

## Relapsing remitting multiple sclerosis in patients under treatment with laquinimod

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Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS), which is pathophysiologically characterized by both inflammatory demyelination and neurodegeneration. Laquinimod is a small molecule, an investigation for oral drug administration being developed for the treatment of relapsing-remitting multiple sclerosis (RRMS). We explore serum IgG antibodies to GM1, GM3 and GD1a gangliosides in our patients under laquinimod treatment. The results show high IgG titers of anti-GM1 antibodies, but low titers of anti-GM3 and anti-GD1a antibodies. There are no data laquinimod to affect the demyelination - in almost all patients the anti-GM1 antibodies titer is positive. It can be concluded that laquinimod has a neuroprotective action. Oral application of laquinimod against MS may reduce brain damage caused by neurodegeneration. Laquinimod has dual properties of immunomodulation and neuroprotection, and is a potentially promising oral treatment of RRMS.

*Key words:* serum anti-ganglioside antibodies, laquinimod, relapsing-remitting multiple sclerosis, neuronal damage, neuroprotection.

### Introduction

Multiple sclerosis (MS) is a chronic, progressive disease affecting the central nervous system (CNS) (both brain and spinal cord). The symptoms are caused by the body's own immune system attacking and damaging the myelin sheaths surrounding nerve fibers. This leads to inflammation within the CNS making the patient to suffer relapses and disease progression. The etiology of the disease is unknown, but it is assumed to depend, like other autoimmune diseases on both genetic and environmental factors [9]. Histopathologically, MS has been characterized by focal inflammatory infiltrates, demyelination, but in some cases remyelination, astrogliosis and variable axonal damage within the CNS [19]. One hallmark of the pathology of MS is inflammation involving B-cells, T-cells, and macrophages, which results in damage of the CNS tissue.

The treatment and the management of MS should be targeted toward relieving symptoms of the disease, treating acute exacerbations, shortening the duration of acute relapse, reducing frequency of relapses and preventing disease progression [12].

The treatment of the disease has two aspects: immunomodulatory therapy (IMT) for underlying the immune disorder and therapies to relieve or modify symptoms. IMT is directed toward reducing the frequency of relapses and slowing progression. Currently, the most disease-modifying agents have been approved for use only in the relapsing forms of MS [12].

Laquinimod may be considered as an immunomodulatory drug. However, murine studies of experimental autoimmune encephalomyelitis (EAE) and results from MRI suggest that this drug may also exhibit indirect and potentially direct neuroprotective effects. In adults with relapsing-remitting multiple sclerosis (RRMS), laquinimod has demonstrated ability to slow disease progression; its beneficial effects have been demonstrated on both clinical endpoints and MRI surrogate markers. It shows a favorable safety profile [5, 7, 8]. Its unique profile together with the convenience of an orally-administered drug, make laquinimod an attractive agent for patients with MS. In order to improve MS therapy, agents are needed to protect effectively against both inflammatory and neurodegenerative components of this disease [9]. Positive results have been reported for five new oral drugs for RRMS: fingolimod, cladribine, teriflunomide, laquinimod and dimethyl fumarate - in phase 3 studies. Several new oral drugs are likely to be approved for RRMS in the near future. Some oral treatments have shown benefit and would generate much interest because of the convenience of such administration. However, the availability of convenient oral drugs will not necessarily translate into clinical effectiveness and safety [10].

Laquinimod (ABR-215062; Molar mass: 356.803 g/mol) is an orally administered quinoline-3-carboxamide small-molecule derivative of the parent compound, the immunomodulator linomide. It was developed as a therapeutic agent against MS because of lack of safety concerns seen with the parent compound. In preclinical studies, evidence has accumulated suggesting that laquinimod may exhibit immunomodulatory and potentially neuroprotective properties [19]. This drug has a high level of oral bioavailability, a small distribution volume and a low rate of total clearance. As a small molecule, laquinimod diffuses freely across the blood–brain barrier (BBB) without any known active transport by extra- and/or intracellular receptors [4]. As such, it can reach the CNS and may exert direct or indirect neuroprotective effects [17]. Mechanisms that have been proposed for neuronal and axonal damage in EAE and MS include effects driven by an inflammatory milieu, mitochondrial dysfunction, and glutamate toxicity. The actual cause of damage may be one or another combination of the described above. Experimental evidence suggests that laquinimod may be able to inhibit some of these effects. Laquinimod's influence on EDSS scores may possibly be due to its ability to affect the CNS directly, thereby reducing the diffuse neurodegenerative effects of MS, which are linked to long-term disability progression rather than peripherally initiated, T-cell-mediated focal lesions that are linked to relapses [9]. Clinical data show laquinimod to be well tolerated in patients with RRMS. In order to clarify of laquinimod's proved neuroprotective effects, the manufacturer is planning to investigate its use in a population with primary progressive MS. Laquinimod also exhibits the effects of cell migration. It may reduce the entry of proinflammatory monocytes into the CNS by lowering the levels of matrix metalloproteinase 9, which regulates the trafficking of monocytes into inflamed tissues [14]. It has a high level of oral bioavailability, a small distribution volume and a low rate of total clearance. The maximum plasma concentration is reached within the first hour following its administration and is less than 5  $\mu$ M after the administration of 0.05 – 2.4 mg of the drug [6]. The experimental studies have shown that laquinimod decreases the activation of microglia [3]. Modulation of astrocytic activation has been postulated as yet another mechanism of action of laquinimod. Downregulation of the astrocytic proinflammatory response appears to

