Effects of prophylactic and therapeutic teriflunomide in transcranial magnetic stimulation-induced motor-evoked potentials in the Dark Agouti rat model of experimental autoimmune encephalomyelitis

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Abbreviations

ARR     Annualized relapse rate
DA      Dark Agouti
EAE     Experimental autoimmune encephalomyelitis
MEP     Motor-evoked potentials
MS      Multiple sclerosis
NIH     National Institutes of Health
RMS     Relapsing multiple sclerosis
SEM     Standard error of the mean
SSEP    Somatosensory-evoked potentials
tcMMEP  Transcranial magnetic motor-evoked potentials
TMS     Transcranial magnetic stimulation
USDA    United States Department of Agriculture
Abstract

Teriflunomide is a once-daily, oral immunomodulatory agent recently approved in the USA for the treatment of relapsing multiple sclerosis (RMS). This study investigated neurophysiological deficits in descending spinal cord motor tracts during experimental autoimmune encephalomyelitis (EAE; a model of MS) and the functional effectiveness of prophylactic or therapeutic teriflunomide treatment in preventing the debilitating paralysis observed in this model. Relapsing–remitting EAE was induced in Dark Agouti rats using rat spinal cord homogenate. Animals were treated with oral teriflunomide (10 mg/kg/day) prophylactically, therapeutically or with vehicle (control). Transcranial magnetic motor-evoked potentials were measured throughout the disease to provide quantitative assessment of the neurophysiological status of descending motor tracts. Axonal damage was quantified histologically by silver staining. Both prophylactic and therapeutic teriflunomide treatment significantly reduced maximum EAE disease scores ($p<0.0001$ and $p=0.0001$, respectively) compared with vehicle-treated rats. Electrophysiological recordings demonstrated that both teriflunomide treatment regimens prevented a delay in waveform latency and a decrease in waveform amplitude versus that observed in vehicle-treated animals. A significant reduction in axonal loss was observed with both teriflunomide treatment regimens compared with vehicle ($p<0.0001$ and $p=0.0014$, respectively). This study suggests that therapeutic teriflunomide can prevent the deficits observed in this animal model in descending spinal cord motor tracts. The mechanism behind reduced axonal loss and improved motor function may be primarily due to reduced inflammation and consequent demyelination observed in these animals, through the known effects of teriflunomide on impairing proliferation of stimulated T cells. These findings may have significant implications for patients with RMS.
Introduction

Multiple sclerosis (MS) is a chronic neuroinflammatory disease characterized by focal demyelination with axonal damage and loss. Experimental autoimmune encephalomyelitis (EAE) is a model of MS, which when induced in the Dark Agouti (DA) rat, mimics the relapsing–remitting nature of MS, together with several aspects of pathology, such as infiltration of T cells and macrophages, glial cell activation, demyelination and axonal loss.

Teriflunomide is a novel, oral immunomodulatory drug recently approved in the USA and Australia as a treatment for relapsing MS (RMS). In the DA rat model of MS it delays the onset and progression of EAE (Merrill et al., 2009). Teriflunomide selectively and reversibly inhibits the mitochondrial enzyme dihydro-orotate dehydrogenase, required for de novo pyrimidine synthesis (Warnke et al., 2009). Blocking de novo pyrimidine synthesis results in the inhibition of cell proliferation, which consequently prevents the expansion of stimulated T and B cells. However, slowly dividing or resting cells, which rely on the salvage pathway for pyrimidine synthesis, are relatively unaffected by teriflunomide, thereby maintaining homeostatic proliferation and availability of cells for immune surveillance (Gold and Wolinsky, 2011).

