

Safinamide and flecainide protect axons and reduce microglial activation in models of multiple sclerosis

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Axonal degeneration is a major cause of permanent disability in the inflammatory demyelinating disease multiple sclerosis, but no therapies are known to be effective in axonal protection. Sodium channel blocking agents can provide effective protection of axons in the white matter in experimental models of multiple sclerosis, but the mechanism of action (directly on axons or indirectly via immune modulation) remains uncertain. Here we have examined the efficacy of two sodium channel blocking agents to protect white matter axons in two forms of experimental autoimmune encephalomyelitis, a common model of multiple sclerosis. Safinamide is currently in phase III development for use in Parkinson's disease based on its inhibition of monoamine oxidase B, but the drug is also a potent state-dependent inhibitor of sodium channels. Safinamide provided significant protection against neurological deficit and axonal degeneration in experimental autoimmune encephalomyelitis, even when administration was delayed until after the onset of neurological deficit. Protection of axons was associated with a significant reduction in the activation of microglia/macrophages within the central nervous system. To clarify which property of safinamide was likely to be involved in the suppression of the innate immune cells, the action of safinamide on microglia/macrophages was compared with that of the classical sodium channel blocking agent, flecainide, which has no recognized monoamine oxidase B activity, and which has previously been shown to protect the white matter in experimental autoimmune encephalomyelitis. Flecainide was also potent in suppressing microglial activation in experimental autoimmune encephalomyelitis. To distinguish whether the suppression of microglia was an indirect consequence of the reduction in axonal damage, or possibly instrumental in the axonal protection, the action of safinamide was examined in separate experiments *in vitro*. In cultured primary rat microglial cells activated by lipopolysaccharide, safinamide potently suppressed microglial superoxide production and enhanced the production of the anti-oxidant glutathione. The findings show that safinamide is effective in protecting axons from degeneration in experimental autoimmune encephalomyelitis, and that this effect is likely to involve a direct effect on microglia that can

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result in a less activated phenotype. Together, this work highlights the potential of safinamide as an effective neuroprotective agent in multiple sclerosis, and implicates microglia in the protective mechanism.

Keywords: safinamide; flecainide; experimental autoimmune encephalomyelitis; neuroprotection; microglia

Abbreviations: EAE = experimental autoimmune encephalomyelitis; ED-1 = endothelin 1; iNOS = inducible nitric oxide synthase

Introduction

Axonal and neuronal degeneration are important causes of permanent neurological deficits in multiple sclerosis (Dutta and Trapp, 2011), but no therapies are known to be neuroprotective in this disease. The cause of the degeneration remains unclear, but accumulating evidence implicates activation of innate immune cells located within the CNS (Trapp *et al.*, 1999; Barnett and Prineas, 2004; Marik *et al.*, 2007; Sharma *et al.*, 2010), associated with a lethal neuronal and axonal energy deficit (Bechtold and Smith, 2005; Trapp and Stys, 2009). In contrast, the currently-approved immunomodulatory therapies principally target aspects of the acquired immune system located outside of the CNS, and do not provide protection against an energy deficit.

The partial blockade of voltage-gated sodium channels has been proposed as an alternative treatment strategy for multiple sclerosis, and several sodium channel blocking agents have been found to be potent agents for axonal protection in different *in vivo* models of multiple sclerosis, including flecainide (Bechtold *et al.*, 2002; Kapoor *et al.*, 2003; Bechtold *et al.*, 2004b), phenytoin (Lo *et al.*, 2002, 2003; Craner *et al.*, 2005; Black *et al.*, 2007), lamotrigine (Bechtold *et al.*, 2006) and carbamazepine (Black *et al.*, 2007). Evidence suggests that inhibition of sodium channel activity by such state-dependent blocking agents can protect axons from degeneration through at least two different mechanisms: first, by directly protecting against the consequences of an energy deficit (Bechtold *et al.*, 2005), and second, by suppressing the activation of innate immune cells in the CNS. Thus, in different types of experimental autoimmune encephalomyelitis (EAE), flecainide has been shown to reduce significantly both microglial activation and inducible nitric oxide synthase (iNOS) expression during disease relapse (Bechtold *et al.*, 2004a), and phenytoin (Craner *et al.*, 2005; Black *et al.*, 2007) and carbamazepine (Black *et al.*, 2007) were found to reduce the abundance of activated microglia. Such effects may be mediated directly through expression of sodium channels on cells involved, such as astrocytes (Sontheimer and Waxman, 1992), some lymphocytes (DeCoursey *et al.*, 1985; Margaretten *et al.*, 1987; Lai *et al.*, 2000; Bortner and Cidrowski 2003), microglial cells (Korotzer and Cotman, 1992; Norenberg *et al.*, 1994; Schmidtmayer *et al.*, 1994; Aronica *et al.*, 2001; Craner *et al.*, 2005; Nicholson and Randall, 2009) and macrophages (Craner *et al.*, 2005; Carrithers *et al.*, 2007). Indeed, a robust increase in sodium channel expression (of the Na_v1.6 subtype) has been found in activated microglia and macrophages in both EAE and multiple sclerosis (Craner *et al.*, 2005) and related to phagocytic and migratory activity (Craner *et al.*, 2005; Black *et al.*, 2009, 2012) and cytokine release (Black *et al.*, 2009). Thus, sodium channel blockade may provide effective neuroprotection

