Published in final edited form as:

NMR Biomed. 2013 December ; 26(12): . doi:10.1002/nbm.3012.

Diffusion tensor imaging detects treatment effects of FTY720 in experimental autoimmune encephalomyelitis mice

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Abstract

Fingolimod (FTY720) is an orally available sphingosine-1-phosphate (S1P) receptor modulator reducing relapse frequency in relapse-remitting multiple sclerosis (RRMS) patients. In addition to immunosuppression, neuronal protection of FTY720 has also been suggested but remains controversial. Axial and radial diffusivities derived from *in vivo* diffusion tensor imaging (DTI) were employed as non-invasive biomarkers of axonal injury and demyelination to assess axonal protection of FTY720 in experimental autoimmune encephalomyelitis (EAE) mice. EAE was induced through active immunization of C57BL/6 mice using myelin oligodendrocyte glycoprotein peptide 35 – 55 (MOG_{35–55}). We evaluated both prophylactic and therapeutic treatment effect of FTY720 at doses of 3 and 10 mg/kg on these EAE mice by daily clinical scoring and end-point *in vivo* DTI. Prophylactic administration of FTY720 suppressed the disease onset and prevented axon and myelin damage as compared with EAE mice without treatment. Therapeutic treatment of FTY720 did not prevent EAE onset but reduced the disease severity improving axial and radial diffusivity toward the control values without a statistical significance. Consistent with previous findings, *in vivo* DTI-derived axial and radial diffusivity correlated with clinical scores in EAE mice. Results support the use of *in vivo* DTI as an effective outcome measure for preclinical drug development.

Keywords

diffusion tensor imaging; axial diffusivity; radial diffusivity; axonal injury; demyelination; experimental autoimmune encephalomyelitis; multiple sclerosis; FTY720

Introduction

Multiple sclerosis (MS) is an inflammation-mediated demyelinating disorder of the central nervous system (CNS) affecting about 400,000 individuals in the United States alone (1,2). It has variable disease courses and complex pathologies. Although inflammatory demyelinating lesions are widely accepted as the hallmark of the white matter pathology,

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axonal degeneration also occurs in MS, even in the earliest stage, and is suggested to lead to irreversible neurological dysfunction (3,4). Most current disease-modifying therapies for MS categorized as immunomodulator primarily target the inflammatory component of the disease without acting directly on the CNS. Although these drugs demonstrated efficacy on reducing relapses in relapsing-remitting MS (RRMS), they have not shown efficacy in primary progressive MS (PPMS) or secondary progressive MS (SPMS) caused by irreversible axonal injury (5). Recently, a sphingosine-1-phosphate receptor modulator known as FTY720 (fingolimod) was shown to ameliorate brain atrophy, reduce the frequency of clinical relapses and MR defined lesions in MS patients (6–9). It is widely recognized that FTY720 effectively reduces lymphocyte infiltration. The beneficial effect of FTY720 on blood-brain-barrier and CNS tissue preservation and repair was also suggested (10–21). However, contradicting reports have also suggested that FTY720 is not axonal or neuronal protective (22,23). Thus, a consensus on CNS tissue protection of FTY720 has yet to be reached.

Diffusion tensor imaging (DTI), by probing the mobility of water molecules, extracts information of the microstructure and pathology of CNS white matter. The DTI-derived axial ($\parallel$, describing water diffusion along fiber tracts) and radial diffusivities ($\perp$, describing water diffusion across fiber tracts), have been demonstrated to reflect axon and myelin integrity respectively (24,25). Specifically, demyelination is associated with an increased $\perp$, likely due to the loss of myelin sheath allowing increased freedom of water movement perpendicular to the axon (24). Axon injury is frequently associated with the decrease in $\parallel$. Though the underlying mechanism is still unclear, the decrease might attribute to the change of the axonal structure (25). Either myelin or axonal damage can result in a decreased white matter anisotropy, thus anisotropy index, such as relative (RA) or fractional anisotropy (FA), is a sensitive biomarker of overall white matter integrity. Previous work on experimental autoimmune encephalomyelitis (EAE) mice has shown that the extent of axonal damage correlated with neurological impairment as assessed by clinical scores (26). Demyelination as reflected by increased $\perp$ showed a much weaker correlation with EAE clinical scores.