Teriflunomide has completed two phase III clinical trials for the treatment of RMS: TEMSO (O’Connor et al., 2011) and TOWER (Kappos et al., 2012). In these studies, teriflunomide 14 mg significantly reduced annualized relapse rate (ARR) (31.5% and 36.3% versus placebo, respectively) and risk of 12-week sustained disability progression (29.8% and 31.5% versus placebo, respectively) in patients with RMS, while the 7 mg dose significantly reduced ARR (31.2% and 22.3% versus placebo) (O’Connor et al., 2011; Kappos et al., 2012). While the anti-
inflammatory mechanism of action of teriflunomide has been extensively studied in vitro, few in vivo studies have been carried out to date. Previous studies have shown that during EAE in the DA rat, changes in the latency and amplitude of somatosensory-evoked potentials (SSEPs) correspond to the disease phases when significant inflammation, demyelination and axonal loss occur pathologically (Merrill et al., 2009). Teriflunomide prevented EAE-associated electrophysiological changes in somatosensory pathways, and histologically, it decreased inflammation, demyelination and axonal loss (Merrill et al., 2009).

Motor-evoked potentials (MEPs) provide a quantitative measure of the neurophysiological status of descending motor tracts (Mazon Pelaez et al., 2005). Transcranial magnetic stimulation (TMS) sends strong magnetic impulses directly into specific brain regions, inducing neurons to fire safely and painlessly (George, 2003). In animal studies, magnetic stimulation applied over the skull stimulates the descending motor tracts, eliciting transcranial magnetic MEPs (tcMMEPs) in peripheral muscle (Magnuson et al., 1999). TcMMEPs are altered in myelin-deficient mice, which demonstrate longer onset latencies and smaller amplitudes, compared with their wild-type counterparts (Zhang et al., 2007). In humans, TMS has been used to study and/or potentially treat various diseases, including depression (Derstine et al., 2010; Fitzgerald and Daskalakis, 2011), epilepsy (Brodbeck et al., 2010), chronic pain (Antal et al., 2010) and spinal cord injury (Lefaucheur, 2006; Saturno et al., 2008). In patients with MS, TMS-induced MEP conduction times are significantly more delayed than in controls (Magnuson et al., 1999).

In this study, tcMMEPs were used to determine the neurophysiological deficits observed throughout EAE disease progression, and the functional effectiveness of prophylactic and
therapeutic treatment with teriflunomide in preventing the debilitating paralysis observed in this model.
Methods

Induction and assessment of EAE

All animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals in compliance with the United States Department of Agriculture Animal Welfare Act, in a fully accredited Association for Assessment and Accreditation of Laboratory Animal Care facility. The studies were carried out in accordance with an animal use protocol approved by the Institutional Animal Care and Use Committee. Six-week-old male DA rats (Harlan laboratories, Indianapolis, IN, USA) were fed with a commercial diet (Purina Mills, Richmond, IN, USA) ad libitum and had free access to water. They were allowed to habituate to the facility for a minimum of 2 weeks or until they reached 180–220 g, when EAE disease was induced by immunization of rats with an encephalitogenic emulsion composed of equal amounts of frozen DA rat spinal cord homogenate (50% w/v in saline) and complete Freund’s adjuvant supplemented with 6 mg/mL Mycobacterium tuberculosis. Each rat received 0.2 mL of the emulsion, subcutaneously, at the base of the tail. Sham-treated animals received the same emulsion minus the rat spinal cord homogenate.

Clinical signs of EAE disease were assessed daily in a non-blinded fashion, beginning on Day 5 post-EAE induction. Neurological deficits were scored according to the following scale: 0 = no clinical disease; 1 = flaccid tail; 2 = hindlimb weakness; 3 = hindlimb paresis; 4 = complete hindlimb paralysis; 5 = death due to EAE.

Compound administration and treatment regimens
Teriflunomide ((2Z)-2-cyano-3-hydroxy-N-[4-(trifluoromethyl)phenyl]but-2-enamide) was suspended in carboxymethylcellulose made up to 0.06% w/v in water, with 0.5% v/v Tween 80 (final concentration). All experimental groups began with 12 rats. Prophylactic treatment with teriflunomide (10 mg/kg, p.o.) or vehicle (as described above minus teriflunomide) started on Day 1 post-EAE induction and continued daily until Day 35.

Therapeutic treatment with teriflunomide (10 mg/kg, p.o.) or vehicle started at onset of EAE disease, when animals reached a functional deficit score of $\geq 1$, and was given once daily until Day 33 (~27 days post-disease onset).