through multiple mechanisms and sites of action. Neuroprotection by such agents has also been reported in rat models of ischaemic brain damage and Parkinson's disease (Caccia *et al.*, 2006). The proposed mechanisms of action include the reduction of neurotoxic levels of synaptic glutamate by inhibiting presynaptic release, reduction in energy demand in situations of energy stress, and avoidance of a pathological intake of calcium ions by reverse operation of the sodium/calcium exchanger (Salvati *et al.*, 1999; Carter *et al.*, 2000; Gangemi *et al.*, 2000; Bechtold and Smith, 2005; Trapp and Stys, 2009).

Based on the observations in EAE, the anticonvulsant state-dependent sodium channel blocking agent lamotrigine was tested for efficacy in neuroprotection in the first clinical trial of this type, in patients with secondary progressive multiple sclerosis (Kapoor *et al.*, 2010). Interpretation of the findings was complicated, but the group treated with lamotrigine performed significantly better in an ambulatory test (25 foot timed walk) in comparison with the control group, and this occurred in conjunction with a statistically significant reduction in circulating axonal neurofilament (Gnanapavan and Giovannoni, personal communication), a potential biomarker of axonal damage (Gnanapavan *et al.*, 2012). These positive findings occurred despite a reduction in brain volume. This effect was not anticipated at trial design (and it confounded the primary outcome measure based on protection from loss of brain volume), but a reduction in swelling would now be expected based on the anti-inflammatory effects of sodium channel blockade (see above) and the knowledge of 'pronounced inflammation' observed in the brain during secondary progressive multiple sclerosis (Frischer *et al.*, 2009).

Balanced against these encouraging findings, consistent with neuroprotection from the clinical trial, is the observation that lamotrigine therapy was less well tolerated by patients than predicted, and only 48% of patients in the treated group took the drug throughout the trial due to a perceived worsening of function. This worsening may be attributable to the well-known suppressive effects of sodium channel blockade on neuronal activity (Kapoor *et al.*, 2010) or to one or more other pharmacological activities of lamotrigine (Zheng *et al.*, 2010). Searching for an agent that might combine the apparently beneficial effects of sodium channel blockade with improved tolerability, we noticed that a potent new sodium channel blocking agent, safinamide, appeared to have little, if any, deleterious effects when administered to patients with Parkinson's disease (Onofrj *et al.*, 2008; Schapira 2010). Safinamide was originally identified as a state-dependent blocker of voltage-gated sodium channels (Salvati *et al.*, 1999) that readily crosses the blood–brain barrier and is active in preclinical seizure models. Later, safinamide was found to be a potent, selective and reversible inhibitor of

monoamine oxidase B (MAOB) and to have symptomatic effects consistent with this pharmacology both in preclinical models and in early testing in Parkinson's disease; safinamide is now in phase III clinical trials for Parkinson's disease. Thus safinamide is a well-tolerated sodium channel blocking agent that is potentially suitable as a neuroprotective therapy in multiple sclerosis.

