

## Brain Penetration of the Oral Immunomodulatory Drug FTY720 and Its Phosphorylation in the Central Nervous System during Experimental Autoimmune Encephalomyelitis: Consequences for Mode of Action in Multiple Sclerosis

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### ABSTRACT

FTY720 [2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride] is an oral sphingosine-1-phosphate receptor modulator under development for the treatment of multiple sclerosis (MS). The drug is phosphorylated *in vivo* by sphingosine kinase 2 to its bioactive form, FTY720-P. Although treatment with FTY720 is accompanied by a reduction of the peripheral lymphocyte count, its efficacy in MS and experimental autoimmune encephalomyelitis (EAE) may be due to additional, direct effects in the central nervous system (CNS). We now show that FTY720 localizes to the CNS white matter, preferentially along myelin sheaths. Brain trough levels of FTY720 and

FTY720-P in rat EAE are of the same magnitude and dose dependently increase; they are in the range of 40 to 540 ng/g in the brain tissue at efficacious doses and exceed blood concentrations severalfold. In a rat model of chronic EAE, prolonged treatment with 0.03 mg/kg was efficacious, but limiting the dosing period failed to prevent EAE despite a significant decrease in blood lymphocytes. FTY720 effectiveness is likely due to a culmination of mechanisms involving reduction of autoreactive T cells, neuroprotective influence of FTY720-P in the CNS, and inhibition of inflammatory mediators in the brain.

FTY720 is an oral sphingosine-1-phosphate (S1P) receptor modulator (Baumruker et al., 2007) under development for the treatment of multiple sclerosis (MS), representing the first of a new class of immunomodulatory agents. Promising results in phase II trials with relapsing MS patients (Kappos et al., 2006) mirror the striking efficacy of FTY720 in MS models of experimental autoimmune encephalomyelitis (EAE), shown by preventive and therapeutic treatment (Brinkmann et al., 2002; Fujino et al., 2003; Webb et al., 2004; Kataoka et al., 2005; Balatoni et al., 2007). FTY720 is converted *in vivo* to its biologically active phosphate ester

metabolite (FTY720-P), which acts as a high-affinity agonist for four of the five known G-protein-coupled S1P receptors, namely S1P<sub>1</sub> and S1P<sub>3-5</sub> (Brinkmann et al., 2002; Mandala et al., 2002). Sphingosine kinase (SPHK) 2 is the primary enzyme required for FTY720-P formation, as we and others subsequently confirmed in SPHK2 knockout mice (Kharel et al., 2005; Zemann et al., 2006). The fact that SPHK1 null mice become lymphopenic after FTY720 administration further supports the view that SPHK2 is sufficient for the functional activation of FTY720 (Allende et al., 2004).

Emerging evidence suggests that the effectiveness of FTY720 in the central nervous system (CNS) extends beyond immunomodulation to encompass other aspects of MS pathophysiology, including an influence on the blood-brain barrier and glial repair mechanisms that could ultimately contribute to restoration of nerve function (Baumruker et al., 2007;

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**ABBREVIATIONS:** FTY720, fingolimod, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; SPHK, sphingosine kinase; CNS, central nervous system; DA, Dark Agouti; CFA, complete Freund's adjuvant; CsA, cyclosporine A; QWBA, quantitative whole-body autoradiography; CSF, cerebrospinal fluid; ANOVA, analysis of variance; AUC, area under the curve; FTY720-P, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol-1-(dihydrogen phosphate); FK506, tacrolimus.

Jung et al., 2007; Osinde et al., 2007). A key consideration behind this concept is the finding that FTY720 distributes to the brain (Meno-Tetang et al., 2006), which contains endogenous SPHK2 for the phosphorylation of FTY720 (Billich et al., 2003). Moreover, neurons and glial cells (astrocytes, microglia, oligodendrocytes) in the brain differentially express S1P receptors (Fig. 1), thus raising the possibility for receptor activation *in situ* by FTY720-P. So far, there is no information on the presence of FTY720-P in the brain or potential concentrations therein. Our primary aim was to investigate the distribution of FTY720 and its phosphorylated form in the CNS after clinically relevant doses in two different EAE models. We provide preclinical evidence that the bioactive metabolite FTY720-P distributes to the CNS white matter, suggesting the potential for functional interaction with glial cells bearing S1P receptors in the brain and spinal cord.