**Transcranial magnetic stimulation**

Magnetic stimulation was used for electrophysiological recordings and all experiments were conducted in restrained awake animals. Approximately 1 week prior to tcMMEPs, animals were handled daily and allowed to acclimatize to the restraint. The restraint fabric was placed over the rat such that the body was immobilized but the head, limbs and tail were exposed. Non-magnetic pins were used to attach the fabric to a wooden board, thus restraining the rat without inflicting injury or pain. After the rat was restrained, electromyography stainless steel needles (27 gauge; Ambu Inc., Glen Burnie, MD, USA) were inserted bilaterally into the gastrocnemius muscles. Reference electrodes were inserted into the distal tendon (Fig. 1a (Magnuson et al., 1999)). A circular coil (40 mm) attached to a Magstim unit (Jali Medical Inc., Woburn, MA, USA) was placed over the head and a short magnetic pulse was delivered while electromyography responses were recorded. After threshold was obtained, the magnetic strength was increased in 20% increments. In our studies, 60% magnetic output was sufficient to reach maximum response in
most of the animals. A Power1401 data acquisition interface, Spike2 software and computer
(Cambridge Electronic Design Ltd, Cambridge, UK) were used to trigger the Magstim unit, and
record and analyze the data.

TcMMEPs were recorded from the same animals at various time points throughout the EAE
disease course; baseline tcMMEP was recorded 2–3 days prior to EAE induction (Days -2 to -3),
and at pre-illness (Day 5), acute attack (Days 10–13), remission (Days 16–18) and relapse-
remission (Days 25–26 and 30–33) phases of disease. TcMMEPs were recorded from the
gastrocnemius muscles of both left and right hindlimbs. The waveform was assessed in terms of
latency (seconds) from the stimulus artifact to the initiation of the waveform (I) and to the negative
peak (N). Amplitude (volts) was the peak-to-peak measurement of the N to positive peak (P)
portion of the tcMMEP (Fig. 1b).

Histology

Rats were sacrificed at Day 35 and Day 33 for the prophylactic and therapeutic studies,
respectively. Immediately after being euthanized with CO₂, each rat was perfused and fixed with
4% w/v paraformaldehyde. The lumbar region of the spinal cord was removed, cryostat sectioned
at 20 µm thickness, and stained with FD NeuroSilver Kit II (FD Neurotechnologies Inc., Elliot
City, MD, USA) for detection of neuronal/axonal damage, according to the manufacturer’s
instructions.

Neurons undergoing degeneration are indicated by dense silver precipitates, appearing as black or
silver grains, in their somata and/or processes which were counted under light microscopy using
the computerized CAST-Grid system (Olympus, Center Valley, PA, USA), as described previously (Merrill et al., 2009).
Statistical analysis

A mixed-effects model using a robust covariance estimate was used to analyze the electrophysiology data, with the rank of latency to the specific peak (I, N) as the response, the rank of latency to the corresponding peak (I, N) at baseline as the covariate, treatment, disease stage, side (left and right hindlimbs) and their two- and three-way interactions as the fixed effects, and animal as the random effect. The Bonferroni–Holm method was used to adjust the multiplicity when comparing the EAE vehicle-treated group with the sham-treated and EAE-teriflunomide groups, respectively, at each disease stage. For the graphical representation of these data, the mean of the left and right sides of hindlimbs was plotted.

For the histology data, Wilcoxon’s test was used to analyze the silver grain (damaged axons) data; the Bonferroni–Holm method was used for multiplicity adjustment.

For the cumulative neurological score and maximum disease score, Wilcoxon’s test was used to analyze the data; the Bonferroni–Holm method was used for multiplicity adjustment.
Results

Neurological deficits

Rats receiving daily administration of teriflunomide starting at Day 1 post-disease induction showed a complete suppression of EAE disease, compared with those receiving vehicle (Fig. 2a). The mean maximal neurological score (± standard error of the mean [SEM]) for vehicle-treated rats, 3.83 (± 0.09), occurred on Day 12 post-disease induction. The corresponding score in the teriflunomide-treated group at the same time was 0 (± 0).

