 9]. Thus, inflammatory damage leads to oligodendrocyte death, axon demyelination, axonal damage, neuronal death and the formation of glial scars. Progressive axonal injury and neuronal loss increase with the course of MS and represent the principal driver of disability.

Key elements that mediate inflammation in MS are microglial cells. Microglia are often considered to be macrophages of the CNS, but a series of recent findings in the mouse have established that they are a unique cell population distinct from macrophages [10]. In normal conditions, microglia present a surveillance state and important roles in normal development, connectivity and plasticity of the CNS. However, they are transformed and activated by a range of signals, such as neuronal death, mechanical injury and toxins [11]. Once activated, they form the first line of defense against infection or injury to the CNS [12]. As a consequence, microglia mediate and trigger a neuroinflammatory response to injury. In MS, this microglial reaction is an early event that often precedes and triggers demyelination and neurodegeneration [13].

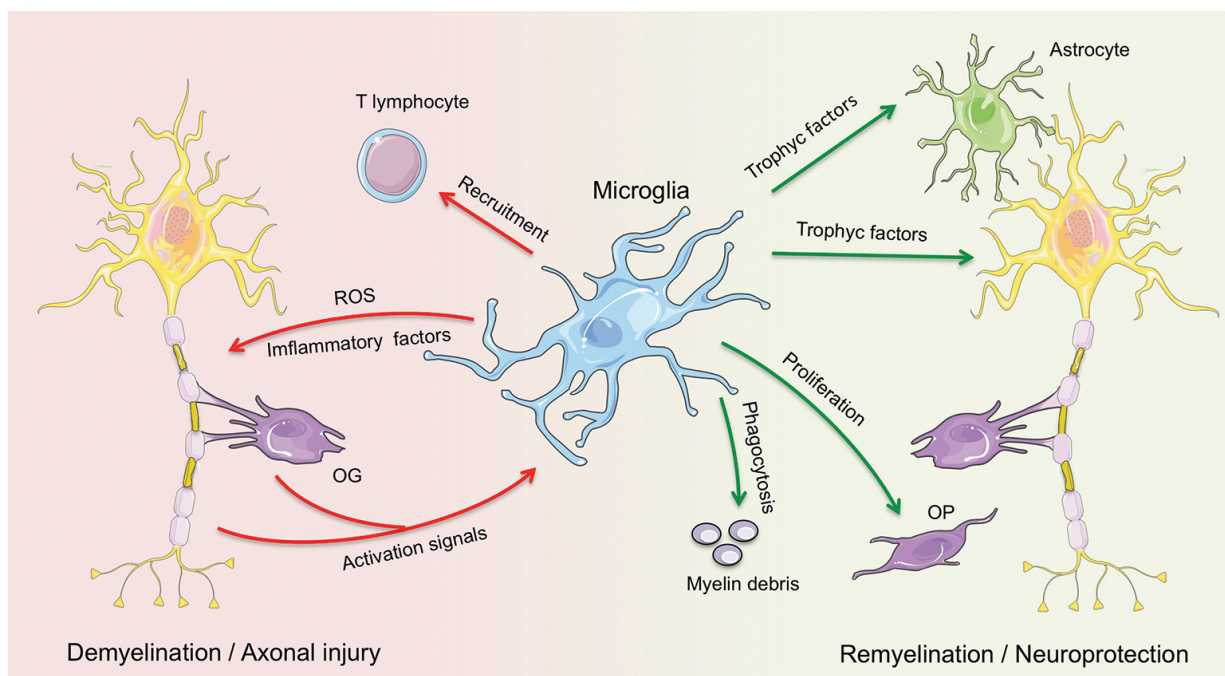


Figure 1. Dual role of microglia in MS. Reactive microglia mediate different pro-inflammatory (red arrows) and neuroprotective processes (green arrows) according to the diversity of signals from the lesioned axons and oligodendrocytes. OG, oligodendrocytes; OP, oligodendrocyte precursors; ROS, reactive oxygen species. (Cell drawings are from SERVIER Medical Art.)

Perivascular microglia are the antigen-presenting cells that recruit myelin-specific T cells and promote the inflammatory process inside the CNS. This process then activates parenchymal microglia by secreting pro-inflammatory and neurotoxic factors such as TNF- α , prostaglandins, interleukin-6 (IL-6), NO or ROS, which elicit myelin damage and neurodegeneration [11, 14]. Whether microglia adopt a phenotype that mostly exacerbates tissue injury or one that promotes brain repair is likely to depend on the diversity of signals from the lesion environment and the response capacity of the cell (**Figure 1**).

As a response to the inflammatory aggression, reparative and regenerative processes are activated and originate MS remissions. This neuroprotective response is led by the activation of microglia and TH2-IL-10 lymphocyte pathways, and results in inhibition of the inflammatory process, up-regulation of antioxidant mechanisms, and secretion of neurotrophic factors. These processes promote remyelination by surviving oligodendrocytes and precursors [15–17]. According to this, the progression of MS symptoms depends on a delicate balance between neuroinflammation and the regenerative process. However, neuronal regeneration is not an easy and common process within the CNS, especially for large myelinated neurons. Although myelin sheath can be partially restored, oligodendrocytes regenerated and axons repaired, and if the inflammatory insult persists, the degenerative damage becomes chronic and cannot be counteracted.