In this work, we started daily FTY720 or vehicle treatment on mice at the time of EAE immunization (prophylactic group) and at the onset of the disease (therapeutic group). All mice were evaluated for neurological deficits daily. At the study end point, mice underwent *in vivo* DTI examinations. DTI-derived parameters ($\parallel$, $\perp$, and RA) of the ventrolateral white matter (VLWM) region in spinal cord lumbar levels of the EAE mice suggested significant preservation of myelin and axon integrity in prophylactic but only moderate improvement in therapeutic treatments of EAE mice with FTY720. The DTI-derived $\parallel$ and $\perp$ highly correlated with the neurological deficits assessed using the widely used clinical score (CS) system (27).

Materials and Methods

Animal Methods

For prophylactic study, EAE was induced in seventeen 7 – 8 weeks old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) via active immunization using 50 μ g myelin oligodendrocyte glycoprotein peptide (MOG_{35–55}) emulsified in incomplete Freund's adjuvant with 50 μ g *Mycobacterium tuberculosis*. The immunized mice were divided into 3 treatment groups (n = 5 for each group) receiving vehicle, 3 or 10 mg/kg FTY720. Daily treatment started on the day of immunization and lasted for the study duration (28 – 33 days post immunization). Additional five mice served as age and sex matched controls and received only incomplete Freund's adjuvant and *Mycobacterium tuberculosis* in the absence of MOG_{35–55} (sham group). Mice were graded daily using a

published 0 – 5 scoring system: 1 = limp tail; 2 = hind limb weakness sufficient to impair righting; 3 = one limb paralyzed; 4 = two limbs paralyzed; 5 = 3 or more limbs paralyzed or the animal is moribund (mice were euthanized if they reach grade 5). At the prophylactic study end point, mice underwent *in vivo* DTI examination from day 28 to day 33 post immunization.

For therapeutic study, EAE was induced in another nineteen 7 – 8 weeks old female C57BL/6 mice using the method described above. Another cohort of five mice undergoing the sham immunization described in the prophylactic study served as the controls. Neurological deficits of these mice were evaluated using the same clinical scoring system on a daily basis. Mice were enrolled, on the 1st day when a clinical sign (clinical score = 0.5) was seen, into vehicle, 3 and 10 mg/kg FTY720 treatment for the duration of the experiment. At the conclusion of the therapeutic study, mice were examined using *in vivo* DTI from day 42 to 47 post immunization. All animal experiments were performed according to protocols approved by the Animal Studies Committee at Washington University and the Animal Care and Use Committee at Pfizer Inc.

Magnetic Resonance Imaging

Mice were anesthetized using isoflurane/oxygen mixture (5% induction and 1% maintenance during imaging), fed to a lab-built nose cone. The mouse vertebral column was immobilized using a custom-built holder to reduce the respiratory motion artifact (28). The body temperature was maintained at 37°C via a circulating warm water pad. A 9 cm Helmholtz coil and a 9 mm × 16 mm saddle surface coil served as transmitter and receiver coils, respectively. Both coils used diode based active decoupling (29). The whole setup was placed in a 4.7 T magnet (Oxford Instrument, Tubney Woods, Abingdon, Oxfordshire, UK.) installed with a 15-cm inner diameter, actively shielded gradient coil (18 G/cm, 200 μs rise time) (MagneX, Oxford, UK). The magnet, gradient coil and gradient power supply were interfaced with a Varian DirectDrive console (Agilent Technologies, Santa Clara, CA, US) controlled by a Linux workstation. Sagittal and coronal scout images were acquired to locate discs and ribs as references to identify vertebral segments (Fig. 1). Eight axial images were collected covering the spinal cord levels L1 to L4 (vertebral levels T11 – L1) using a multi-echo spin-echo diffusion-weighted sequence with the following acquisition parameters: repetition time, ~ 1500 ms (varied by the respiratory rate of the animal); echo time, 40ms; number of excitations, 2 – 3 (depend on the SNR of the non-diffusion weighted image); slice thickness, 1 mm; field of view, 1×1 cm²; data matrix, 128×128 (zero filled to 256×256). Diffusion sensitizing gradients were applied in six orientations: (G_x, G_y, G_z) = (1,1,0), (1,0,1), (0,1,1), (-1,1,0), (-1,0,1), and (0,-1,1) with a gradient strength of 11.6 G/cm, duration () = 5 ms, and separation () = 20 ms, resulting in a b value of 1000 s/mm². Acquisition time was about 1.5 hours. DTI parameter maps were calculated for ADC, $\parallel$, $\perp$, and RA as previously described (24,25).