preserve oligodendrocytes, axons and myelin. Laquinimod was also able to regulate synaptic transmission by increasing inhibitory postsynaptic currents and, at the same time, by reducing excitatory postsynaptic currents, pointing to its novel, potentially neuroprotective properties [17]. Since MS patients must be on medication throughout their lifetime, an oral treatment creates a substantial advantage compared with existing products in the market, all of which must be injected. If applied once daily, an oral tablet of investigated laquinimod (Nerventra) acts as a CNS-active immunomodulator with a novel mechanism of action being developed for the treatment of RRMS. The global Phase III clinical development program which evaluates oral laquinimod in MS includes two pivotal studies, ALLEGRO and BRAVO. Phase III of laquinimod trial, CONCERTO, is evaluating two doses of the investigational product (0.6 mg and 1.2 mg) in approximately 1,800 patients for up to 24 months. Following late-stage studies, Teva™ is conducting this phase of the trial, CONCERTO, evaluating two doses of (0.6 mg and 1.2 mg) in 2,100 patients for up to 24 months. The primary outcome measure is the time to confirm disability progression as measured by the Expanded Disability Status Scale (EDSS). Treatment with laquinimod reduces development of active MRI lesions in relapsing MS [15].

MS treatment needs accessible markers for early neuronal injury and rapid onset of therapy. Over the past ten years the importance of potential applicability of antibodies as biological markers for the diagnosis, classification, disease activity and prediction of clinical courses in MS has evolved [1]. Gangliosides are a family of acidic glycosphingolipids highly concentrated in the nervous system, where they represent about 10% of the total lipid content [21]. GM1 is a main myelin ganglioside and the GD1a is one of the major ganglioside fractions in the CNS [22]. MS treatment needs accessible markers for early neuronal injury and rapid onset of the therapy. Therefore, the MS therapy should include the prevention of neuronal degeneration and dysregulation of neuron-glia interactions, as soon as the diagnosis is made. In the last decade an enormous effort has been applied to discover biological markers of neuronal damage capable to predict the course of the disease and effective response to therapy [2, 13]. Over the past ten years the importance of potential application of antibodies as biological markers for the diagnosis, classification, disease activity and prediction of clinical courses in MS has evolved [1].

The aim of the present study was to correlate the neuronal injury with serum levels of anti-GM1, anti-GM3 and anti-GD1a antibodies in patients with RRMS under treatment with laquinimod.

## Materials and Methods

The samples are from Neurology Clinic of First MHAT, Sofia. Sera were obtained from 10 patients with clinically defined MS with relapsing – remitting form of the disease. They were with a long duration of the disease and in clinical relapse during sample collection. None of MS patients received another immunosuppressive treatment at the time of venipuncture. An informed consent was obtained from each patient. Because our Laquinimod patients are in chronic relapsing remitting form of the disease in our experiments we worked with anti-gangliosides IgG antibodies.

**ELISA Protocol.** The serum anti-GD1a, GM1, GM3 IgG antibodies were estimated by the enzyme-linked immunosorbent assay (ELISA) [16]. We determined antiganglioside antibodies (AGAs) of the IgG class against GD1a ganglioside. AGAs were found in low titers in some healthy subjects we estimated a reference range for the healthy controls. MS patients were considered strongly positive only if the optical density of their sera exceeded

$x \pm 2$  SD of the healthy controls. Briefly, 1000 ng of GD1a, GM1 and GM3 ganglioside in 100 ml of methanol were separately pipetted into three 96 well microtitre plates. After air drying, the wells were blocked with BSA-PBS (1% bovine serum albumine in phosphate-buffered saline) for 1h. After washing six times with PBS, 100 ml of sera diluted 1:20 to 1:5000 in BSA-PBS, were added to each well and incubated overnight. After that the plates were washed thoroughly six times with PBS. Binding was detected following a 2h incubation period with BSA-PBS diluted (1/3200) peroxidase-conjugated goat anti-human IgG antibodies and with BSA-PBS diluted (1/4800) peroxidase conjugated goat anti-human IgM antibodies. All incubation steps were performed at 4°C. After washing with PBS, colour development was achieved in a substrate solution containing 15 mM 0-phenilendiamine and 0.015% H<sub>2</sub>O<sub>2</sub> in 0.1M sodium acetate buffer, pH 5.0 at room temperature. The reaction was stopped after 30 min by addition of 50 ml of 1N H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) was measured and read spectrometrically at 490 nm with an ELISA reader (TECAN, Sunrise TM, Austria). Non specific antibody binding (OD value in a well not containing GD1a ganglioside) was subtracted for each measurement. Adult patients were considered strongly positive only if the mean OD of their sera exceeded  $x \pm 2$ SD (standard deviation) of the healthy controls. Determinations were carried out in triplicate. The Student's test was used to determine statistical differences between the groups using p value of less than 0.05 as the level of confidence. The data were presented as a mean value (M)  $\pm$  standard error of mean.