According to these processes, the therapeutic approaches for MS should follow three different but not exclusive strategies: (a) to suppress the autoimmune reaction by inhibiting the initial immune response and lymphocyte infiltration into the CNS, (b) to prevent neurodegenerative chronic damage by inhibiting neuroinflammation and fostering neuronal survival or (c) to promote myelin repair and neuroregeneration.

2.2. Molecules with proposed neuroprotective and antioxidative activity

Until recently, MS pharmacotherapy has been dominated by immunomodulatory drugs, which were developed on the basis that the disease was primarily an autoimmune disease. This hypothesis postulates that T lymphocytes specific for myelin antigens initiate an inflammatory reaction in the CNS, which ultimately leads to demyelination and subsequent neuronal loss. However, since the approval of oral dimethyl fumarate to treat RRMS, with a proposed antioxidative neuroprotective mechanism of action, the therapeutic landscape for MS is rapidly evolving. Currently, the development of drugs with primarily CNS neuroprotective effects is a pharmaceutical priority.

Pursuit of neuroprotective therapies in MS provides new valid alternatives that could significantly impact on disease progression and neurodegenerative changes, including the promotion of myelin sheath restoration through the remyelination process [18]. Thus, fingolimod, laquinimod, Anti-LINGO antibody, (-)-epigallocatechin-3-gallate (EGCG) and diazoxide emerge as neuroprotective drugs for the treatment of MS (**Table 1**). Nevertheless, the neuroprotective effect of these compounds has not been fully established and requires further investigation.

Compound	Proposed mechanism of action	Effects	Clinical phase	Key adverse effects
Dimethyl fumarate	Nrf2 up-regulation Induction of IL-4-Th2 response	Anti-inflammatory activity Decreased lymphocyte entry into CNS Protective effects on oligodendrocytes, myelin, axons and neurons	Phase III: DEFINE and CONFIRM Approved	Moderate infections Elevated liver enzymes Leucopenia
Fingolimod	Decreased expression of S1p1 on lymphocytes and other cells	Decrease of T lymphocytes infiltrated in the CNS Anti-inflammatory and neuroprotective microglia phenotypes	Phase III: TRANSFORMS and FREEDOMS Approved	Bradycardia and AV block Severe viral infections Macular oedema
Laquinimod	Unknown Inhibition of NF-kB activation? Inhibition of IL-17 expression?	Decreased lymphocyte entry into CNS Axon protection Decrease of pro-inflammatory cytokines Increased BDNF levels	Phase III studies CONCERTO Results expected by 2017	Elevated liver enzymes Infections
Anti-LINGO antibody BIIB033	Blockade of Nogo signaling	promotes axonal integrity/remyelination functional recovery	Phase I NCT01052506 NCT01244139 Phase II: SYNERGY Results expected by 2016	Hypersensitivity reactions Elevated liver enzymes
(-)- Epigallocatechin-3-gallate	Inhibition of NF-kB activation Free radical scavenger.	Improves muscle metabolism during moderate exercise in MS patients	Phase II SunIMS completed. No results available NCT01417312 completes	Elevated liver enzymes (1 case)
Diazoxide	Activation of K _{ATP} channels Maintenance of mitochondrial homeostasis and function	Anti-inflammatory activity Neuronal, axonal and myelin protection Modulation of antigen presentation	Phase II: NEUROADVAN completed	Autoimmune hypothyroidism (1 case)

Table 1. Neuroprotective drugs for the treatment of RRMS.

2.2.1. *Dimethyl fumarate*

Fumaric acid esters (FAEs) are a group of simple low-molecular structured compounds that has been used for long time in the treatment of moderate to severe psoriasis [19]. Due to their immunomodulatory potential, FAEs were also evaluated as a potential treatment for RRMS. At preclinical stages, FAEs showed promising results in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (MOG-EAE) mice, ameliorating the disease course [20]. In vivo, the mechanism of action of dimethyl fumarate is not completely understood. For some authors, fumarate treatment induces IL-4-producing Th2 dendritic cells [21]. In the human and mice, type II dendritic cells subsequently produce the anti-inflammatory cytokines IL-10 and IL-4 instead of pro-inflammatory IL-12 and IL-23 [21]. This anti-inflammatory activity also causes apoptosis of activated T cells preserving the CNS from influx of activated lymphocytes.

Regarding the neuroprotective role of fumarate, other authors found in vitro and in vivo effects, potentially via up-regulation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Dimethyl fumarate also exerts protective effects on oligodendrocytes, myelin, axons and neurons, and reduces oxidative stress as measured by protein nitrosylation [22]. Nonetheless, as recently shown, the protective effect of dimethyl fumarate could depend on pre-existing tissue expression of Nrf2. Actually, Nrf2 is intrinsically higher in astrocytes and macrophages from active MS lesions and increased Nrf2 levels have been recently reported in oligodendrocytes from active MS lesions [23].