Histological analysis

Immediately after *in vivo* imaging, mice were subjected to intra-cardiac perfusion fixation using 0.01M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS. Mouse vertebral columns were excised, post-fixed overnight, decalcified for 48 hours, and embedded in paraffin. The embedded tissues were sectioned using a sliding microtome at a thickness of 5 μm. Sections were deparaffinized, rehydrated and then were blocked using 1% bovine serum albumin (BSA) in 1% PBS for 30 min at room temperature. The sections were then incubated with polyclonal anti-(myelin basic protein) (MBP; 1:1000 dilution; Sigma Chemical Company, St. Louis, MO, USA) or monoclonal anti-(phosphorylated neurofilaments) (SMI31; 1:800 dilution; Convance, Emeryville, CA, USA) antibodies at 4°C overnight. After rinse in PBS for 30 min, the sections were incubated again with Alexa

Fluor 488 conjugated goat-anti-mouse IgG (H+L) (1:1000 dilution; Invitrogen Co. Camarillo, CA. USA) antibody for 1 h at room temperature to visualize immunoreactive materials. After washing in PBS for 30 min, the slides were mounted in Vectashield Medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratory, Burlingame, CA, USA). Images were acquired with a fluorescence microscope (Nikon, USA).

MR data analysis

Ventrolateral white matter (VLWM) area was drawn manually on the raw MR images as the region of interest (ROI) using ImageJ software (<http://rsbweb.nih.gov/ij/>) with customized tools. The boundary between white matter and cerebrospinal fluid was identified on the B0 image (no diffusion weighting) and that of white and grey matter was identified on one of the diffusion weighted image (-1,1,0). DTI-derived λ_1 , λ_2 and RA maps were used as references while defining VLWM ROIs (Fig. 2). The ROI-averaged λ_1 , λ_2 and RA were calculated for each slice from every mouse at the study end point. A linear repeated measures model was used to test the differences in λ_1 , λ_2 and RA between each pair of treatment groups within each study. Twelve tests were involved for each dependent variable (λ_1 , λ_2 and RA), and the p values were corrected for multiple testing (Tukey's HSD). The correlations between DTI-derived parameters (λ_1 and λ_2) and clinical scores were performed with a Kendall's tau correlation analysis. Data are expressed as mean $\pm$ standard deviation.

Results

After immunization, the vehicle groups followed a typical disease course: disease started at days 15 to 20 post immunization and progressed for about 10 days marked by rising clinical scores (Fig. 3a and c). In the prophylactic treatment groups, FTY720 at both doses suppressed the disease throughout the study course with the mean end-stage clinical scores of 1 ± 1 and 1 ± 2 for 3 and 10 mg/kg treated groups respectively (Fig. 3a and b, Table 1). Nevertheless, therapeutic treatment of FTY720 on EAE mice at the disease onset did not inhibit the disease progression. Hint of treatment effect could still be seen in decreased mean end-point clinical scores (without reaching statistical significance). The 3 mg/kg therapeutic regimen delayed the time reaching disease peak and lowered the mean end-stage clinical score to 2 ± 1 ; 10 mg/kg dose ameliorated disease severity and decreased mean end-stage clinical score to 2 ± 1 (Fig. 3c and d, Table 1).

At the end of each study, *in vivo* DTI was applied to exam the spinal cords at lumbar levels. DTI-derived RA, λ_1 , and λ_2 were used to assess the VLWM integrity. Three color-coded DTI maps (λ_1 , λ_2 and RA) of spinal cord L4 level are shown for a representative mouse from each of the four groups (Fig. 4a and Fig. 5a). In both studies, improvement of white matter integrity by FTY720 treatment can be appreciated on the DTI maps. Statistical analysis revealed that prophylactic treatment at 3 and 10 mg/kg was effective in improving λ_1 in VLWM (Table 1 and Fig. 4b): significant 28% and 24% increases in λ_1 were seen in 3 mg/kg and 10 mg/kg groups respectively, comparing to that of the vehicle group. Therapeutic treatment was less effective examining the mean λ_1 of FTY720 treated and vehicle treated groups (Fig. 5b). Even though a marginal 6% increase in 3 mg/kg group and a 9% increase in 10 mg/kg group were observed in λ_1 comparing to vehicle group, they were statistically indistinguishable from each other (Table 1 and Fig. 5b).