## Materials and Methods

**Animals.** For EAE, female RT1<sup>1</sup> Lewis rats from Charles River (Sulzfeld, Germany) and Dark Agouti (DA) rats from Harlan Winkelmann (Borchen, Germany) were kept under standardized light- and climate-controlled conditions with free access to food and water. Age-matched rats were acclimatized for at least 1 week before distribution into the experimental groups. For autoradiography, male pigmented LE/CR WIGA rats (Charles River; 198–238 g) were housed individually in metabolism cages. All experiments conformed to Novartis animal care regulations and were approved by the Austrian and Swiss health authorities in compliance with international animal welfare standards according to the European Communities Council Directive and the guidelines set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

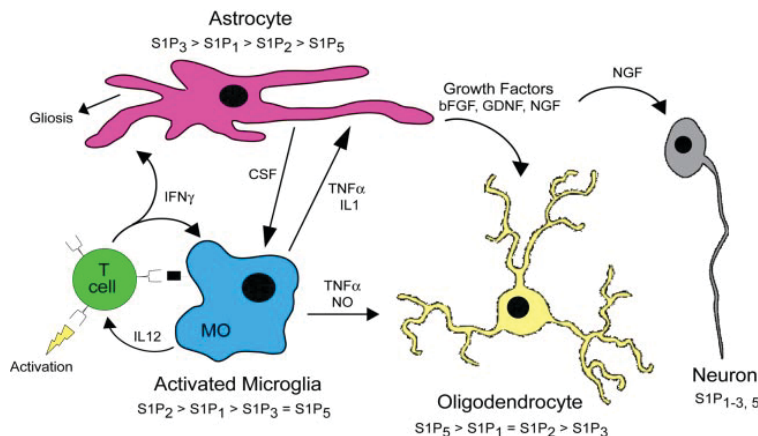
**EAE Induction and Clinical Scoring.** Animals were lightly anesthetized by isoflurane inhalation (0.5% Forane; Abbott Laboratories, Vienna, Austria) and given a single intradermal 200- $\mu$ l inoculation in the dorsal base of the tail root. The immunization mixture for DA rats consisted of syngeneic CNS antigen (4 parts brain to 6 parts of spinal cord) in phosphate-buffered saline emulsified 1:1 in incomplete Freund's adjuvant supplemented with 200  $\mu$ g of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; DIFCO, BD Diagnostics, Oxford, UK); the adjuvant is henceforth referred to as complete Freund's adjuvant (CFA). For Lewis rats, the inoculum contained guinea pig spinal cord in phosphate-buffered saline emulsified 1:1 in CFA. As an adjuvant control for all EAE studies, animals were injected with CFA alone and vehicle-treated. The rats were weighed every other day and scored daily for neurological signs as

follows: 0, no clinical deficit; 1, complete loss of tail tonus; 2, limb weakness or ataxia; 3, full paralysis of hind or forelimbs; or 4, tetraparalysis or moribund. Animals with a score of 4 were sacrificed if weight loss indicated little chance of recovery, in accordance with animal welfare standards. Mortality due to sacrifice or spontaneous EAE-related death was indicated ( $\dagger$ ) and recorded as a 4 on the given day; this death score continued to be included in the clinical assessment, but body weight measurements were not carried forward.

**Test Compounds for *In Vivo* Evaluation.** Unlabeled and <sup>14</sup>C-labeled FTY720, as well as cyclosporine A (CsA), were supplied by Novartis Pharma AG (Basel, Switzerland). The radiochemical purity of [<sup>14</sup>C]FTY720, which was labeled in position 2, was shown by high-pressure liquid chromatography to be 98% with a specific activity of 35  $\mu$ Ci/mg. FTY720 was dissolved in water, and CsA was dosed in the Neoral vehicle. Both drugs were freshly prepared and given *p.o.* once daily by gavage at a dosing volume of 5 ml/kg body weight. For prophylactic and therapeutic treatment, oral dosing started on day 0 at immunization and at the peak of disease in fully established EAE, respectively.

**Peripheral Leukocyte Counts.** Rats were lightly anesthetized by isoflurane inhalation and 100  $\mu$ l of blood from the retro-orbital venous plexus was collected in EDTA-coated tubes (Sarstedt AG, Nümbrecht, Germany). Automated differential leukocyte analysis was performed on the HESKA Vet ABC-Diff Hematology Analyzer (Heska Corp., Fort Collins, CO).