Two randomized, placebo- and active-controlled, double-blind, parallel-group Phase III studies (DEFINE and CONFIRM) of dimethyl fumarate (Code name BG-12) have been conducted (funded by Biogen Idec). The primary end point of these studies was the proportion of patients who had a 2-year relapse. Secondary end points included the annualized relapse rate (ARR), the time to confirmed progression of disability as measured by Expanded Disability Status Scale (EDSS), and findings on brain magnetic resonance imaging scan (MRI) such as the number of gadolinium-enhancing lesions. Both studies described highly significant superiority of BG-12 to placebo on almost all end points in patients with RRMS [24]. In the DEFINE and CONFIRM extension study (ENDORSE), a minimum of 5 years of treatment with the drug was associated with continued benefit and no new/worsening tolerability signals. Thus, in March 2013, the Food and Drug Administration (FDA) approved dimethyl fumarate as a new first-line oral treatment for patients with RRMS. In September 2013, it was also approved in Canada and Australia and, in 2014, the European Commission has approved the use of the drug for the treatment of RRMS in Europe.

2.2.2. *Fingolimod*

Fingolimod became the first drug approved for relapsing forms of MS in the USA in 2010. However, some safety issues were identified during the drug development process, after completion of trials and in the first months of clinical use in the United States. These issues led to the approval of fingolimod as a second-line drug by the European Medicines Agency (EMA) after the FDA had licensed it as a first-line agent. In addition, contradictory results regarding

efficacy on progression of disability in MS patients were found in the two pivotal Phase III trials that allowed fingolimod marketing in most countries [18].

Although fingolimod was primarily believed to be a pure immunosuppressive compound, recent findings revealed direct effects on the CNS [25]. When incorporated to the organism, fingolimod is rapidly phosphorylated by sphingosine kinases [26] and the product of this phosphorylation, phosphofingolimod, is a potent modulator of S1P receptors. Administration of fingolimod and similar S1P1 modulators produce marked beneficial effects on different animal models of MS, especially when administered preventively. The proposed mechanism of action for fingolimod is the inhibition of encephalitogenic T-cell responses and/or their migration into the CNS [27, 28]. According to this, EAE animals treated with fingolimod showed a dramatic decrease of T lymphocytes infiltrated in the CNS and even a reversible peripheral lymphocytopenia. These animals also showed better myelin preservation and decrease of pro-inflammatory cytokine production [29].

In the CNS, both neurons and glia profusely express S1P receptors. Some of the beneficial effects of fingolimod in EAE and MS could be attributed to S1P receptor modulation in these cells and result in neuroprotection. Activation of neuronal S1P1 and S1P3 receptors promotes neurogenesis and increases neurite outgrowth, although high and continuous activation of neuronal S1P receptors could lead to overactivation of the glutamatergic system with deleterious effects [30]. In microglia, fingolimod promotes anti-inflammatory and neuroprotective phenotypes, but the exact mechanism of action involved remains unclear [31]. Furthermore, one of the most discussed direct effects of fingolimod on the CNS is on myelin repair and oligodendrocyte regeneration [32, 33].

One Phase II study, followed by an active extension, and two clinical Phase III placebo-controlled and active comparator-controlled clinical trials (named TRANSFORMS and FREEDOMS, sponsored by Novartis) have been performed with fingolimod in patients with RRMS. These studies have demonstrated the efficacy of fingolimod in treating RRMS [34, 35]. More recently, FREEDOMS II, a third Phase III trial of fingolimod, was conducted predominantly in USA and Canada. The trial replicated the findings of FREEDOMS regarding the ARR and MRI outcomes, but the significant effect on reducing EDSS score progression was not confirmed. On the other hand, disability showed a statistically significant change in favour of fingolimod treatment in the FREEDOMS II study [36]. Finally, the recently completed INFORMS trial showed no significant benefit of fingolimod on neurological disability in primary progressive MS patients treated for at least 3 years [37].

2.2.3. *Laquinimod*

Laquinimod (N-ethyl-N-phenyl-5-chloro-1,2-dihydroxy-1-methyl-2-oxo-quinoline-3-carboxamide) is structurally related to linomide, which was tested for efficacy in MS more than one decade ago, and discontinued after serious adverse events occurred in a Phase III trial [38, 39]. After an extensive screening of a large number of chemically modified quinoline-3-carboxamides, laquinimod was selected to have less toxicity and better efficacy than linomide

in various experimental autoimmune inflammatory-mediated animal models, including experimental autoimmune neuritis in Lewis rats [40].