Comparing to mean λ_1 of vehicle group in the prophylactic study, a significantly 34% reduction was seen in 3 mg/kg FTY720 treatment group (Fig. 4c). Prophylactic 10 mg/kg treatment lowered mean λ_1 by 29%, but no statistical significance was detected. Therapeutically, no statistically significant effect in λ_1 was observed with either 3 mg/kg or 10 mg/kg FTY720 treatment (Table 1 and Fig. 5c).

Anisotropy index RA, was significantly improved by prophylactic FTY720 treatment (by 45% in 3 mg/kg group and 38% in 10 mg/kg group) comparing to vehicle treatment (Fig. 4d). However, RA of neither 3 mg/kg nor 10 mg/kg therapeutically treated group was significantly different from that of the vehicle treated group, despite its sensitivity to white matter change (Fig. 5d). Each mouse's mean $\parallel$ and $\perp$ of VLWM were found correlated strongly with their end-point clinical scores at all examined spinal cord levels (Table 2).

To verify *in vivo* DTI findings, immunohistochemistry (IHC) was also performed on the paraffin sections of spinal cords from representative mice at L4 level. In the prophylactic study (Fig. 6), it is apparent that the density and morphology of SMI-31-positive axons was comparable between two treated cords and the sham cord. The SMI-31-positive axon density of the vehicle treated mouse, on the other hand, was dramatically reduced (Fig. 6a). The sham cord and the 3 mg/kg FTY720 treated cord showed comparable MBP-positive myelin density in the L4 level (Fig. 6b). However, the representative mouse with 10 mg/kg dose presented a mildly decreased myelin density in its L4 cord. The vehicle treated mouse, unsurprisingly, has the lowest myelin density in the VLWM region. In the therapeutic study, a substantial reduction in SMI-31-positive axons was seen in the vehicle treated mouse cord compared with the sham mouse cord (Fig. 7a). Therapeutic FTY720 treatment at both doses improved SMI-31-positive axon density. However, loss of intact axons was noticed especially at the proximity of the meninges. Similarly, drastic reduction of MBP-positive myelin population was seen in the vehicle treated mouse cord (Fig. 7b). Therapeutic FTY720 treatment at both doses provided partial preservation of VLWM myelin, but moderate loss can still be seen in the representative cords.

Discussion

In order to monitor EAE disease progression and to evaluate the efficacy of potential axonal protective agents, spinal cord white matter integrity needs to be carefully assessed throughout the treatment course. Immunohistochemistry provides a direct and definitive diagnosis of CNS tissue pathology. However, it can only be performed at study end points due to its invasive nature, thus not suitable for monitoring the dynamic change of the underlying pathology. The 5-point clinical scoring system, as a behavior outcome measure, has been widely used to assess the neurological deficits in EAE mice on a daily basis. Nevertheless, it is not capable of differentiating between axon injury, demyelination, or inflammation underlying the neurological impairment.

Conventional relaxivity-based MRI techniques have been extensively used to detect the CNS lesions in MS patients. Gdolinium complexes enhanced T1-weighted imaging, allowing for visualization of BBB breakdown, is routinely used to monitor disease progression (30). However, the breakdown of the BBB is an indirect indicator of CNS inflammation, and to a lesser extent, reflects axon or myelin integrity. A recent study has shown that by labeling macrophages in EAE mice using ultra-small super-paramagnetic iron oxide (USPIO) particles, its population within CNS can be tracked by *in vivo* MRI (31). Nevertheless, so far none has demonstrated the capability of differentiating or quantifying the underlying axonal injury vs. demyelination (32). Thus a more pathology-specific biomarker is needed to quantify axonal damage in order to monitor disease progression and to determine the efficacy of neuronal protective agents. DTI allows the visualization of white matter structural changes resulting from axonal damage and demyelination. Herein, we demonstrate that the axonal protection of FTY720 can be noninvasively assessed by DTI-derived axial ($\parallel$) and radial diffusivity ($\perp$). Previous works in our lab using MOG-EAE model revealed that white matter $\parallel$ highly correlated with both clinical impairment and histological markers of axonal damage in EAE mice (26,33). In line with previous conclusions, our results showed that $\parallel$ strongly correlated with clinical scores (Table 2).