In this study we explored the effects of safinamide in protecting axonal structure and function in two commonly used animal models of multiple sclerosis. We also examined the effects of safinamide treatment on microglial/macrophage activation in this model, and compared these with the effects of an older drug, flecainide, that we have previously shown to be potent in axonal protection (Bechtold *et al.*, 2004b). Flecainide is a classical sodium channel blocking agent, with no recognized MAOB activity. We report that both safinamide and flecainide provide significant axonal protection in models of multiple sclerosis, in conjunction with a significant reduction in endothelin 1 (ED-1) and iNOS expression, namely markers of the activation of the innate immune system within the CNS.

To explore whether the suppression of microglia was likely to be an indirect consequence of the reduction in axonal damage, or possibly instrumental in the axonal protection, the action of safinamide was examined in separate experiments *in vitro*. In isolated microglial cells activated by the addition of lipopolysaccharide, safinamide was potent in suppressing superoxide production, and it increased the microglial concentration of the antioxidant glutathione. Collectively, these studies suggest that sodium channel blockade remains a viable and potentially important treatment in multiple sclerosis with a mechanism of action that includes direct axonal protection (Garthwaite *et al.*, 1999; Kapoor *et al.*, 2003) and direct effects on cells of the innate immune system.

Materials and methods

Two sodium channel blocking agents were assessed, safinamide and flecainide. The effects of safinamide were studied regarding both axonal protection and microglial/macrophage activation, at one time point, 16 days after the onset of neurological deficit. Axonal protection by flecainide is already established (Bechtold *et al.*, 2002, 2004b), therefore experiments with this drug were restricted to examine its effects on microglial/macrophage activation at two stages during the disease course.

Induction of experimental autoimmune encephalomyelitis

Two types of EAE were induced, using either a homogenate of syngeneic spinal cord (spinal cord homogenate; SCH), or recombinant myelin oligodendrocyte glycoprotein (rMOG) as immunogen. The spinal cord homogenate was prepared from freshly dissected spinal cords obtained from dark agouti rats (150–200 g, Harlan) and made into an emulsion using complete Freund's adjuvant (Sigma-Aldrich) in a 1:1 ratio. Male dark agouti rats (150–200 g, Harlan) were inoculated with a 200 μ l subcutaneous injection of the prepared emulsion. Recombinant MOG EAE was induced by immunizing female dark agouti rats (150–200 g, Harlan) with a subcutaneous injection of a 200 μ l emulsion consisting of 100 μ g of recombinant MOG (1 mg/ml,

K. Malpass, University of Aberdeen, UK), and 100 μ l incomplete Freund's adjuvant (Sigma-Aldrich) at the base of the tail. All animals with EAE were weighed and assessed daily by a blinded observer for the magnitude of neurological deficit (see below). All drug dosing and assessment of neurological deficit was performed blind to the treatment regimen. All the experiments were approved by the local ethics committee (University College London) and were licensed under the Animals (Scientific Procedures) Act of 1986 of the UK.

Safinamide administration

The effect of safinamide was examined in two independent spinal cord homogenate EAE trials, both of which involved treatment with safinamide at a high, clinically relevant dose (90 mg/kg/day safinamide or saline vehicle; $n = 7$ /treatment in Trial 1 and $n = 15$ /treatment in Trial 2). Three independent trials were also performed in the recombinant MOG EAE model: one with a high dose of safinamide (90 mg/kg/day; $n = 15$ /treatment), and two trials involving high and low (90 mg/kg/day and 30 mg/kg/day, respectively) doses of safinamide ($n = 15$ /treatment/trial). Following the induction of EAE, the animals were matched in pairs, or triplets (depending on the number of treatment groups involved), based on the similarity of the timing and severity of neurological deficit once they reached a neurological score ≥ 2 . Each animal of a pair was randomly assigned to receive either safinamide at high-dose (90 mg/kg/day in saline) or vehicle (saline), whereas each animal of a triplet was assigned to receive either safinamide at high (90 mg/kg/day in saline), or low (30 mg/kg/day) dose, or vehicle (saline). To provide consistent administration of drug, solutions were added under sterile conditions to osmotic mini-pumps (Alzet) that were implanted (blindly regarding content) subcutaneously in the dorsal flank of the animal under general anaesthesia (2% isoflurane in oxygen). Following a delay of ~ 1 day, the osmotic pumps released the drug or vehicle at a rate of 5 μ l/h for at least 2 weeks (manufacturer's guidelines). The neurological deficit for each animal was assessed daily on a 10-point scale, receiving 1 point for each of the following signs: loss of tail tip muscle tone; loss of total tail muscle tone; tail paralysis; decreased toe spread; unsteady gait; 1 point/hind limb dragged (maximum 2 points); 1 point/hind limb paralysed (maximum 2 points); moribund.