The precise mechanism by which laquinimod induces these beneficial effects is yet to be fully elucidated. Preclinical studies in EAE mice have shown that laquinimod might protect myelin and axons by decreasing pro-inflammatory cytokines such as IL-17 by T cells [41]. In addition, suppression of the nuclear factor-kappaB (NF- κ B) pathway that concordantly led to the activation of apoptosis of immunocompetent cells was also induced by laquinimod, proving the anti-inflammatory potency of the drug [42]. Regarding the effects in the CNS, laquinimod treatment prevented the loss of brain-derived neurotrophic factor (BDNF) induced by the disease process. In EAE mice, laquinimod induced an elevation of BDNF expression in various brain regions to reach similar BDNF concentration as that of naïve controls [43]. In the human, a Phase II study undergoing laquinimod treatment showed a significant and specific elevation of serum BDNF levels compared to the placebo group after 3 months of treatment [44]. Whether laquinimod directly affects BDNF expression within the neurons, or induces bystander mechanisms that arrest the inflammatory progression and facilitates neuroprotection, needs to be further clarified.

Two Phase III clinical trials of laquinimod in RRMS (the ALLEGRO and BRAVO trial sponsored by TEVA) have been completed. In the first one, treatment with laquinimod as compared with placebo was associated with a modest reduction in ARR, decreased the risk of disability progression and reduced the number of gadolinium-enhancing lesions detected by MRI. However, the failure to meet primary end point in the BRAVO study led the manufacturer to delay requesting FDA approval. On May 2013, laquinimod was approved in Russia as a treatment for RRMS.

Nevertheless, in 2013 Teva Pharmaceutical and Active Biotech started the third Phase III trial of laquinimod in patients with RRMS. The study is designed to evaluate the safety and efficacy of laquinimod with a primary end point of time to confirmed disability progression, as measured by the EDSS (CONCERTO trial, ClinicalTrials.gov Identifier: NCT01707992). CONCERTO results are expected to be available towards mid-2017.

2.2.4. *Anti-LINGO antibody*

Nogo receptor-interacting protein (LINGO-1) is a transmembrane signal-transducing molecule, selectively expressed by oligodendrocytes and neurons and that associates with the Nogo-66 receptor (NgR1) complex [45]. NgR1 binds myelin-associated inhibitors of axonal regeneration such as Nogo-A, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein [46]. In response to CNS injury and demyelination, oligodendrocyte precursor cells and stem cells from the white matter become activated, migrate to the demyelinated area, proliferate and differentiate into oligodendrocytes that will remyelinate damaged axons. In chronic MS lesions, despite the presence of oligodendrocyte precursors in demyelinated plaques and the close proximity between pre-myelinating oligodendrocytes and demyelinated axons, axonal remyelination failed. This suggests that the differentiation process may be locally blocked by inhibitory factors [47].

In animal models, LINGO-1 expression is up-regulated in rat spinal cord injury, experimental EAE, 6-hydroxydopamine neurotoxic lesions and glaucoma models [48, 49]. Several works have reported the blockade of Nogo signalling as a therapeutic approach for neurological disorders such as spinal cord injury, traumatic brain injury, stroke, schizophrenia, amyotrophic lateral sclerosis and MS [50]. Animal models provide evidence that LINGO-1 is a potent inhibitor of axonal remyelination and regeneration in vivo. For example, transgenic mice overexpressing full-length LINGO-1 under the neuronal promoter of synapsin showed a significant reduction in the number of myelinated axons in the spinal cord and brain at P8 [51]. Other authors showed that neurite outgrowth inhibitor Nogo-A is involved in autoimmune-mediated demyelination in vivo [52].

BIIB033 is a monoclonal antibody that inhibits LINGO-1 and promotes axonal integrity/remyelination in MOG-EAE mice [49]. In preclinical experiments, LINGO-1 antagonist antibodies did not alter EAE onset but did significantly mitigate disease severity across all stages of disease progression. This functional recovery in EAE correlates with improved axonal integrity as determined by magnetic resonance diffusion tensor imaging, and with newly formed myelin sheaths as determined by electron microscopy [53].

Biogen Idec Inc. started two different human studies with BIIB033 in 2010 (ClinicalTrials.gov Identifier: NCT01244139 and NCT01052506, respectively). Both studies finished in 2012 and BIIB033 showed favourable safety profile, desirable pharmacokinetic in healthy adults and MS patients and predicted brain penetration [53]. Based on these promising results, in 2013, Biogen started a Phase II dose-response study to assess the efficacy, safety, tolerability and pharmacokinetics of BIIB033 in 400 RRMS patients when used concurrently with Interferon β -1a (Avonex[®]) (SYNERGY, ClinicalTrials.gov Identifier: NCT01864148). SYNERGY results are expected to be available along 2016.

2.2.5. (-)-Epigallocatechin-3-gallate

Green tea consumption has been associated to differences in the incidence of some diseases such as different kinds of cancer or cardiovascular pathologies [54]. Its composition includes, among other bioactive molecules, vitamins, pro-vitamins and antioxidants. Some of the more abundant components are tea polyphenols, also known as catechins, especially the (-)-epigallocatechin-3-gallate (EGCG) [55]. EGCG has been profusely studied as an anticancer compound, as long as it can cause apoptosis and arrest cell cycle of tumoural cells [56]. As long as EGCG presents antioxidative properties, it can act as a chelator of neurotoxic metals and inhibit pro-inflammatory processes [57].