Meanwhile, immunohistochemistry of the representative vehicle treated cords show substantially decreased SMI-31 positive as well as MBP positive axons (Fig. 6 and Fig. 7). In prophylactic study, treatment effect was demonstrated in preservation of VLWM axons and normalization of group mean μ . In therapeutic study, axonal protective effect of FTY720 was reflected by higher mean μ in both FTY720 treated groups comparing to that of vehicle treated group. This improvement in μ was supported by higher SMI-31 positive axon density seen in the treated cords than in the untreated cord.

In current study, μ was also found highly correlated to EAE clinical scores. This strong correlation could reflect conduction deficits resulting from both demyelination and axon damage. It is possible that myelin damage reflected by increased μ was secondary to axon injury, which is the primary cause of the neurological impairment. Significantly lowered μ (suggesting improved myelin integrity) was observed in prophylactic 3mg/kg treatment group. Even though without reaching a statistical significance, prophylactic 10 mg/kg treatment group and both therapeutic treatment groups also improved mean μ comparing to vehicle treatment groups. Consistent with this observation, higher density of MBP-positive myelin was seen in the representative cords from prophylactically and therapeutically treated groups compared to that in the untreated EAE mice cords. This result suggests that FTY720 also preserves myelin integrity of the EAE mice examined in the present study, although a direct effect has yet to be determined. Results also support that DTI-derived μ could be a suitable myelin integrity biomarker.

Spinal cord lesions are common in MS patients leading to the impairment of sensorimotor functions (34). Previous MRI examinations of MS patients have shown the correlation between their spinal cord cross-sectional area and impairment assessed by Expanded Disability Status Scale (EDSS) (35,36). With the development of phased array coils and fast diffusion weighted sequences, DTI has shown promise for monitoring MS-affected human spinal cord (37–41). Diffusion anisotropy (fractional and relative anisotropy) and mean diffusivity (apparent diffusion coefficient) were frequently assessed as overall white matter integrity marker and successfully detected abnormalities in normal appearing white matter (NAWM) (38,41–44). Directional diffusivity (μ and μ) has been used to differentiate axonal damage versus demyelination (38,44,45). The capability of DTI to detect abnormalities in NAWM supports its use as a potential quantitative diagnostic modality for MS. Current findings further support that DTI may be used to detect occult spinal cord pathology, correlating with sensorimotor impairment, and assess therapeutic effect in MS.

The full mechanism of FTY720 action in MS is so far still not known with certainty. The predominant view is that acting as a functional antagonism of S1P, FTY720 inhibits egress of lymphocytes from lymph nodes and their recirculation, thus protects CNS from lymphocytic infiltration and attack (16,46). These immunologic effects were thought to account for the reduced acute relapses, MRI lesion activity, and disability progression in clinical trials (6,8). In fact, within the identified S1P receptors family, S1P_{1–3} are ubiquitously expressed, S1P₄ is primarily expressed in lymphoid cells, and S1P₅ is expressed in the spleen and CNS. The phosphorylated FTY720 binds 4 of 5 S1P receptors (S1P₁, S1P_{3–5}) with high affinity (47). As a result, the non-specific interaction between FTY720 and S1P receptors mediated a wide range of biological functions in the CNS, immune and cardiovascular system. (48) Recent studies suggested that FTY720 penetrates BBB (13) and the phosphorylated FTY720 formed *in situ* might have effects on astrocytes, microglia, and oligodendrocyte progenitor cells (OPC) via S1P mediated pathways (49,50). It is also suggested that S1P plays important roles in regulation of neuronal growth, differentiation, survival, and function (51), even though few studies indicated direct action of FTY720 on neuron cells. In a recent study on a cuprizone-induced, non T-cell mediated

MS model, FTY720 was found to attenuate injuries to oligodendrocytes and myelin *in vivo*, through protection mechanism independent of its effect on lymphocyte trafficking (19).

In the therapeutic study, slightly better functional outcome marked by lower end-point CS (without statistical significance) was seen in 10 mg/kg group comparing to 3 mg/kg group. Similar finding was also reported by a study using the same EAE model and treatment regimen (11). However, in the prophylactic study, a better treatment outcome was seen in 3 mg/kg group indicated by both function measurement and DTI biomarkers. This could arise from the fact that the effect of phosphorylated FTY720 on differentiation of OPC (52) is concentration dependent. More specifically, the differentiation of OPC into oligodendrocytes was stimulated by low concentrations of phosphorylated FTY720 (53), but inhibited by high concentration of phosphorylated FTY720 (54–56). In therapeutic study, this “toxicity” of higher dosage may be reduced by shortened treatment duration.

