Title: Neuroprotective and Remyelinating Potential of Siponimod (BAF312) evaluated in a Xenopus Model of Conditional Demyelination and a Mouse Model of Experimental Autoimmune Encephalomyelitis-Optic Neuritis

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Background
Siponimod, a potent and highly selective sphingosine 1-phosphate receptor modulator, has recently been approved for treatment of relapsing forms of MS and active SPMS1.

Objective
To assess remyelination and neuroprotective potential of siponimod in a Xenopus remyelination and a mouse optic neuritis (EAEON) model using histological analysis and longitudinal visual system readouts.

Methods
We used a conditional demyelination transgenic Xenopus laevis model (MBP-GFP-NTR), in which oligodendrocyte apoptosis can be induced by metronidazole (MTZ) treatment2. After MTZ withdrawal, remyelination was assessed with or without siponimod (0.1nM-1µM). In a pharmacokinetics study, brain siponimod levels were analysed. EAEON was induced in female C57BL/6J mice immunized with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) and continuously fed with vehicle- or siponimod-loaded pellets at 10 or 30 mg/kg of food, either prophylactically or therapeutically, over 90 days3. Sections of the optic nerve (Xenopus and mouse) were used to detect de- and remyelination, as well as inflammatory infiltrates. In mice, thickness of retinal layers and visual function were assessed by optical coherence tomography and optokinetic response, respectively. Circulating lymphocytes (flow-cytometry) and siponimod blood and brain levels were analysed at the end of the experiment.
Results
Treatment of demyelinated tadpoles with siponimod (1nM) improved remyelination by a factor of 2.3±0.2 fold in comparison to control. The dose-response of siponimod efficiency to accelerate remyelination showed a bell-shaped curve with a maximum remyelination effect at concentrations of siponimod ranging between 1nM and 3nM. In the EAEON mouse model, prophylactic siponimod treatments attenuated the EAEON clinical scores by about 80% and 95%, respectively, and reduced the retinal neurodegeneration and the loss of visual function. Interestingly, therapeutic treatment starting at day 14 of EAEON had no impact on optic nerve immune cell infiltrates but resulted in increased myelin levels and protection of inner retinal layers also in a bell-shaped dose-response curve with significant protective effects only at the lower dose.

Conclusions
Our data suggest that while siponimod strongly impacts immune cells at higher concentrations (linear dose-response), its effects on remyelination and neuroaxonal survival are dose dependent following the dynamics of a bell-shaped dose-response curve in both animal models.

References
Figures

Figure 1 Confocal illustrations of GFP expressing oligodendrocytes in the optic nerve of transgenic MBP-GFP-NTR Xenopus laevis (stage 50 tadpole) before (D0), at the end of metronidazole exposure (D10) and after 3 days (R3) and 8 days (R8) spontaneous recovery (A). Semithin transversal sections stained with luxol-fast blue (B) and electron micrographs of transversal ultrathin sections (C) of optic nerve of transgenic MBP-GFP-NTR Xenopus laevis tadpole before (D0), at the end of metronidazole exposure (D10) and after 3 days (R3) and 8 days (R8) of spontaneous recovery. Quantification of the number of myelinated axons of the semithin section between D0 and R8 (D).

Figure 2 Dose response of siponimod remyelination potency. Stage 52-53 transgenic MBP-GFP-NTR Xenopus laevis tadpoles were exposed for 10 days in metronidazole (10µM) before being returned in normal water or water containing increasing concentrations of siponimod. Remyelination was assayed by counting the number of GFP+ oligodendrocytes per optic nerve in vivo on day 3 (R3) of the repair period (see figure 1). Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software). Data are expressed as mean ± SEM, n = 5–8 tadpoles per group. P-values were calculated using 1-way ANOVA followed by Dunn’s post-hoc test to compare siponimod concentrations to control condition.
Figure 3 Longitudinal changes siponimod blood concentrations and lymphocyte counts in C57Bl/6J mice. (A) Siponimod blood concentrations over 14 days and (B) lymphocyte counts after 14 days in mice during continuous siponimod treatment via drinking water (n=3 per group). (C) Siponimod concentration over 28 days and (D) lymphocyte counts in blood after 28 days in mice, fed continuously with siponimod-loaded food pellets (n=6 per group). All graphs represent the pooled mean ± SEM with *p<0.05; **p<0.01; ***p<0.001, area under the curve compared by ANOVA with Dunnett’s post hoc test for time courses and with *p<0.05, **p<0.01, by ANOVA with Dunnett’s post hoc test for scatter plots compared to control untreated mice.

Figure 4 Clinical EAE score (A), degeneration of the inner retinal layers (B) and visual function by spatial frequency in cycles per degree (c/d) (C) of female C57Bl/6J EAE mice over 90 days of EAE. Brn3a stained RGCs after 90 days of EAE of Sham, MOG EAE and siponimod treated mice, scale bar = 50 µm (D). The bar graph shows the RGC density 90 days after immunization (E). Siponimod was administered either on the day of immunization (d0), 14 days (d14) or 30 days (d30) post immunization (dpi). All graphs represent the pooled mean ± SEM (out of two independent experiments each with n = 6 animals per group) with *p<0.05; **p<0.01; ***p<0.001, area under the curve compared by GEE or ANOVA with Dunnett’s post hoc test for time courses compared to untreated MOG EAE. **p<0.01, ***p<0.001, by ANOVA with Dunnett’s post hoc test compared to MOG EAE untreated mice for the bar graph.
Figure 5 Leucocytes were isolated 90 days after Sham injection or EAE immunization and directly analysed. The cell population was gated and percentages of CD45+ cells were analyzed for CD3 and B220 cells (A). Quantitative analysis of the flow-cytometry measurement (B). Siponimod blood concentration 90 days after EAE Immunization (C). Graph represents the pooled mean ± SEM (n = 6 animals per group out of two independent experiments) with *p<0.05, ***p<0.001, by ANOVA with Dunnett’s post hoc test compared to MOG EAE untreated mice.
**Figure 6** Longitudinal sections of optic nerves of C57Bl/6J mice were stained for Iba1, CD3 and MBP 90 days after MOG35-55 immunization; white arrows indicate areas of demyelination (A). Quantitative analyses of microglial activation (Iba1) by fluorescence intensity measurement, T-cell infiltration (CD3 score) and myelin status (MBP score) (B). One optic nerve per mouse was included. All graphs represent the pooled mean ± SEM, (n = 6 animals per group out of two independent experiments) with *p<0.05, **p<0.01, ***p<0.001 by ANOVA with Dunnett’s post hoc test compared to MOG untreated mice.