Efficacy studies in the EAE models for MS have shown that, alone or in combination with approved treatments for MS, oral EGCG ameliorated disease course and decreases EAE severity, preventing and reversing disability. This positive effect has been mainly attributed to the inhibition of NF- κ B-mediated inflammation, cell proliferation, TNF- α secretion and Th1/Th2 response. EGCG enhanced axonal preservation and inhibited neuronal apoptosis in treated animals [58]. The neuroprotective profile of EGCG has been attributed to its activity as a free radical scavenger [58]. Although this effect may be related with the inhibition of NF-

κ B pathway and glial reactivity, this indirect neuroprotection is also of main interest for the treatment of MS and other neurodegenerative diseases.

Several epidemiologic studies and clinical trials have been performed to test the preventive and therapeutic effects of EGCG in neurodegenerative diseases such as Alzheimer's, Parkinson's or Huntington's diseases [59]. In general, these studies show no EGCG-related prevention of neuronal and glial cell death in patients [59]. In 2013, a multicentric Phase II national study to investigate the anti-inflammatory and neuroprotective effects of EGCG in 120 RRMS patients was started (SunIMS Study, ClinicalTrials.gov identifier: NCT00525668, sponsored by Charite University, Berlin, Germany). At the moment, no results are available for this study.

Finally, to analyse the metabolic effects of EGCG and to assess the importance of lipid oxidation in fuel muscle's energy metabolism and its relationship with muscle weakness and fatigue in RRMS patients, the same institution completed a clinical research trial in 2013 (ClinicalTrials.gov identifier: NCT01417312). Results showed that EGCG given to MS patients over 12 weeks improves muscle metabolism during moderate exercise to a greater extent in men than in women, possibly because of sex-specific effects on autonomic and endocrine control. These results indicate that EGCG could be a promising treatment for MS, with a good- and well-known safety profile and an interesting combinability with other treatments. However, as the EGCG pass through the blood-brain barrier in humans is still controversial, its bioavailability remains to be characterized [60].

3. Effects of diazoxide in multiple sclerosis

3.1. K_{ATP} channels and neuroinflammation

Adenosine triphosphate (ATP)-dependent potassium (K_{ATP}) channels play important roles in many cellular functions by coupling cell metabolism to electrical activity. First detected in cardiac myocytes, they are also expressed in a wide number of cell types such as pancreatic β -cells, skeletal and smooth myocytes, neurons and microglia. In these cells, K_{ATP} channels regulate potassium fluxes across the cell membrane when glucose is available in sufficient conditions, which couple the electrical activity of the cell to energy metabolism [61, 62]. An increase in the cellular ATP concentration leads to channel closure and membrane depolarization. On the contrary, metabolic inhibition opens K_{ATP} channels and suppresses electrical activity.

Functional K_{ATP} channels in the cell membrane are assembled as a heterooctameric complex [63] from two structurally distinct subunits: the regulatory sulphonylurea receptor (SUR) and the pore-forming inwardly rectifying potassium channel (Kir) subunit 6.1 or 6.2. While ATP inhibits the K_{ATP} channel by directly binding to the cytoplasmic Kir6 domains, activators, such as potassium channel openers (KCOs), and inhibitors, such as sulphonylurea drugs [64], bind SUR to modulate the channel. Similar K_{ATP} channels have also been described in the mitochondria, located on the inner membrane of these organelles where they play a crucial role in the maintenance of mitochondrial homeostasis and function [65].

In recent years, K_{ATP} channels have attracted increasing interest as targets for drug development. Their pivotal role in a plethora of physiological processes has been underscored by recent discoveries linking potassium channel mutations to various diseases. The second generation of KCOs with an improved in vitro or in vivo selectivity has broadened the chemical diversity of K_{ATP} channel ligands. On the other hand, considering the unique role that K_{ATP} channels play in the maintenance of cellular homeostasis, KCOs add their potential in promoting protection against metabolic stress to the already existing pharmacotherapy. Studies in animal models showed KCO effects in several pathological situations such as hypertension, cardiac ischaemia or asthma, which indicates a broad therapeutic potential for KCOs. For example, in the smooth muscle of blood vessels and pancreatic β -cells, diazoxide (7-chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide) binds with similar affinities to SUR1 and SUR2B subunits of K_{ATP} channels, increases membrane permeability to potassium ions and induces hyperpolarization [64]. In these cells, diazoxide-induced hyperpolarization inhibits the opening of voltage-gated calcium channels, and results in vasorelaxation and inhibition of insulin secretion [66]. As a consequence, diazoxide has been used since the 1970s for treating malignant hypertension and hypoglycaemia in the United States, Canada, and most European countries [67].