## Results and Discussion

Our observations in the titers of the anti-gangliosides GM1, GM3 and GD1a antibodies in sera of patients under treatment with laquinimod show elevated titer of anti-GM1 antibodies but lack of anti-GM3 and anti-GD1a antibodies (**Table 1**). There are no data laquinimod to affect the demyelination.

As we know from our previous studies, the presence of anti-GM1 IgG antibodies in the patient sera is in correlation with demyelination and of IgG anti-GD1a - with neurodegeneration, respectively [23, 24]. There are three hypotheses about the presence of anti-GM3 IgG antibodies in the patient sera. The increased titers of the last are usually considered to indicate neurodegeneration and BBB damage. It is correlated with BBB destruction, which may lead to severe metabolic abnormalities (e. g. diabetes) or with appearance of cancer cells [11].

A considerable increase of GD1a in the serum after the first attacks of RRMS and primary progressive MS (PPMS) was determined and is generally connected with neuronal damage [11, 23, 24]. Autoantibodies against gangliosides GM1 and/or GD1a are associated with acute motor axonal neuropathy and acute motor-sensory axonal neuropathy, whereas antibodies to GD1b ganglioside are detected in acute sensory ataxic neuropathy. Antibodies to GM1 and GD1a gangliosides have been proposed to disrupt the nodes of Ranvier in motor nerves via complement pathway [18]. Serum antibodies to different gangliosides have also been identified in some subtypes and variants of Guillain-Barré Syndrome (GBS). These observations, correlated and integrated with electrophysiological and pathological findings in humans indicate that the GBS subtypes acute motor conduction block neuropathy, acute motor axonal neuropathy, acute motor and sensory neuropathy, but also acute sensory neuropathy and possibly also a chronic disorder as multifocal motor neuropathy represent a spectrum of the same immunopathologic process. Being nodal axolemma and paranode the focus to the nerve injury, these immune-mediated neuropathies could be more properly classified as nodo-paranodopathies [20].

**Table 1.** Titers of the anti-gangliosides GM1, GM3 and GD1a antibodies in sera of patients under treatment with laquinimod. Only patients from 1 to 7 with GM1 antibody titer show very high OD with p value < 0.05. Their sera are considered highly positive.

Patients	serum anti-GM1 antibody titer	serum anti-GM3 antibody titer	serum anti-GD1a antibody titer
1. RRMS without relapse	+	-	+/-
2. RRMS without relapse	+	-	+/-
3. RRMS without relapse	+	-	+/-
4. RRMS without relapse	+	-	-
5. RRMS without relapse	+	-	-
6. RRMS without relapse	+	-	-
7. RRMS without relapse	+	-	-
8. RRMS without relapse	+/-	-	-
9. RRMS without relapse	+/-	-	-
10. RRMS without relapse	+/-	-	-

**Numerical value of the titer:** (-)  $\leq 0.047$ ; normal is 0.047; ( $\pm$ ) is 0.062; (+) is 0.077; (++) is 0.107

## Conclusions

In conclusion, our study indicates, for the first time, that the changes in serum IgG anti-GM1, anti-GM3 and anti-GD1a ganglioside antibodies in MS patients under treatment with laquinimod reflect with the CNS neuronal injury. Increased relative anti-GD1a portion in the serum of patients with MS indicates continuous neuronal damage. Therefore, GD1a and GT1b gangliosides can serve as biomarkers for these early pathological CNS changes in MS. The results from these trials could further inform about the clinical benefit of laquinimod in patients with a persisting, but still insufficient need of safe and at the same time effective oral compounds with neuroprotective effects. Reflecting the results, we conclude that some patients may proceed to make relapses, regardless of the fact that they are continuously treated with a suitable medicine, and the serum titers of GM1, GM3 and GD1a IgG anti-ganglioside antibodies demonstrate that all these medicines are not 100% effective. The admission of laquinimod should not be interrupted. Laquinimod should be admitted for long period of time. So far we do not have enough patients receiving laquinimod. We continue to follow our patients and to look for new ones. There are no data for laquinimod to affect the demyelination – almost all patients are positive on GM1. It can be concluded that laquinimod has a neuroprotective action. Oral laquinimod for MS may reduce brain damage caused by neurodegeneration. For the first time, IgG antibodies against GM3 ganglioside in serum were examined and it could be concluded that patients before, during and after this therapy have no metabolic disorders. Laquinimod had potential benefits in reducing relapse rates and was safe for most of the patients with RRMS, subjected on short-term treatment. The most common adverse events included headache, back pain, arthralgia, diarrhea, cough, urinary tract infection, elevated alanine aminotransferase, insomnia, nausea, abdominal pain and sinusitis.

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