Teriflunomide administered therapeutically daily, starting at onset of EAE disease symptoms, reduced neurological deficits at the acute-attack phase of EAE and demonstrated reduced disease severity throughout the observation period compared with that seen in vehicle-treated rats (Fig. 2b). The mean maximal neurological score (± SEM) for vehicle-treated rats was 3.89 (± 0.12); this occurred on Day 12 post-disease induction. In teriflunomide-treated rats, the mean maximal neurological score was 1.85 (± 0.27), occurring on Day 11 post-disease induction. At the end of the experiment (Day 33 post-induction) there were greater residual deficits in vehicle-treated rats compared with teriflunomide-treated rats. This was reflected in a higher neurological score in this group (2.64 ± 0.47) compared with rats treated therapeutically with teriflunomide (0.44 ± 0.15).

Both prophylactic and therapeutic teriflunomide treatment of EAE rats significantly reduced cumulative neurological disease scores by 99.5% and 75.7%, respectively (p<0.0001 for both regimens), and maximum disease scores by 94.9% and 40.6%, respectively (p<0.0001 and p=0.0001), compared with those seen in vehicle-treated rats (Fig. 3).
TcMMEP alterations

Latency delay may arise from inflammation, demyelination, conduction block, and/or axonal damage/loss. Example tcMMEP recordings from individual animals followed through various phases of EAE disease are shown in Fig. 4 and illustrate the increase in latency to onset of waveform response during the acute phase of EAE. A clear reduction in N-P amplitude is also observed, particularly during the acute and relapse phases of EAE, and to a lesser extent during disease remission. These changes are largely eliminated or reversed by prophylactic (a) or therapeutic (b) treatment with teriflunomide (Fig. 4).

Latency to I and N

Compared with sham-treated animals, EAE rats treated with vehicle demonstrated significant alterations in tcMMEPs. Specifically, the latency to onset of waveform response (I) and first negative deflection (N) were significantly increased in these animals (Fig. 5a). The latency to I was 0.0065 seconds pre-disease onset increasing to 0.0077 seconds during the acute phase of disease. Similarly, the latency to N increased from 0.0081 to 0.0093 seconds. Electrophysiological recordings demonstrated that prophylactic treatment with teriflunomide prevented a delay in the latency of waveform parameters (I and N) that occurred in vehicle-treated EAE animals. This effect was significant at Days 11, 18, 26 and 33 post-EAE disease induction, relating to the acute, remission and relapse-remission phases of the disease (Fig. 5a [i] and [ii]; \( p < 0.05 \)).

With therapeutic teriflunomide treatments, again, the vehicle-treated animals showed significant alterations in tcMMEPs. The latency to I was 0.0060 seconds pre-disease onset, which increased to...
0.010 seconds during the relapsing–remitting phase of disease. Similarly, the latency to N increased from 0.0077 to 0.012 seconds. Therapeutic teriflunomide treatment also had a significant effect on EAE-induced alterations in tcMMEPs. Teriflunomide prevented a delay in the latency to I, a significant effect during the relapse phase of disease at Days 26 and 33 post-disease induction, compared with that seen in EAE vehicle-treated rats (Fig. 5b [i]; p<0.05). Rats receiving therapeutic teriflunomide treatment also showed a greater effect on latency to N; a significant difference compared with EAE vehicle-treated rats was seen at Days 11, 18, 25 and 33 (Fig. 5b [ii]; p<0.05).

**N-P amplitude**

A significant decrease in the amplitude of N-P was observed during the relapse phase (Days 25 and 33) of EAE disease in vehicle-treated animals, compared with sham-treated animals (p<0.05). Prophylactic teriflunomide treatment prevented this decrease (Fig. 6a; p<0.05).