The modulation of S1P receptors by FTY720 in both the immune system and CNS could produce a combined beneficial anti-inflammatory and potential neuronal protective effects contributing to its treatment efficacy in MS. Even though the exact mechanism of action of FTY720 is yet to be determined, the current report demonstrated the feasibility of employing *in vivo* DTI to assess the white matter integrity during the course of treatment.

In summary, we have demonstrated that *in vivo* DTI is readily applicable to monitor treatment effect of EAE mice using FTY720 as the example. Our results showed that the improvement of axon and myelin integrity towards the control state in both prophylactic and therapeutic studies is consistent with recent *in vivo* findings suggesting CNS protection effect of FTY720 (10–13). The excellent linear correlations between DTI metrics ($\parallel$ and $\perp$) and clinical scores again indicate the potential of DTI derived biomarkers of axon and myelin integrity as an outcome measure for assessing the efficacy of therapeutic interventions in EAE. The histology results reflect our *in vivo* findings, supporting the sensitivity and specificity of DTI metrics for EAE pathologies.

Acknowledgments

This study was supported in part by funding from the National Institutes of Health R01-NS047592, P01-NS059560, and a Pfizer Washington University Collaborative Project: 009756-0001.

Abbreviations

ADC	apparent diffusion coefficient
BBB	blood-brain-barrier
CNS	central nerve system
DTI	diffusion tensor imaging
EAE	experimental autoimmune encephalomyelitis
FA	fractional anisotropy
MOG	myelin oligodendrocyte glycoprotein
MD	mean diffusivity
MS	multiple sclerosis
NAWM	normal appearing white matter
RRMS	relapsing-remitting MS

RA	relative anisotropy
ROI	region of interest
S1P	sphingosine 1-phosphate
VLWM	ventrolateral white matter

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Figure 1. Sagittal and coronal scout images of mouse vertebral column

Mouse vertebral discs can be seen on the sagittal image while ribs can be seen on the coronal images. Discs and ribs were used as references to determine target axial image plan.

Figure 2. Region of interest (ROI) of spinal cord ventrolateral white matter (VLWM). ROIs of spinal cord L4 level were shown on B0 image, diffusion-weighted (DW) image, map, $\lambda_{\parallel}$ map and RA map. The boundary between white matter and cerebrospinal fluid was identified on the B0 image (no diffusion weighting) and that of white and grey matter was identified on one of the raw diffusion weighted image (-1,1,0). DTI-derived $\lambda_{\parallel}$, $\lambda_{\perp}$ and RA maps were used as references while defining VLWM ROIs.

Figure 3. EAE disease course (a, prophylactic and c, therapeutic) and group-averaged clinical score at end point (b, prophylactic and d, therapeutic)

In prophylactic study, FTY720 in both doses prevented the EAE onset and suppressed the disease throughout the course of study (a). Mean clinical scores of both treated groups decreased to values less than 1 without statistical significance (b). In therapeutic study, 3 mg/kg treatment after the disease onset delayed the disease peak while 10 mg/kg treatment ameliorated the disease severity (c). Lower mean CS was also seen in both treatment groups (without reaching statistical significance). Open circle: non-treated EAE group; open square: FTY720 3 mg/kg group; open triangle: FTY720 10 mg/kg group. Error bar represents standard error in Figure 3a and 3c, standard deviation in Figure 3b and 3d.