**Autoradiography of <sup>14</sup>C-Labeled FTY720.** Quantitative whole-body autoradiography (QWBA) and light microscopic autoradiography were performed to assess the uptake and tissue distribution of [<sup>14</sup>C]FTY720 radioactivity in male pigmented rats ( $n = 6$ ) following seven oral doses at 7.5 mg/kg/d. At 8, 24, and 168 h after the last dose, the animals were deeply anesthetized with isoflurane and submerged in a dry ice-hexane bath at  $-70^{\circ}\text{C}$  for at least 20 min. The frozen carcasses were rapidly shaven and stored below  $-20^{\circ}\text{C}$  until embedment in an ice-cold aqueous solution of 2% carboxymethylcellulose. They were then frozen for approximately 30 min in a dry ice-hexane mixture at  $-70^{\circ}\text{C}$ , followed by an overnight stabilization at  $-20^{\circ}\text{C}$ . Lengthwise sections (40  $\mu$ m thick) were obtained in a cryomicrotome (Leica Microsystems, Nussloch, Germany) at  $-20^{\circ}\text{C}$ . Whole-body autoradiograms were obtained by autoradioluminography. Briefly, sections with a paper backing were placed on Fuji BASIII imaging plates (Fuji Photo Film, Tokyo, Japan) for 1 day at room temperature in a lead shielding box. After exposure (detection of approximately 1.5 dpm/mg), the imaging plates were first kept in the dark for 3 to 5 min and then transferred to a Fuji BAS 2000 TR phosphorimaging device (Fuji Photo Film) for scanning at a 100- $\mu$ m step with a 1024 gradation. Images were prepared by re-exposing the sections onto Super Resolution storage phosphor screens (PerkinElmer, Shelton, CT) for 1 day at room temperature and



**Fig. 1.** Cartoon depicting hierarchy of S1P receptor expression on rat glial subpopulations (Rao et al., 2003; Tham et al., 2003; Toman et al., 2004; Yu et al., 2004): implications for FTY720-P-mediated repair in the CNS. bFGF, basic fibroblast growth factor; CSF, colony-stimulating factor; GDNF, glial-derived neurotrophic factor; IFN $\gamma$ , interferon  $\gamma$ ; IL1, interleukin-1; IL12, interleukin-12; M $\phi$ , macrophage/microglia; NGF, nerve growth factor; NO, nitric oxide; TNF $\alpha$ , tumor necrosis factor  $\alpha$ .

scanning at a 42- $\mu$ m step (Cyclone PhosphorImager; Packard Instrument, Meriden, CT). The image files were processed using Photoshop Elements 2.0 software (Adobe Systems, San Jose, CA). Levels of radioactivity in the tissues were determined by comparative densitometry, as described previously (Schweitzer et al., 1987).

For light microscopy, brain and spinal cord samples from all animals were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 days at 4°C. Postfixation was performed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C. The tissues were dehydrated in graded acetone solutions and embedded in Epon. Semithin sections were dipped in Ilford L4 emulsion (Ilford, Moberley, Cheshire, UK) in the dark at 4°C. Dipped sections were developed in Kodak D19 (Eastman Kodak, Rochester, NY) after 31 weeks exposure, stopped in distilled water, fixed in Ilford Hypam rapid fixer (Ilford), and counterstained with toluidine blue. Light microscopic examination of sections was performed independently by three pathologists. Labeling was identified by more silver grains over the cells than the background without tissue.

**Quantification of FTY720 and FTY720-P in the Blood, Brain, and Cerebrospinal Fluid.** Plasma, whole blood, brain, and cerebrospinal fluid (CSF) were collected from EAE rats at 24 h after the last FTY720 dose to obtain trough levels. Concentrations of FTY720 and FTY720-P were determined by high-pressure liquid chromatography (Agilent 1100; Agilent, Waldbronn, Germany) with mass spectrometric detection as described previously for serum and other tissues (Zemann et al., 2006). For measurements in plasma, heparinized blood, or CSF, 20- to 100- $\mu$ l aliquots were spiked with internal standards (final concentration, 0.5  $\mu$ g/ml) and extracted with chloroform/methanol at acidic pH; extracts were dried and reconstituted in methanol/0.2% formic acid. Samples were chromatographed on a Luna C8 column (3  $\mu$ , 2  $\times$  50 mm; Phenomenex, Torrance, CA) equipped with a C4 wide-bore precolumn. The analytes were eluted with a gradient (eluent A, 10 mM ammonium acetate containing 0.08% HCOOH in water; eluent B, 10 mM ammonium acetate containing 0.08% HCOOH in MeOH; 50–98% B in 14 min) at a flow of 0.4 ml/min at 40°C. Analytes were detected by electrospray-ionization liquid chromatography with tandem mass spectrometry using an API 4000 QTrap instrument (MDS Sciex, Concord, ON, Canada). The optimal collision energies for FTY720 and FTY720-P were 23 and 25 V, respectively. The multiple reaction monitoring transitions monitored for FTY720 and FTY720-P were *m/z* 308/255 and 388/255, respectively.

