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In vitro assessment of the direct effect of laquinimod on basic functions of human neural stem cells and oligodendrocyte progenitor cells

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ABSTRACT

Laquinimod is an orally active immunomodulatory small molecule that has shown clear clinical benefit in trials for relapsing–remitting multiple sclerosis and in experimental rodent models that emulate multiple sclerosis (MS). Studies in healthy mice, and in mice with experimental autoimmune encephalomyelitis, have demonstrated that laquinimod is capable of entering the central nervous system. It is therefore important to determine if laquinimod is capable of a direct influence on basic functions of neural stem cells (NSC) or oligodendrocyte progenitor cells (OPC)—cells critical for myelin repair in MS. In order to address this question, a series of experiments was conducted to determine the effect of exogenous laquinimod on viability, proliferation, migration and differentiation of human NSC and OPC *in vitro*. These data show, for the first time in cells of human origin, that direct, short-term interaction between laquinimod and NSC or OPC, in an isolated *in vitro* setting, is not detrimental to the basic cellular function of these cells.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune and neurodegenerative disease of the central nervous system (CNS). The disease affects approximately 400,000 individuals in the United States and about 2.5 million worldwide, with evidence of increasing incidence over the last 50 years [16,18,19,24,31]. Pathologically, MS is characterized by focal leukocyte infiltration into the CNS by multiple immune cell types, including autoreactive, myelin-specific T cells [28,38]. Targeted myelin destruction leads to demyelination, axonal loss and clinical neurological presentation of the disease. In the earlier stages of MS there is evidence of endogenous repair in the form of remyelination, which leads to axonal protection and restoration of nerve signaling [15,30]. The process of remyelination involves the recruitment of neural stem cells (NSC) and oligodendrocyte progenitor cells (OPC) to lesion sites from either the subventricular zone or neighboring parenchyma, possibly in response to the release of chemoattractant molecules present in the active lesion [7–9,21,35,40]. Once *in situ* these cells undergo differentiation and remyelination occurs, but, as the disease progresses remyelination fails for reasons that are still unclear. Possible mechanisms of remyelination failure may involve overall neural cell dysfunction, persistent inhibitory signals or inappropriate cell migration [3,12,13,21]. Despite the increasing availability of MS drug treatments in the last 15 years, current FDA approved therapies are only moderately effective in reducing relapse rate and stopping disease progression in the MS population as a whole, and are not targeted at the direct protection or repair of the CNS. While new therapies addressing this issue are in great demand it is important to confirm that existing and new immunomodulatory agents, especially those capable of crossing the blood–brain barrier, are not detrimental to the stem cell repair process.

Laquinimod, *N*-ethyl-*N*-phenyl-5-chloro-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-3-quinoline-carboxamide, is an orally active immunomodulatory small molecule that has been shown to prevent the onset of disease, suppress established disease and reduce relapse rate in experimental autoimmune encephalomyelitis (EAE; an established animal model of MS) [6,26,32,41]. Clinical studies in humans have also demonstrated efficacy in reducing active lesions and brain atrophy on MRI and slowing clinical disease progression [10,11]. The mechanism of action of laquinimod may involve a modest shift in immune function from a pro-inflammatory Th1 to an anti-inflammatory Th2/3, rather than a general immune suppression [17,33,37,39,41]. Alterations in immune cell trafficking and a reduction in acute axonal damage have also been proposed as mechanisms of action [5,25,39]. Intriguingly, laquinimod may also

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have direct effects in the CNS. Using ^{14}C-labeling in mice it was shown that laquinimod, at a dose of 1–25 mg/kg, is capable of entering the CNS to ~7–8% in healthy and 13% in mice with EAE [39]. We therefore estimate that the concentration of laquinimod in the CNS to be in the region of 100 nM (0.1 μM). These data suggest that laquinimod is capable of directly affecting the functions of neural cells, especially NSC and OPC. To address this possibility, we conducted a series of experiments to test the effect of direct exposure of laquinimod on NSC and OPC viability, proliferation, migration and differentiation, using human embryonic derived NSC and OPC as an *in vitro* model system.

2. Experimental procedures

2.1. Laquinimod

Research grade laquinimod Na was provided by Teva Pharmaceutical Industries (Netanya, Israel) and was prepared fresh for each experiment by reconstitution in distilled water at a stock concentration of 100 mM. Laquinimod was diluted to appropriate working concentrations in complete NSC and OPC culture media.

2.2. Differentiation of hESC into NSC and OPC

Human NSC and OPC were derived from the human embryonic stem cell (hESC) line WA09 according to previously published methods [21]. For NSC cultures, hESC-derived cell lines that met specific NSC criteria (morphology, phenotype and ability to further differentiate to neural cell lineages), were maintained in neural-induction media (NIM) (DMEM/F12 supplemented with N2 (Invitrogen), 2 μg/ml heparin (Sigma) and 0.5 mM sodium pyruvate) containing 10 ng/ml basic fibroblast growth factor (bFGF, R&D Systems) and 20 ng/ml epidermal growth factor (EGF, R&D Systems) in tissue culture plates treated with growth factor reduced matrigel (GFRM;1:30 dilution, BD Biosciences).