Figure 4. DTI parameter maps and values of each group in prophylactic study end point
 Color-coded DTI maps of spinal cord L4 level are shown for a representative mouse from each of the four groups (a). Improvement in VLWM integrity was seen in treated mice cords marked by normalized $\lambda_{||}$, $\lambda_{\perp}$ and RA. Group-averaged $\lambda_{||}$ (b), $\lambda_{\perp}$ (c) and RA (d) show the same trend. Mean RA and $\lambda_{||}$ of both FTY720 treated groups are significantly higher than that of vehicle treated group ($P < 0.05$). Mean $\lambda_{\perp}$ of 3 mg/kg treatment group is significantly lower than that of vehicle treated group ($P < 0.05$). The 10 mg/kg treatment group mean does not show statistical difference. No statistical difference exists between control group and either of the FTY720 treated groups in RA, $\lambda_{||}$, or $\lambda_{\perp}$. Error bar represents standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Figure 5. DTI parameter maps and values of each group in therapeutic study end point
 Color-coded DTI maps of spinal cord L4 level are shown for a representative mouse from each of the four groups (a). Treated groups still shown increased $\parallel$, increased RA, and decreased $\perp$ in the VLWM region compared to those of vehicle treated EAE group (a). However, no statistical improvement was detected in either FTY720 treated group in $\parallel$ (b), (c), or RA (d), ($P > 0.05$). Error bar represents standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Figure 6. SMI-31 (a) and MBP (b) staining of L4 spinal cord sections from representative mice in prophylactic study

Substantially decreased SMI-31-positive axons (a) and MBP-positive myelin rings (b) can be seen in the vehicle treated mouse cord. The density and morphology of SMI-31-positive axons in both representative FTY720 treated cords resemble those in sham cord (a).

Comparable myelin density between sham mouse cord and FTY720 3mg/kg treated mouse cord can also be seen. On the other hand, the 10 mg/kg treated cord show mild decrease in myelin density (b). Scale bar: 50 μm .

Figure 7. SMI31 (a) and MBP (b) staining of L4 spinal cord sections from representative mice in therapeutic study

Drastic loss of SMI31-positive axons (a) and MBP-positive myelin rings (b) can be observed in the vehicle treated mouse cords. Therapeutic treatment at both doses helped preserving axon density in the VLWM (a). However, axon loss proximity to the meninges can still be seen in 3 and 10 mg/kg treated cords (a). Similarly, FTY720 treated cords show partially improved myelin density comparing to vehicle treated cord (b). Scale bar: 50µm.

Mean $\bar{\mu}$, RA, and clinical scores (CS) of each group in prophylactic and therapeutic studies.

Table 1

Studies	Groups	Mean $\bar{\mu}$ $\pm$ STD ($\mu\text{m}^2/\text{ms}$)	Mean $\bar{\epsilon}$ $\pm$ STD ($\mu\text{m}^2/\text{ms}$)	Mean RA $\pm$ STD	Mean CS $\pm$ STD
Prophylactic	Sham (n = 5)	1.78 $\pm$ 0.09	0.27 $\pm$ 0.02	0.94 $\pm$ 0.04	0 $\pm$ 0
	3 mg/kg (n = 6)	1.70 $\pm$ 0.13	0.27 $\pm$ 0.03	0.93 $\pm$ 0.06	0.67 $\pm$ 1.40
	10 mg/kg (n = 6)	1.65 $\pm$ 0.24	0.29 $\pm$ 0.07	0.88 $\pm$ 0.16	0.83 $\pm$ 1.60
	Vehicle (n = 5)	1.33 $\pm$ 0.05	0.41 $\pm$ 0.06	0.64 $\pm$ 0.08	2.90 $\pm$ 1.47
Therapeutic	Sham (n = 5)	1.76 $\pm$ 0.05	0.25 $\pm$ 0.02	0.97 $\pm$ 0.02	0 $\pm$ 0
	3 mg/kg (n = 7)	1.55 $\pm$ 0.08	0.34 $\pm$ 0.05	0.80 $\pm$ 0.08	2.36 $\pm$ 0.69
	10 mg/kg (n = 5)	1.59 $\pm$ 0.08	0.35 $\pm$ 0.08	0.81 $\pm$ 0.11	1.80 $\pm$ 1.30
	Vehicle (n = 7)	1.46 $\pm$ 0.17	0.42 $\pm$ 0.09	0.68 $\pm$ 0.15	3.07 $\pm$ 0.61

Table 2Correlation between $\ddot{e}_{||}$ and of each spinal cord level and clinical scores

Vertebral level (Spinal cord level)	T11 (L1)	T12 (L2)	T13 (L3)	L1 (L4)
$\ddot{e}_{ }$ and CS correlation coefficient	0.70	0.71	0.74	0.79
(P value)	(<0.0001)	(<0.0001)	(<0.0001)	(<0.0001)
$\ddot{e}_{\perp}$ and CS correlation coefficient	-0.72	-0.74	-0.73	-0.69
(P value)	<0.0001	<0.0001	<0.0001	<0.0001