For CNS determination, half of a rat brain (approximately 750 mg) was emulsified 1:1 in 2 ml of MeOH/H<sub>2</sub>O (1:1) with a glass homogenizer (Potter S; Braun, Melsungen, Germany). The homogenate was divided for separate determination of FTY720 and FTY720-P, then aliquots were spiked with their respective internal standards (35  $\mu$ l) to give a final concentration of 0.5  $\mu$ g/ml. Methanol (400  $\mu$ l) was added to 0.5 ml of homogenate, followed by 20 min of mixing on a

rotary shaker and 5 min of sonication at room temperature. Supernatants were harvested after centrifugation at 12,000g; the pellet was extracted once with 200  $\mu$ l of methanol, then the supernatant was harvested after centrifugation. Combined supernatants were subjected to solid-phase extraction using STRATA-X-C 33  $\mu$ m Cation Mixed-Mode Polymer Phase columns (Phenomenex). The columns were eluted with 3 ml of MeOH/1% NH<sub>4</sub>OH. The eluate was dried, its residue was dissolved in a 35:65 mixture of eluents A and B (see above), and then samples were chromatographed as described above.

An alternative method for blood or homogenized brain comprised two extractions with acetonitrile/methanol/ethyl acetate (5:3:2), dissolution of the residue of the evaporated organic phase in methanol/water/ammonia (50:48.75:1.25), and chromatography on a Zorbax SB-C8 0.5- $\times$  75-mm column (3.5  $\mu$ m; Agilent Technologies) at 50°C with a linear gradient from 0 to 95% B within 10 min at 20  $\mu$ l/min (solvent A, 0.2% HCOOH in water; B, 0.2% HCOOH in acetonitrile). In this case, a Micromass Micro triple quadrupole mass spectrometer with electrospray source (Waters AG, Rapperswil, Switzerland) was used, and multiple reaction monitoring transitions were monitored for FTY720 and FTY720-P at *m/z* 308/105 and 388/255, respectively. Both analytical methods yielded identical results.

**Statistical Analysis.** A one-way analysis of variance (ANOVA) was used to compare all data sets using SigmaStat for Windows, version 3.11 (Systat Software Inc., Richmond, CA). Differences between groups were analyzed using the post hoc Tukey test for pairwise multiple comparison. For EAE, area under the curve (AUC) values for body weight loss and clinical grade scores were evaluated during the entire prophylactic treatment period or after the initiation of therapeutic dosing. Probabilities (*p*)  $\leq$  0.05 were considered to be statistically significant.

## Results

**FTY720 Provides Sustained Protection in EAE.** Two-week therapeutic treatment with 0.03 to 0.9 mg/kg FTY720 dose dependently inhibited progression of established disease in the DA rat model of chronic EAE compared with vehicle controls, which exhibited sustained neurological deficits throughout the 2-month observation (Table 1; Fig. 2). Evidence for rapid and full disease suppression was consistently observed after administration of 0.3 mg/kg FTY720, whereas the minimum effective dose providing almost complete protection even 1 month after discontinuation was 0.1 mg/kg (*p* = 0.00002). A plateau in efficacy appeared to be reached by 0.3 mg/kg since there was no difference in the cumulative disease score between this dose and the 3-fold higher one of 0.9 mg/kg (Table 1). The very low dose of 0.03