For OPC cultures, enriched populations of human OPC that met specific OPC criteria, including phenotype, morphology, expression of myelin proteins and ability to become myelinating cells were maintained in ITB media (a 50:50 mix of ITTSPP media [DMEM/F12 media supplemented with 25 μg/ml human insulin, 50 μg/ml human apotransferrin, 6.3 ng/ml progesterone, 10 μg/ml putrescine, 50 ng/ml sodium selenite, 40 ng/ml triiodothyronine and 0.5 mM sodium pyruvate] and B27 media [DMEM/F12 supplemented with 2X B27 (Invitrogen)]) containing 20 ng/ml EGF on GFRM-coated tissue culture plates.

2.3. Measurement of cell viability

Cell viability and apoptosis were determined using fluorescein diacetate and propidium iodide (FDA/PI) staining and by caspase activation using CaspaTag Pan-Caspase In Situ Assay Kit, Fluorescein (Millipore) as previously described [20–22]. For FDA/PI quantification NSC and OPC were seeded at a concentration of 15,000 cells/well to GFRM-coated 48-well plates. For CaspaTag assay cells were seeded at 50,000 cells/well in 12-well plates treated with GFRM. Twenty-four hours later cultures were dosed, in duplicate, with increasing log doses of laquinimod-Na or the positive cell death control staurosporine (500 nM, Sigma). Cells were incubated for 24 h, 48 h and 7 days at 37 °C 5% CO_2, with media replenishment every 3 days. Following FDA/PI staining cell counts (living vs. dead cells) were determined using a Nikon Eclipse 2000 inverted fluorescence microscope from three random fields of view per well. For CaspaTag assay, caspase and PI staining was quantified using a BD FACS Caliber Flow Cytometer using instrument settings and fluorochrome compensations defined in preliminary experiments.

2.4. Measurement of cell proliferation

Proliferation was measured using [^{3}H] thymidine incorporation into cells harvested by an automated cell harvester (Tomtec Harvester 96 Mach III M). Cells were seeded onto a 96-well plate at 15,000 cells/well (NSC) and 25,000/well (OPC) for 24h time point and at 8000 cells/well for 4 day time point. Twenty-four hours later cells were treated with increasing concentrations of laquinimod-Na. For NSC and OPC cultures, proliferation media containing either 20 ng/ml EGF and 10 ng/ml bFGF, 20 ng/ml EGF or 10 ng/ml PDGF, 10 ng/ml bFGF and 20 ng/ml EGF were used as a positive control. For 24h proliferation 0.2 μCi [^{3}H] thymidine/well, and for 4 day assay, 1 μCi [^{3}H] thymidine/well was added to the adhered cells immediately following laquinimod dosing. Radioactivity (CPM) was counted using a Wallac Trilux 1450 Microbeta liquid scintillation counter and data expressed as percentage change from control (basal media only).

2.5. Chemotaxis of NSC and OPC

The migratory capacity of NSC and OPC-enriched cultures was assessed using Boyden chambers (8 μm, Corning Costar) as previously described [21]. Briefly, 60,000 cells were seeded to inserts and migration towards lower chambers containing varying concentrations of laquinimod or the control chemoattractants CXCL12 or PDGF-AA was measured. To determine if direct interaction of cells with laquinimod promoted or hindered cell migration to positive stimuli (such as CXCL12 or PDGF-AA), cells were incubated with varying concentrations of laquinimod for 30 min prior to adding to the upper chamber inserts of the migration chamber. Cell counts were measured using a phase contrast microscope from five random fields of view per treatment. Appropriate controls with no chemokine, irrelevant soluble factor, no migrated cells and migrated cells were carried out within each experiment. Results were calculated as a percentage of the control (media only) and the validity of the results determined by performing three independent experiments.

2.6. Laquinimod differentiation studies

2.6.1. NSC neuronal differentiation

Differentiation of NSC to immature neurons (as measured by doublecortin (DCX) expression and morphology change) was achieved by culturing NSC on poly-D-lysine/laminin coated tissue culture plates in NIM in the absence of growth factors for at least 7 days. To enhance differentiation, NSC were treated with BDNF and cAMP in NIM media as described by Zhang et al. [42]. Cells were treated with laquinimod at day 1 of the differentiation protocol and media, with and without laquinimod, were replenished every 2 days. DCX positive cells and total number of DAPI positive cells per field of view were counted from at least three random fields of view per well and the experiment was repeated at least three times. Differentiated cells are expressed as percentage DCX/nestin positive or as DCX only positive.

2.6.2. OPC differentiation

For differentiation studies, enriched OPC populations were cultured in ITB media without EGF for 24 h and subsequently dosed with laquinimod for 10 days, with media changes every 2 days. Cultures were either lysed for quantification of oligodendrocyte proteins CNPase or myelin basic protein (MBP) by Western blot, or analyzed directly by immunofluorescence analyses using the immature oligodendrocyte marker O4. Cell counts (O4-positive vs. dapi-positive nuclei) were measured using a 20× objective on a Nikon Eclipse 2000 inverted fluorescence microscope from three random fields of view per well.

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