The concentration of safinamide circulating in the blood was determined in a separate series of naïve animals (weight and strain matched to those used in the EAE studies; $n = 3$ /dose) implanted with pumps loaded with 15 mg/ml or 150 mg/ml safinamide in saline (rated to release safinamide at either 9 mg/kg/day or 90 mg/kg/day) by assay of plasma samples taken 3, 7 and 14 days after pump implantation.

Flecainide administration

The effects of flecainide were studied in EAE induced by immunization with spinal cord homogenate. During immunization, the animals were electronically tagged (Avid) with a subcutaneous implant and randomly assigned to receive either flecainide acetate (Tambacor; 3 M, in 2.5% glucose containing 20 mM HEPES, pH 7.4, 30 mg/kg/day) or vehicle. Drug was administered by subcutaneous injection every 12 h. From 7 days post-inoculation, prior to the onset of any neurological deficits, until time of sacrifice. The animals were weighed and assessed daily for the magnitude of neurological deficit, receiving one point for each of the following signs: loss of tail tip muscle tone; loss of total tail muscle tone; tail paralysis; decreased toe spread; unsteady gait; 1 point/hind limb dragged (maximum 2 points); 1 point/hind limb paralysed (maximum 2 points); moribund.

Electrophysiological examination

At the termination of the experiments with safinamide (16 days after the onset of neurological deficit, i.e. 25–28 days post-inoculation) the animals ($n = 60$) were anaesthetized with a combination of 90 mg/kg of ketamine (Fort Dodge Animal Health Ltd) and 10 mg/kg of xylazine (Animal Care Ltd) administered by intraperitoneal injection, and prepared for an electrophysiological examination by shaving the back and making a small skin incision over the lower thoracic vertebrae. Sensory compound action potentials were evoked by electrical stimulation of the dorsal column axons using a stimulating cathode inserted through the paravertebral muscle with the tip resting on the vertebrae at the level of T10/T11 vertebral junction, and the stimulating anode located over the shoulder blade. A ground electrode was inserted percutaneously over the pelvis. Averaged ($n = 10$) sensory compound action potentials were recorded differentially with the active electrode placed at the base of the tail, and the indifferent electrode inserted through the tail tip. The recordings were obtained blind to the treatment received. Electrophysiological examination was performed in all the animals treated with safinamide in trials SCH2 and rMOG1 (Table 1). In each trial, the records from all the animals receiving the same treatment were averaged, and the averaged records compared.

Termination of trials

Safinamide

For trials involving safinamide, perfusion occurred 16 days following the onset of treatment (i.e. 16 days following implantation of the pump, 25–28 days post-inoculation).

Flecainide

For trials involving flecainide, perfusion occurred either during the first peak of disease (9–13 days post-inoculation; $n = 41$) or during the relapse phase (18–22 days post-inoculation; $n = 27$). At each phase of disease, tissue was collected when rats were judged to have achieved the peak deficit score. For analysis of the relapse phase of EAE, only rats that had exhibited a deficit score ≥ 6 during the first peak of disease (acute phase) were included. Therefore, all rats had previously experienced severe disability. Of such animals, 94% experience a relapse or enter a state of chronic disability (Bechtold and Smith, unpublished observations).

Tissue collection

Tissues were fixed for histological examination by perfusion through cardiac puncture with rinse (0.9% saline containing 10 mM HEPES, 0.05% lignocaine, 2 U/ml heparin, 0.02% NaNO_2), followed by 4% paraformaldehyde solution.

Immunohistochemistry

For immunohistochemical examination, the spinal cords were excised and stored in 4% paraformaldehyde solution at 4°C for 4 h before being transferred to 30% sucrose solution for cryoprotection. The spinal cords were transected at the L4/L5 junction and a short length (~3 mm long in the caudal direction) embedded in tissue-freezing medium (Jung Tissue Freezing Medium; Leica Microsystems) which was subsequently frozen in isopentane cooled in liquid nitrogen. Frozen transverse sections were cut at 10 μm and thaw-mounted onto glass slides (safinamide trials), or cut at 25 μm for collection as free-floating sections into PBS containing TritonTM X-100 (PBST; flecainide trials). Immunohistochemistry was conducted using standard procedures with the appropriate biotinylated secondary