In the therapeutic study, vehicle-treated animals showed a significant decrease in the amplitude of N-P at Day 25 post-EAE induction, compared with sham-treated animals (p<0.05), but the effect was not consistent at the second recorded time point during the relapse phase. This is probably a result of animals entering second relapse at different times, an effect not uncommon in the EAE disease model. Compared with EAE vehicle treatment, therapeutic teriflunomide treatment prevented this decrease and led to a significant increase in the amplitude of N-P at Days 11, 18 and 25 post-EAE induction (Fig. 6b; p<0.05).
Histopathology

Silver staining of the motor tracts in the lumbar region of the spinal cord at Day 35 of EAE revealed substantial axonal damage in vehicle-treated rats, which was reduced in rats given prophylactic or therapeutic teriflunomide (Fig. 7a [i] and b [i]). Quantification of damaged motor axons revealed a significant protective effect of both teriflunomide treatment regimens, compared with vehicle, resulting in an approximate 88% reduction in axonal damage for prophylactic teriflunomide and a 96% reduction in axonal damage for therapeutic teriflunomide ($p<0.0001$ and $p=0.0014$, respectively; Fig. 7a [ii] and b [ii]).
Discussion

Teriflunomide has shown promising results in both phase II and III clinical trials in patients with RMS (O'Connor et al., 2006; O'Connor et al., 2011), suggesting that it has potential as a future oral treatment for the disease. This study demonstrates for the first time the functional benefit of both prophylactic and therapeutic teriflunomide treatment on descending motor tracts during relapsing–remitting EAE in the DA rat model of MS.

The DA rat model permits behavioral, functional and histopathological assessment of relapsing–remitting EAE, which shows many pathological similarities to RMS in humans (McFarland and Martin, 2007). The results presented here confirm previous findings demonstrating that prophylactic and therapeutic administration of teriflunomide reduces maximal and cumulative EAE disease scores, and that EAE rats demonstrated a delay in latency and a decrease in the amplitude of the SSEP (Merrill et al., 2009). The in vivo SSEP recordings used in the previous study had some limitations: the procedure was terminal, invasive and required use of anesthesia, which has been reported to affect evoked responses (Agrawal et al., 2009; Sloan et al., 2010). Conversely, the magnetic stimulation technique used in the current study is a non-invasive translational method that effectively measures MEP in awake, non-anesthetized rats (Linden et al., 1999; Zhang et al., 2007). Passing a high-voltage current through a coil generates a magnetic field, which when placed in close proximity to inducible tissue such as the brain, will cause neurons to fire (Hovey, 2008; Bolognini and Ro, 2010). The major advantage of using magnetic stimulation over electrical stimulation to evoke responses is that it does not require surgically implanted electrodes for longitudinal-type studies and is not painful when applied to the animal.
TcMMEPs have been used to evaluate neuronal transmission in animal models of spinal cord injury (Magnuson et al., 1999) and epilepsy (Vahabzadeh-Hagh et al., 2011). This method is particularly well suited to the EAE model because it provides information on the physiological status of myelinated cortical motor neuronal projections and synaptic transmission (Mazon Pelaez et al., 2005), which are functionally affected during inflammatory demyelination (Bannerman and Hahn, 2007; Vogt et al., 2009). Another important benefit of this technique is that individual animal responses can be tracked throughout the course of the experiment, from pre-induction (baseline) through the progression of EAE disease. In this study, all recordings were performed in restrained, fully awake animals. For the first time, we were able to demonstrate that TMS is a valuable method to evaluate motor function in EAE-induced animals throughout all disease stages, and by using tcMMEPs as the endpoint in a longitudinal study we were able to demonstrate that teriflunomide improved motor deficits associated with EAE.

Teriflunomide treatment initiated 1 day post-disease induction (prophylactically), or at disease onset (therapeutically), reduced neurological deficits associated with EAE and prevented latency delays and amplitude changes in tcMMEPs. The most relevant findings of this study were the delay in latency of tcMMEPs in vehicle groups, but not in prophylactic or therapeutic teriflunomide-treated groups. Patients with MS exhibit a delay in the latency of TMS-induced MEPs, which correlates with disability (Michels et al., 1993; Schmierer et al., 2002; Conte et al., 2009; Kale et al., 2009). Latency delays can result from inflammation and demyelination, and are observed during the acute, relapsing and remitting phases of EAE. During acute attack, inflammation is notable in various areas along the spinal cord of rats (unpublished results) and mice (Batoulis et al., 2012). In humans, episodic neuritis (Plant, 2008) is observed in acute-stage MS. A decrease in amplitude is largely associated with axonal loss (Chalk et al., 1994; Chalk et
Subsequent to the deficits observed during the initial acute attack, most of the animals recorded at the first-remission phase of EAE in our study demonstrated an increase in amplitude in both teriflunomide-treated groups. During the remission phase of EAE, it has been reported that numerous regenerative and cell repair processes occur following the pro-inflammatory phase of EAE, which work to repair and/or enhance the survival of affected neurons (Zhu et al., 2003; Barbizan and Oliveira, 2010; Seger et al., 2010). However, in EAE vehicle-treated animals, we observed a reduction in amplitude at first relapse and then a more notable reduction during the relapse-remission phase of EAE disease; this timeframe has been associated with increased axonal loss (Merrill et al., 2009).