Our laboratory has described the expression of K_{ATP} channels in microglia [68–70], through which they control the release of a diversity of inflammatory mediators, such as NO, IL-6 or TNF- α [71, 72]. We also evidenced that reactive microglia increase the expression of the K_{ATP} -channel components Kir6.1, Kir6.2, SUR1 and SUR2B [69, 73]. With this increased microglial expression, KCOs may modulate neuroinflammation. In this line, we and other authors have documented that pharmacological activation of K_{ATP} channels with diazoxide can exert CNS neuroprotective and anti-inflammatory effects against excitotoxicity, ischaemia, trauma and neurotoxicants [71, 72, 74, 75]. For example, any rat brain microinjection of glutamate analogues triggers a persistent process that leads to progressive atrophy with a widespread neuronal loss and a concomitant neuroinflammation [76]. This neurodegenerative process is reduced by diazoxide oral treatment, which ameliorated microglia-mediated inflammation and reduced neuronal loss [73].

Controlling the extent of microglial activation may offer prospective clinical therapeutic benefits for inflammation-related neurodegenerative disorders [73]. In this context, increased expression of K_{ATP} channels by activated microglia and the specific anti-inflammatory actions of diazoxide reveal the use of this drug as a therapeutic agent to treat MS inflammatory processes.

3.2. K_{ATP} channel openers and experimental autoimmune encephalomyelitis

We analysed the putative neuroprotective effects of diazoxide on an EAE murine model of MS by its oral administration in the classical EAE MOG35-55 mouse model for preclinical studies of MS [72]. The doses tested (0.8 and 0.05 mg/kg) were below those that induce blood glucose increase (>1 mg/kg) and both of them ameliorated clinical signs of EAE, being 0.8 mg/kg more effective [72]. Then, this dose was tested in two different experimental designs: (a) a preventive paradigm, in which diazoxide was administrated daily starting on the same day animals were immunized and (b) a palliative paradigm, in which mice were treated with diazoxide daily

starting when they reached a clinical score of ≥ 1 . In both cases, the treatment showed similar effectiveness and mice treated with diazoxide obtained a lower clinical score during the chronic phase of EAE. However, in the preventive paradigm, diazoxide could not delay EAE onset or reduce the number of animals developing EAE. This indicates that diazoxide effects could be mediated mainly by neuroprotection rather than by immunosuppression. The histopathological analysis of injured spinal cords confirmed that diazoxide elicited a significant reduction in myelin and axonal loss accompanied by a decrease in glial activation and neuronal damage, but it did not affect the number of infiltrating lymphocytes positive for CD3 and CD20.

We then analysed the neuroprotective properties of diazoxide *in vitro* and *ex vivo* [77]. In this study, diazoxide effectively protected NSC-34 motoneurons against oxidative, excitotoxic and inflammatory insults. It also enhanced the expression and nuclear translocation of Nrf2 in these cells as well as in the spinal cord of EAE animals orally administered with diazoxide. This demonstrated that one of the mechanisms of actions implied in the neuroprotective role of diazoxide is mediated by the activation of Nrf2 expression and nuclear translocation. Finally, diazoxide decreased neuronal death in organotypic hippocampal slice cultures after excitotoxicity and preserved myelin sheath in organotypic cerebellar cultures exposed to pro-inflammatory demyelinating damage. Thus, diazoxide is a neuroprotective agent against oxidative stress-induced damage and cellular dysfunction.

We finally studied the putative actions of diazoxide on autoimmune key processes during EAE such as antigen presentation and lymphocyte activation and proliferation [78]. For this, we analysed K_{ATP} channel expression in CD4-positive T cells and the proliferative of lymphocyte response in the EAE model. When we used whole splenocytes to test whether diazoxide could modulate lymphocyte proliferation, results showed a significant inhibition of the lymphocyte proliferative rate both *in vitro* and *in vivo*. Also, the expression of dendritic cell activation markers such as CD83, CD80, CD86 or major histocompatibility complex class II was reduced in cultures treated with diazoxide. However, we observed no inhibition of cell proliferation when isolated CD4-positive T lymphocytes were used instead of whole splenocytes. Diazoxide also failed to inhibit the expression of lymphocyte activation markers. These results suggest that although diazoxide does not directly suppress lymphocyte activation and proliferation, it could modulate lymphocyte activity by regulating antigen presentation. These discrete effects indicate again that diazoxide treatment attenuates EAE pathology with no immunosuppressive effects.

Taken together, at the doses studied, our results demonstrate novel actions of diazoxide as an anti-inflammatory drug that present beneficial effects on EAE through neuroprotection. At the functional level, diazoxide is a neuroprotective agent against oxidative stress-induced damage and cellular dysfunction (**Figure 2**). It attenuates EAE pathology not by causing lymphocyte suppression but by modulating immune communication, decreasing glial harmful activation and promoting myelin and neuronal protection. Thus, treatment with this widely used and well-tolerated drug may be a useful therapeutic intervention in ameliorating MS disease.

