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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ABSTRACT
Teriflunomide is a once-daily oral immunomodulatory agent recently approved in the United States 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 multiple sclerosis) 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 daily) 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 wave-form latency and a decrease in wave-form amplitude compared with 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). The results of this study suggest 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 the 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 neuro-inflammatory 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 United States 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, which is 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 the availability of cells for immune surveillance (Gold and Wolinsky, 2011).

Teriflunomide has completed two phase III clinical trials for the treatment of RMS: the TEMSO (Teriflunomide Multiple Sclerosis Oral trial) (O’Connor et al., 2011) and TOWER (an Efficacy Study of Teriflunomide in Patients with Relapsing Multiple Sclerosis) (Kappos et al., 2012). In these studies, 14 mg of teriflunomide significantly reduced the annualized relapse rate (31.5 and 36.3% versus placebo, respectively) and the risk of 12-week sustained disability progression (29.8 and 31.5% versus placebo, respectively) in patients with RMS, and the 7-mg dose significantly reduced the annualized relapse rate (31.2 and 22.3% versus placebo) (O’Connor et al., 2011; Kappos et al., 2012). Whereas the anti-inflammatory mechanism

ABBREVIATIONS: DA, Dark Agouti; EAE, experimental autoimmune encephalomyelitis; I, initiation of the wave form; MEP, motor-evoked potential; MS, multiple sclerosis; N, negative peak, P, positive peak; RMS, relapsing multiple sclerosis; SSEP, somatosensory-evoked potentials; tcMMEP, transcranial magnetic motor-evoked potentials; TMS, transcranial magnetic stimulation.
of action of teriflunomide has been extensively studied in vitro, to date, few in vivo studies have been carried out. 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 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.

Materials and 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 U.S. 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) were fed with a commercial diet (Purina Mills, Richmond, IN) 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 nonblinded fashion, beginning on day 5 post-EAE induction. Neurologic deficits were scored according to the following scale: 0 = no clinical disease; 1 = flaccid tail; 2 = hindlimb weakness; 3 = hindlimb paraparesis; 4 = complete hindlimb paralysis; 5 = death attributable to EAE.

Compound Administration and Treatment Regimens. Teriflunomide ((2Z)-2-cyano-3-hydroxy-N-[4-(trifluoromethyl)phenyllbut-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 PO) 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 PO) or vehicle started at onset of EAE disease, when animals reached a functional deficit score of ≥1 and was given once daily until day 33 (~27 days after disease onset).

Transcranial Magnetic Stimulation. Magnetic stimulation was used for electrophysiological recordings, and all experiments were conducted in restrained awake animals. Approximately 1 week before 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) 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) 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 Power 1401 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 before EAE induction (days −2 to −3), and at preillness (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 wave form was assessed in terms of latency (seconds) from the stimulus artifact to the initiation of the wave form (I) and to the negative peak (N). Amplitude (volts) was the peak-to-peak measurement of the N to the 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.
Neurons undergoing degeneration are indicated by dense silver precipitates, appearing as black or silver grains, in their somata or processes, which were counted under light microscopy using the computerized CAST-Grid system (Olympus, Center Valley, PA), 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), their two- and three-way interactions as the fixed effects, and the 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 neurologic score and maximum disease score, Wilcoxon’s test was used to analyze the data; the Bonferroni-Holm method was used for multiplicity adjustment.

Results

Neurologic Deficits

Rats receiving daily administration of teriflunomide starting at day 1 after disease induction showed a complete suppression of EAE disease compared with those receiving vehicle (Fig. 2A). The mean maximal neurologic score (±S.E.M.) for vehicle-treated rats, 3.83 (±0.09), occurred on day 12 after 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 neurologic deficits at the acute-attack phase of EAE and demonstrated reduced disease severity throughout the observation period compared with those seen in vehicle-treated rats (Fig. 2B). The mean maximal neurologic score (±S.E.M.) for vehicle-treated rats was 3.89 (±0.12); this occurred on day 12 after disease induction. In teriflunomide-treated rats, the mean maximal neurologic score was 1.85 (±0.27), occurring on postinduction day 11. At the end of the experiment (postinduction day 33), vehicle-treated rats showed greater residual deficits compared with teriflunomide-treated rats as reflected in a higher neurologic 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 neurologic disease scores by 99.5 and 75.7%, respectively (P < 0.0001 for both regimens) and maximum disease scores by 94.9 and 75.7%, 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, or axonal damage or 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 wave-form 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 wave-form response (I) and first negative deflection (N) were
significantly increased in these animals (Fig. 5A). The latency to I was 0.0065 seconds before 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 wave-form 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\textsuperscript{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 before 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 postdisease induction, compared with that seen in EAE vehicle-treated rats (Fig. 5Bi; $P < 0.05$). 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. 5Bii; $P < 0.05$).

**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. 7, Ai and Bi). 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. 7, Ai and Bi).
Discussion

Teriflunomide has shown promising results in both phase II and III clinical trials in patients with RMS (O’Connor et al., 2006, 2011), suggesting that it has potential as a future oral treatment of 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 pathologic 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 noninvasive translational method that effectively measures MEP in awake, nonanesthetized 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 (Jalinous, 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 its application is not painful to the animal.

TeMMEPs 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 physiologic 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 preinduction (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 teMMEPs 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 after disease induction (prophylactically) or at disease onset (therapeutically)
reduced neurologic deficits associated with EAE and prevented latency delays and amplitude changes in tcMMEPs. The most relevant finding of this study was 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, 1995; Felts et al., 1997; Merrill et al., 2009). 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 after the proinflammatory phase of EAE, and these processes work to repair 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 time frame has been associated with increased axonal loss (Merrill et al., 2009).

This method has limitations, particularly with regard to assessing amplitude changes. Wave-form amplitude is one of the most problematic indexes of wave-form morphology; intersubject and intra-subject variability is common. 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, whereas 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 the number of damaged axons and neurons was observed in the vehicle-treated group compared with teriflunomide-treated and sham-treated controls. This observation supports our observations that although latency is significantly increased in vehicle-treated animals, sufficient axons are still conducting. These findings, along with the tcMMEP results, point toward 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 mechanisms behind the reduction in axonal damage and improved neuronal function observed in this EAE model remain 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 under way 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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Wrote or contributed to the writing of the manuscript: Iglesias-Bregna, Hanak, Ji, Liu, Zhang, McMonagle-Strucko.

Fig. 7. Silver stain of the motor tracts in the lumbar region of the spinal cord, magnified 20×. (i) and quantification of axonal damage (ii) in EAE and sham-treated DA rats treated with vehicle or teriflunomide (10 mg/kg PO) (A) prophylactically and (B) therapeutically. ***P < 0.0001; **P = 0.0014 EAE-teriflunomide versus EAE-vehicle.

References


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