There are limitations to this methodology, in particular with regard to assessing amplitude changes. Waveform amplitude is one of the most problematic indexes of waveform morphology, with common inter- and intra-subject variability. In addition, the synchrony of EAE disease progression becomes a major factor after the remission phase; relapse rates are highly individualized and the recording day may not coincide with the relapse phase of EAE. Nevertheless, increases in amplitude were observed in animals receiving therapeutic teriflunomide and were maintained into the first relapse, while increases in amplitude in vehicle-treated animals were absent by the first relapse. Increases in amplitude were also observed during the relapse-remission phase in animals receiving prophylactic teriflunomide, whereas vehicle-treated animals displayed a decrease in amplitude during the relapse-remission phase. Taken together, these findings suggest that teriflunomide treatment can reduce axonal loss associated with the relapsing–remitting phase of EAE.

Silver-stain technique is designed for the detection of degenerating neurons in fixed tissue sections of the central nervous system. A significant increase in number of damaged axons and neurons...
was observed in the vehicle-treated group, compared with teriflunomide-treated and sham-treated controls. This supports our observations that while latency is significantly increased in vehicle-treated animals, there are sufficient axons still conducting. These findings, along with the tcMMEP results, point towards an additional beneficial effect of teriflunomide on axonal integrity during neuroinflammation.

The data presented here extend our knowledge of teriflunomide in the EAE model. Together with data from previous studies (Merrill et al., 2009), it is clear that both sensory and motor pathways are affected during chronic-relapsing EAE, and that therapeutic administration of teriflunomide can prevent the changes observed in both ascending and descending tracts of the spinal cord. The exact mechanism behind the reduction in axonal damage and improved neuronal function observed in this EAE model remains to be defined. Given the documented ability of teriflunomide to inhibit the proliferation of activated lymphocytes, it is likely that the therapeutic benefits of teriflunomide involve the reduction of inflammatory lymphocyte activity and consequent demyelination. Further studies are underway to determine whether direct neuroprotective effects of teriflunomide may also contribute to the therapeutic benefit. Irrespective of the underlying mechanism, attenuation of axonal damage and improvement of motor function in teriflunomide-treated-EAE rats are relevant findings that may have significant implications for patients with RMS.
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Author contributions

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References


**Footnotes**

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Legends for Figures

Fig. 1. Schematic representation of experimental set-up for measuring (A) transcranial motor-evoked potentials (tcMMEPs) and (B) sample recording of a tcMMEP

(A) Magnetic stimulation applied over the skull of a restrained animal delivers a short magnetic pulse that elicits a tcMMEP. These are recorded from the gastrocnemius muscle of both left and right hindlimbs (Reproduced from Magnuson et al. (Magnuson et al., 1999), with kind permission from Elsevier and Dr David Magnuson, University of Louisville School of Medicine, Louisville, KY, USA)

(B) Measurements taken were latency to I (onset of waveform response), latency to N (first negative deflection) and amplitude of N-P (first positive deflection)

Fig. 2. Mean neurological score (± SEM) of EAE and sham-treated DA rats treated with vehicle or teriflunomide (10 mg/kg, p.o.) (A) prophylactically daily starting from 1 day following EAE induction and (B) therapeutically daily from onset of EAE disease

TcMMEPs were recorded at baseline (prior to EAE induction), and at pre-illness, acute attack, remission, and relapse-remission phases of EAE