TABLE 1  
FTY720 therapeutic treatment effect in DA rat model of EAE<sup>a</sup>

Treatment Groups: Oral Dose: from Days 12 to 25	EAE Onset <sup>b</sup>		Maximum Weight Loss <sup>c</sup>		Maximum Disease <sup>d</sup>		Cumulative Disease Score from Days 13 to 56 <sup>e</sup>	
	Day	%	Day	Score	Day	AUC	<i>p</i> vs. Vehicle	
<i>mg/kg</i>								
Vehicle ( <i>n</i> = 17)	8.4 $\pm$ 0.4	23.6 $\pm$ 0.02	20.7 $\pm$ 0.7	2.9 $\pm$ 0.2	22.6 $\pm$ 1.5	79.0 $\pm$ 12.6		
FTY720, 0.03 mg ( <i>n</i> = 8)	8.8 $\pm$ 0.3	16.4 $\pm$ 0.03	21.9 $\pm$ 1.0	2.3 $\pm$ 0.4	28.5 $\pm$ 2.6	64.0 $\pm$ 14.7		N.S.
FTY720, 0.1 mg ( <i>n</i> = 19)	8.4 $\pm$ 0.3	12.0 $\pm$ 0.02	20.4 $\pm$ 0.2	1.2 $\pm$ 0.3	24.0 $\pm$ 1.9	17.0 $\pm$ 3.7		0.00002
FTY720, 0.3 mg ( <i>n</i> = 19)	9.0 $\pm$ 0.3	9.6 $\pm$ 0.01	20.2 $\pm$ 0.2	0.3 $\pm$ 0.1	24.2 $\pm$ 2.0	7.8 $\pm$ 2.5		<0.00001
FTY720, 0.9 mg ( <i>n</i> = 9)	8.3 $\pm$ 0.2	15.4 $\pm$ 0.02	20.2 $\pm$ 0.1	0.6 $\pm$ 0.4	25.7 $\pm$ 3.7	7.7 $\pm$ 3.3		0.0005
CsA, 25 mg ( <i>n</i> = 18)	9.0 $\pm$ 0.3	16.9 $\pm$ 0.02	28.4 $\pm$ 2.4	3.3 $\pm$ 0.1	33.9 $\pm$ 0.6	52.8 $\pm$ 6.7		N.S.

*n*, number of rats per group; N.S., not significant.

<sup>a</sup> Syngeneic antigen-induced EAE (Fig. 2); data shown as mean  $\pm$  S.E.M.

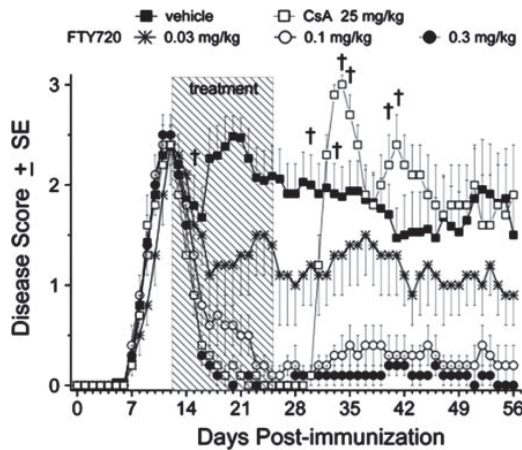
<sup>b</sup> Based on disease score  $\geq$  1.

<sup>c</sup> Highest weight loss starting 1 week after dosing (days 20–56) compared with adjuvant control.

<sup>d</sup> Initial peak of disease severity starting 1 week after dosing (days 20–56).

<sup>e</sup> Level of significance (*p*) determined by ANOVA of AUC values for clinical scores from days 13 to 56 compared with the positive control.





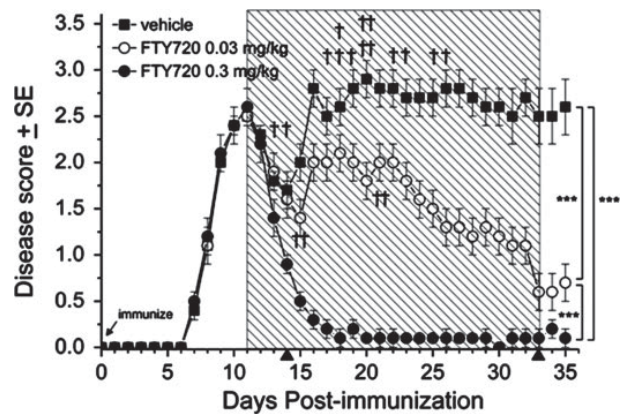
**Fig. 2.** Sustained efficacy of FTY720 after cessation in DA rat model of EAE. Representative disease course in syngeneic antigen-induced EAE in DA rats, depicted by the vehicle control (■, *n* = 17) and shown as mean score ± S.E. Oral therapeutic dosing started at the peak of established disease on day 12 and continued for 2 weeks. CsA at 25 mg/kg (□; *n* = 18) suppressed EAE signs during treatment, but animals became severely paralyzed, and 22% died (†) upon drug discontinuation. In sharp contrast, FTY720 at 0.1 (○; *n* = 19) and 0.3 mg/kg (●; *n* = 19) significantly prevented wasting and recurrence of neurological deficits, as detailed in Table 1. Although 0.03 mg/kg FTY720 (\*; *n* = 8) tended to reduce the disease burden, it was not statistically different to the vehicle (Table 1). Nevertheless, this very low dose as well as the other FTY720 treatments completely protected against EAE-related deaths, compared with three in the vehicle (days 15, 30, and 42) and four in CsA-treated animals (days 34, 35, 40, and 41).