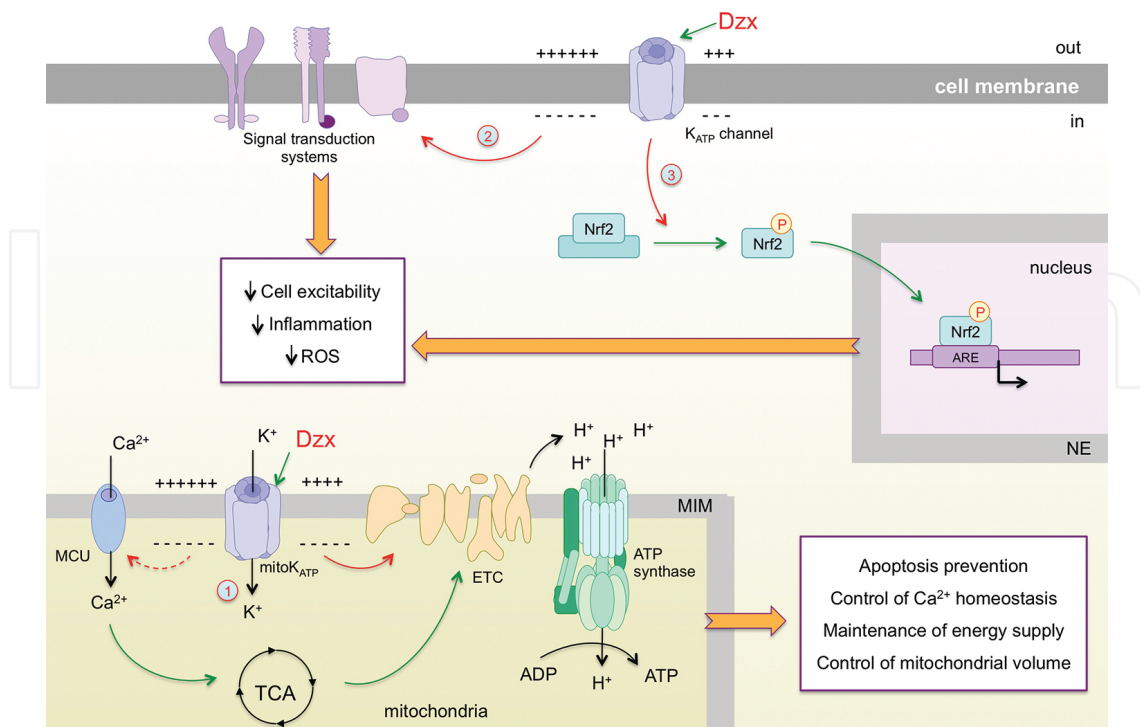


Figure 2. Diazoxide modifies microglial reactivity in brain. Drawing of the effects of diazoxide in the microglial reaction during MS. (1) Diazoxide (Dzx) binds to mitochondrial K_{ATP} channels, induces depolarization of the mitochondrial internal membrane (MIM) and potentiates the H⁺ gradient generated by the electron transport chain (ETC). This enhances both ATP synthesis and calcium concentration by activation of ATP synthase and the mitochondrial calcium uniporter (MCU), respectively. Calcium in the mitochondria also increases the tricarboxylic acid cycle (TCA) flux by activation of deshydrogenases and enhances ATP production [73]. Dzx also activates K_{ATP} channels from the plasma membrane, which modifies the cell response to activation signals, (2) activates Nrf2 and (3) through a mechanism that remains to be described. ARE, antioxidant response element; NE, nuclear envelope.

3.3. Effects of diazoxide in MS patients

Based on the positive preclinical studies and considering that diazoxide has been on the market for decades with an excellent safety profile, two Spanish biotechnological companies, Neurotec Pharma and Advancell, performed a clinical development programme to assess diazoxide efficacy and safety in MS patients. NEUROADVAN trial (ClinicalTrials.gov identifier: NCT01428726) was initiated in 2011 and ended in 2014. NEUROADVAN was a Phase IIa, multinational, double-blind, placebo-controlled clinical trial to evaluate the efficacy and safety of diazoxide. The drug was orally administered at two different doses and compared in 103 patients with RRMS versus placebo (1:1:1). The total duration of the treatment was 6 months. Additionally, patients were allowed to continue during an optional extension period, in the same arm of the study, in a blinded way, until study finalization.

Men and women aged 18–55 years with RRMS (McDonald criteria 2010 [79]) and an EDSS score of 0–5.0 were eligible for the study. The inclusion criteria required at least one relapse in the previous 2 years or the presence of at least one Gad1 lesion in the previous year. During 24 weeks, patients received one daily oral tablet with 0.3 or 4 mg diazoxide. MRI scans were

performed at baseline and every 4 weeks until the end of the study. Patients enrolled in the follow-up study were subjected to an additional scan at week 48.