Prophylactic study: for vehicle-treated rats, n = 12 at baseline, and Days 5 and 11; n = 11 at Days 18, 26 and 33; for teriflunomide-treated rats, n = 12 at all recording time points; for sham vehicle-treated rats, n = 12 at baseline and Days 5, 11 and 18; n = 11 at Days 26 and 33

Therapeutic study: for vehicle-treated rats, n = 13 at baseline, and Days 5 and 11; n = 11 at Day 18; n = 12 at Days 26 and 33; for teriflunomide-treated rats, n = 13 at all recording time points; for
sham vehicle-treated rats, \( n = 12 \) at baseline, and Days 5, 11 and 18; \( n = 11 \) at Day 26; \( n = 7 \) at Day 33

DA Dark Agouti, EAE experimental autoimmune encephalomyelitis, p.o. per os, SEM standard error of the mean, TMS transcranial magnetic stimulation.

**Fig. 3.** Mean (± SEM) cumulative neurological disease score (i) and maximum disease score (ii) in EAE DA rats treated with vehicle or teriflunomide (10 mg/kg, p.o.) (A) prophylactically and (B) therapeutically

\[ ***p \leq 0.0001 \text{ EAE-teriflunomide versus EAE-Vehicle} \]

DA Dark Agouti, EAE experimental autoimmune encephalomyelitis, p.o. per os, SEM standard error of the mean.

**Fig. 4.** TcMMEPs of the same animal recorded at baseline and during acute attack, remission and first-relapse phases in EAE and sham-treated DA rats treated with vehicle or teriflunomide (10 mg/kg, p.o.) (A) prophylactically and (B) therapeutically

Cursor line is drawn at onset response at baseline recordings

DA Dark Agouti, EAE experimental autoimmune encephalomyelitis, p.o. per os, tcMMEPs transcranial magnetic motor-evoked potentials

**Fig. 5.** Latency to (i) I and (ii) N in tcMMEPs recorded at 60% magnetic output in EAE and sham-treated DA rats treated with vehicle or teriflunomide (10 mg/kg, p.o.) (A) prophylactically and (B) therapeutically
DA Dark Agouti, EAE experimental autoimmune encephalomyelitis, p.o. per os, tcMMEPs transcranial magnetic motor-evoked potentials.

**Fig. 6.** Amplitude of N-P of tcMMEPs recorded at 60% magnetic output during the first-relapse phase in EAE and sham-treated DA rats treated with vehicle or teriflunomide (10 mg/kg, p.o.) (A) prophylactically and (B) therapeutically

DA Dark Agouti, EAE experimental autoimmune encephalomyelitis, p.o. per os, SEM standard error of the mean, tcMMEPs transcranial magnetic motor-evoked potentials.

**Fig. 7.** Silver stain of the motor tracts in the lumbar region of the spinal cord, magnified 20x (i) and quantification of axonal damage (ii) in EAE and sham-treated DA rats treated with vehicle or teriflunomide (10 mg/kg, p.o.) (A) prophylactically and (B) therapeutically

***p ≤ 0.0001 and **p ≤ 0.01 EAE-Teriflunomide versus EAE-Vehicle

DA Dark Agouti, EAE experimental autoimmune encephalomyelitis, p.o. per os.
Figure A: Neurological score progression during EAE induction and treatment with Teriflunomide.

Figure B: Neurological score progression during EAE induction and treatment with Teriflunomide for a different time frame.

Key:
- EAE-Vehicle
- EAE-Teriflunomide
- Sham-Vehicle
A (i) Cumulative Neurological Disease Score (Mean ± SEM)

- EAE-Vehicle
- EAE-Teriflunomide

**p < 0.0001**

A (ii) Maximum Disease Score (Mean ± SEM)

- EAE-Vehicle
- EAE-Teriflunomide

**p < 0.0001**

B (i) Cumulative Neurological Disease Score (Mean ± SEM)

- EAE-Vehicle
- EAE-Teriflunomide

**p < 0.0001**

B (ii) Maximum Disease Score (Mean ± SEM)

- EAE-Vehicle
- EAE-Teriflunomide

**p = 0.0001**