mg/kg also tended to diminish the overall disease burden and prevent a marked rebound. In contrast, severe paralysis reoccurs following cessation of classic immunosuppressive agents, such as CsA at 25 mg/kg (Fig. 2) or FK506 at 4 mg/kg (data not shown), that were used as reference compounds. All doses of FTY720 completely prevented moribund incidence compared with 18 and 22% with vehicle and CsA, respectively (Fig. 2).

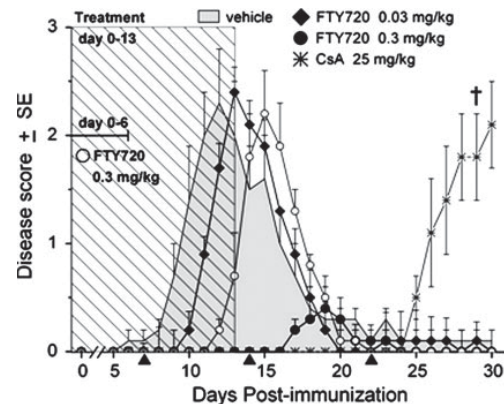
**Prolongation of Low-Dose FTY720 Steadily Reduces EAE Signs after Therapeutic Treatment.** To further explore the long-term efficacy of low-dose FTY720 in a more clinically relevant setting, 0.03 to 0.3 mg/kg was administered for 3 weeks in DA rats with established EAE (Fig. 3). By the last week of therapeutic treatment, three different studies consistently demonstrated a significant decrease in disease signs with 0.03 mg/kg versus the vehicle. Higher doses of 0.1 mg/kg (data not shown) and 0.3 mg/kg were even more efficacious. Moreover, the mortality rate was markedly reduced with FTY720 at 0.03 mg/kg (8.9% death), with complete protection at 0.1 and 0.3 mg/kg compared with 29.2% deaths in the positive control.

In contrast, 2-week prophylactic treatment with 0.03 mg/kg FTY720 in the Lewis rat model of acute EAE merely delayed the onset of paralysis by approximately 1 day but had no protective effect on disease development (Fig. 4). Increasing the 2-week dose to 0.3 mg/kg markedly prevented neurological deficits during the 1-month study (*p* = 0.0003); however, restricting the treatment to days 0 to 6 failed to stop EAE induction, with clinical signs appearing 1 week after drug cessation.

**Influence of FTY720 on Circulating Lymphocyte Counts during EAE.** Given that the above disparities in



**Fig. 3.** Prolonged therapeutic treatment with low-dose FTY720 progressively reduces EAE burden in DA rats. Change in clinical scores, along with EAE-related deaths (†), during the disease course in DA rats immunized with syngeneic CNS antigens. Data were pooled from three EAE studies and expressed as mean ± S.E. Animals received daily treatment p.o. with vehicle (■, *n* = 48) or FTY720 for 3 weeks, starting from day 11. Prolongation of 0.03 mg/kg FTY720 dosing (○, *n* = 45) gradually reduced the EAE burden and weight loss such that animals no longer exhibited ataxia or limb paralysis (*p* < 0.001), in contrast with vehicle controls, which displayed sustained disease throughout the 5-week observation period. As expected, the 0.3 mg/kg dose (●; *n* = 33) rapidly suppressed the EAE grade to a level near baseline, similar to the adjuvant controls that were injected with CFA alone and vehicle-treated (data not shown). Both doses of FTY720 profoundly prevented mortalities, except for 4 in the 0.03 mg/kg group (days 15 and 21), compared with 14 in the vehicle (days 13, 14, 17, 18, 19, 20, 22, 23, 25, and 26). Level of significance was determined by ANOVA of AUC values from days 12 to 33 versus the positive control. Blood was collected on days 14 and 33 (▲) for differential leukocyte analysis, as shown in Fig. 5B. \*\*\*, *p* < 0.001.