The primary efficacy end point was that the number of new Gad1 lesions appeared on T1-weighted sequences from weeks 4 to 24. This end point has been validated in many previous 6-month MS trials and was based on the diazoxide effects on microglia activation and blood-brain barrier closing. Secondary MRI end points included cumulative number of lesions on T2-weighted sequences for all MRIs, cumulative number of lesions on T2-weighted sequences in the 6 months of the study (compared with the baseline MRI); cumulative number of combined unique active lesions (CUALs), addition of new or enlarged lesions on T2-weighted sequences that do not enhance with gadolinium and new Gad1 lesions for all MRIs; cumulative number of CUALs, addition of new or enlarged lesions on T2-weighted sequences that do not enhance with gadolinium and new Gad1 lesions in the 6 months after starting therapy (compared with the baseline MRI); number of patients without Gad1 lesions in T1-weighted sequences in the 6 months after starting therapy; and percentage brain volume change (PBVC). Secondary clinical end points also included the measurement of relapse-free status, relapse rate, number of relapses requiring corticosteroid treatment, time to first relapse during the trial, change in EDSS scale and quality of life. Secondary safety end points were monitored during the 6 months of therapy up until 15 days after the last dose of diazoxide and included incidence, nature and severity of adverse events (AEs). Control of glucose levels, glycosylated haemoglobin and blood pressure were also monitored.

The results of the clinical trial showed no differences in the diazoxide groups in the primary end point or in the other MRI variables associated with the presence of new lesions [80]. However, an interesting decrease in the PBVC in the patients who received diazoxide compared with placebo was found. The number of new T2/Proton Density lesions converting to black holes was not different between arms. Finally, in accordance with the small sample size of the trial, no differences were detected in clinical variables of relapse-free status, relapse rate, number of relapses requiring corticosteroid treatment, change in EDSS or quality of life.

There were six serious adverse events during the study but only a case with autoimmune hypothyroidism was considered to be related to the therapy that was not discontinued. Regarding the described clinical effects of diazoxide on glycaemia, the detected glucose blood levels were always within the limits of normality. This confirms that the diazoxide doses used in this trial were lower than those that induce hyperglycaemic and hypotensive effects.

As commented above, at the doses tested, diazoxide does not seem to have a significant effect that impedes the appearance of new Gad1 lesions. However, although patients were randomly distributed within the groups, at the beginning of the study those patients receiving diazoxide presented a higher number of Gad1 lesions than patients included in the placebo group. This initial higher disease activity in the group treated with diazoxide was maintained throughout the trial. These findings confirm the need to perform an extensive and accurate selection of patients, in terms of the activity of the disease, which must be carried out prior to the clinical trial.

Regarding the effects of diazoxide on brain atrophy, PBVC progressed more slowly in treated patients in a dose-dependent manner. This is consistent with a recent study that validates the measurement of brain atrophy as an outcome for Phase II trials in RRMS [81]. However, taking into account that treated patients had a more active disease, and that diazoxide presents vasodilator actions and peripheral resistance, the reduced atrophy found in patients treated with diazoxide may also be secondary to fluid shifts and not a true protection against brain injury. Nevertheless, the effects in slowing the progression of brain atrophy would require further validation.

To sum up, NEUROADVANCE study indicates that diazoxide is a safe drug, well tolerated in patients with MS, but it was not possible to find evidence of efficacy in preventing the formation of new inflammatory lesions.

4. Conclusion

Neurodegenerative changes in MS such as demyelination and axonal loss take place early in the disease and independently from acute immuno-mediated inflammation. The reason why these focal demyelinating inflammatory lesions appear within the CNS still needs to be clarified, but diverse clinical presentations indicate the existence of different patterns of inflammation and neurodegeneration. Nevertheless, the molecular mechanisms underlying axonal damage in acute inflammation and chronic demyelination are potentially amenable to therapy. In this context, the pursuit of neuroprotective therapies in MS should provide new valid alternatives to significantly impact on disease progression. In this pursuit, the standard view of neuroprotection, which has long been mostly focused on neurons, is no longer valid. Instead, this view has been replaced by an integrative approach that recognizes the importance of dynamic interactions between immune cells, microglia, astrocytes and neurons.

A proper evaluation of neurodegenerative aspects in MS remains difficult. Also, for all mentioned drugs the neuroprotective effect has not been fully established. These issues could be better understood by applying well-established and new clinical and imaging parameters that require further preclinical and clinical investigations. Moreover, molecular characterization of the different MS clinical presentations must allow a pharmacogenomics classification of patients and development of personalized therapies. In this line, promising future approaches will combine high-throughput techniques such as proteomics and genomics to investigate further MS neurodegenerative processes, in particular neuroinflammation.

5. Disclosures

JFEP and MP have applied for a PCT application 'Diazoxide for use in the treatment of a central nervous system (CNS) autoimmune demyelinating disease' (application number PCT/EP2011/050049).

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Author details

Juan Espinosa-Parrilla, Marco Pugliese, Nicole Mahy and Manuel J Rodríguez*

*Address all correspondence to: marodriguez@ub.edu

Department of Biomedical Sciences, Institute of Neuroscience, IDIBAPS, University of Barcelona, and Center for Biomedical Research Network in Neurodegenerative Diseases (CIBERNED), Barcelona, Spain

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