**Fig. 4.** Efficacy of FTY720 prophylaxis in EAE is more dependent on dose and duration compared with therapeutic treatment. Clinical scores ± S.E. in Lewis rat model of EAE (nine animals per group) showing typical monophasic disease in the vehicle control (■). Preventive therapy with FTY720 was effective when 0.3 mg/kg was given for 2 weeks (●) but not when dosing was restricted to the first 7 days (○). A 10-fold lower dose of 0.03 mg/kg (◆) failed to prevent disease development. As expected, 25 mg/kg CsA (\*) was fully protective during treatment, yet paralysis occurred almost 2 weeks after drug cessation, resulting in a death (†). Blood was collected at 0, 6, and 24 h and on 3 subsequent days (▲) for differential leukocyte analysis, as shown in Fig. 5A.

EAE-efficacy appeared to be less related to the FTY720 dose but more to its duration, we sought to investigate the temporal relationship between peripheral lymphocyte counts and EAE treatment regimens. Earlier studies showed that

0.03 and 0.3 mg/kg FTY720 can decrease the peripheral lymphocyte count by approximately 20 and 70%, respectively, within 6 h after a single oral dose in naive Lewis rats (Brinkmann et al., 2002), resulting in an ED<sub>50</sub> of 0.09 ± 0.01 mg/kg by 48 h. Likewise during EAE, 0.3 mg/kg FTY720 already reached maximum reduction (approximately 90%) of lymphocytes by 6 h in the Lewis rat (Fig. 5A). Furthermore, it was already recognized that daily doses of 0.03 mg/kg FTY720 for 1 week can reduce lymphocyte counts by up to 80% compared with placebo-treated Lewis rats in a heart allograft model (Nikolova et al., 2000). We have extended these findings to EAE and demonstrate that 0.03 mg/kg therapeutic dosing for 3 weeks in DA rats leads to a highly significant reduction in circulating lymphocytes by days 14 and 33 versus vehicle (Fig. 5B), i.e., 52 and 67%, respectively. At the early time point, 0.3 mg/kg FTY720 led to over twice the reduction in lymphocyte numbers compared with 0.03 mg/kg (*p* < 0.001), but by day 33, there was no difference between these two doses (Fig. 5B).

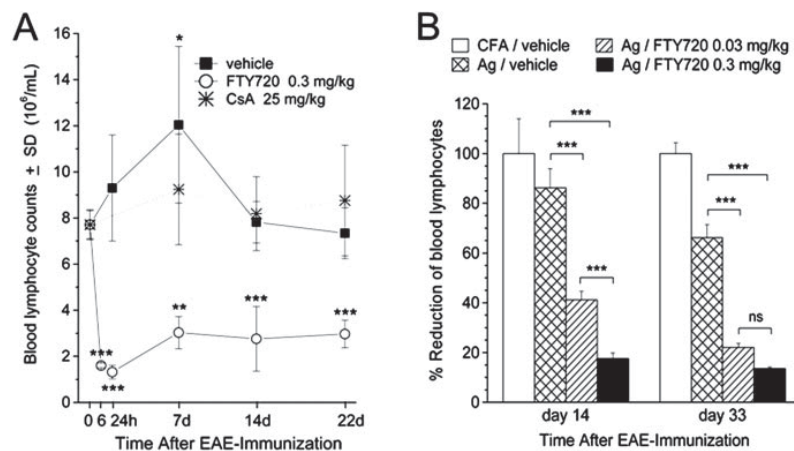
Taking into account that 7-day preventive dosing with 0.3 mg/kg FTY720 failed to suppress EAE (Fig. 4), yet lymphocytes were reduced by 75% versus vehicle (Fig. 5A), we agree with previous suggestions (Webb et al., 2004) that FTY720 or its phosphate are apt to exert additional effects beyond the induction of peripheral lymphopenia. For example, although 0.03 mg/kg FTY720 decreased the circulating lymphocytes by at least 50% shortly after drug initiation (Fig. 5B), this low dose failed to significantly prevent EAE signs when treatment is limited to 2 weeks in a prophylactic setting (Fig. 4). To explore whether additional effects of FTY720 and FTY720-P in the brain are possible at all, based on available drug concentrations in that tissue, we examined the distribution of FTY720 in the rat and determined levels of FTY720/FTY720-P in the brain.

**Quantitative Whole-Body Autoradiography and Myelin Sheath Distribution of [<sup>14</sup>C]FTY720.** First, QWBA was used to investigate the distribution of [<sup>14</sup>C]FTY720-related radioactivity in vivo and, in particular, its uptake into the CNS. Pigmented rats received [<sup>14</sup>C]FTY720 for 1 week at a high oral dose of 7.5 mg/kg/d. By 24 h after the seventh and last dose (Fig. 6A), elevated amounts of extravascular radioactivity were detected in the adrenal cortex, kidney (cortex-medullary junction), nasal turbinates, pitu-

itary gland, preputial gland, and stomach (glandular mucosa); maximal levels of radioactivity occurred in the brain, epididymis, eye (ocular membranes, vitreous body), and testis. At 168 h, residual radioactivity was still observed in most of these tissues, equivalent to approximately 1.4% of the administered dose, but the highest concentrations were found in the brain (reticular nucleus, corpus callosum, cerebellar white matter), preputial gland, and spinal cord (Fig. 6B). The distribution pattern after multiple doses was similar to that after a single dose (data not shown), especially the preferential localization to brain and spinal cord at 168 h. The accumulation factor (i.e., ratio between the tissue concentration after multiple versus single dosing) was 3.2 for brain and 3.7 for spinal cord at 24 h after the seventh dose compared with the single dose.

Light microscopic evaluation showed that the <sup>14</sup>C labeling in brain and spinal cord was confined to the myelin sheets (Fig. 7). Neurons were free of grains, except for some background labeling.

**Levels of FTY720 and FTY720-P in Blood and Brain.** Given that exposure of the CNS after oral dosage of FTY720 to normal rats was shown in the above QWBA study and in a previous pharmacokinetic analysis (Meno-Tetang et al., 2006), we next sought to determine concentrations of FTY720 and its phosphorylated form in blood and brain of rats diseased with EAE. In fact, levels of FTY720-P in brain have so far not been reported at all. Samples from both acute and chronic models of EAE (in Lewis and DA rats, respectively) were obtained 24 h after the last dose to determine trough levels. FTY720 and FTY720-P were found both in the blood and brain, with ratios between approximately 0.8 and 3 (Table 2). Brain levels of both forms were considerably higher (by factors of 10 to 27 after 23 doses) than those in blood, as seen before for FTY720 in normal rats (Meno-Tetang et al., 2006). Brain concentrations of the two forms were of comparable magnitude. The observed amounts of both FTY720 and its phosphorylated form in the DA rat brain increased with the oral dose of FTY720, as did the blood levels; thus, the brain/blood ratio was relatively constant in DA rats (21–27 for FTY720; 14–17 for FTY720-P). We also followed the time course of FTY720 and FTY720-P concentrations in blood and brain (Table 2). Although blood levels were constant over 21



**Fig. 5.** FTY720 rapidly reduces the peripheral lymphocyte count, with sustained activity at a low subtherapeutic dose during EAE. A, time course of lymphocyte count in the Lewis rat model of EAE following prophylactic treatment from days 0 to 13 with vehicle (■), 0.3 mg/kg FTY720 (○), or 25 mg/kg CsA (\*); *n* = 6 per group, represented as mean ± S.D. At 6 h postimmunization, FTY720 had already reduced the lymphocyte number to the maximal extent. CsA, despite full efficacy during treatment (Fig. 4), did not significantly alter the lymphocyte count. Level of significance was based on ANOVA comparison with naive animals at day 0. B, reduction of peripheral lymphocytes during early and late EAE in DA rats therapeutically treated from days 11 to 33 with vehicle (□; *n* = 21) or FTY720 at 0.03 (▩; *n* = 26) and 0.3 (■; *n* = 13) mg/kg. Data were pooled from three EAE studies (Fig. 3) and expressed as mean percentage ± S.E. of lymphocytes in the adjuvant control (□; *n* = 13). ANOVA was performed against the vehicle of antigen-immunized animals. \*, *p* ≤ 0.05; \*\*, *p* ≤ 0.01; \*\*\*, *p* ≤ 0.001.

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