reagents and ABC amplification (Vector Laboratories). Briefly, in the safinamide study, slides were rehydrated in PBS and endogenous peroxidase activity was quenched in 0.3% H_2O_2 and methanol for 20 min prior to washing with Triton in PBS (pH 7.4). Sections from the safinamide study were incubated with the appropriate blocker for 1 h, followed by overnight incubation with primary antibodies: anti-CD68 (ED-1, Serotec 1:200), anti-CD11b/c (OX-42, Vector, 1:200). In the flecainide study, endogenous peroxidase activity was blocked with 0.2% sodium azide and 10% H_2O_2 in PBST for 20 min, followed by 70% methanol for 15 min. Sections were washed, blocked in 5% normal serum for 30 min, and incubated overnight in antibody specific for iNOS (1:2500, Transduction Labs), or anti-CD68 (ED-1, Serotec, 1:2,000). For all treatments, immunoreactivity was revealed with diaminobenzidine (DAB). In all studies, omission of either the primary or secondary antibody resulted in no deposition of reaction product.

Light microscopy and quantitative analysis

Photomicrographs of the L4–L5 spinal cord from all the animals were taken using a light microscope (Axiophot microscope; Zeiss) connected to a Nikon D300 camera (Nikon). Photomicrographs of sections labelled by immunohistochemistry from the safinamide trials were analysed using ImageJ (<http://rsbweb.nih.gov>, United States Department of Health and Human Services, Maryland, USA). An intensity threshold was applied to distinguish all the labelled cells within the spinal cord white matter, and then the total area of spinal white matter was determined and the percentage area occupied within this space by ED-1 or OX-42 positive cells was recorded. In the flecainide study, SigmaScan digital analysis software was used to assess the spinal cord cross-sectional area occupied by cells positive for ED-1 or iNOS in a minimum of four sections per animal.

High resolution microscopy

In safinamide trials, a transverse block of tissue (0.5 mm in length) was taken at the junction of the L4–L5 dorsal root entry zones, post-fixed in 3.5% glutaraldehyde in 0.15 M phosphate buffer, and prepared for high resolution light microscopy by embedding into TAAB resin (TAAB Laboratories) using a standard protocol as previously described (Redford *et al.*, 1995). Following resin embedding, semi-thin sections (700 nm) were cut, collected onto slides, and stained with thionin and acridine orange. These sections were then examined at high magnification. The regions of axonal pathology (containing a mixture of demyelination and degeneration, or degeneration alone) were quite clearly demarcated and they were quantified by drawing around them using ImageJ software. The dorsal columns, right and left lateral columns, and the ventral columns were treated separately, and the areas of these white matter tracts were then determined to allow calculation of the percentage of the area occupied by demyelinated and degenerated axons, and the area occupied by degenerated axons alone.

Preparation and use of cultures of primary rat microglia

Primary cultures of rat microglia were prepared from whole brain (minus the cerebellum) of Sprague Dawley rat pups (6-days-old) bred and reared in-house from stock animals obtained from Charles River UK. Rats were sacrificed under Schedule 1 kill methods approved by the Animals Scientific Procedures (1986) Act. Cultures were prepared as previously described (Mead *et al.*, 2012) and used after 1 day *in vitro*. Microglia were activated with lipopolysaccharide (1 $\mu\text{g}/\text{ml}$ final concentration) or phorbol-12-myristate-13-acetate (PMA;

Table 1 Summary of *in vivo* experiments

Trial	EAE model and treatment			Behavioural		Axonal function		Microglia/macrophage activation		Axonal survival/protection	
	Group size; Drug; Dose (mg/kg/day)	Duration (dpo/dpi)	White matter pathology assessed	Neurological deficit scores	Therapy beneficial	Electrophysiological assessment	Therapy beneficial	Immunoreactivity assessed	Therapy beneficial	High resolution assessment	Therapy beneficial
SCH1	n = 7; Saf; (90) n = 7; Vehicle	16 dpo	Dorsal column	Yes	Yes	No	N/A	No	N/A	Yes	Yes
SCH2	n = 15; Saf; (90) n = 15; Vehicle	16 dpo	Dorsal column	Yes	Yes	Yes	Yes	No	N/A	Yes	Yes
SCH3	n = 33; Flec; (30) n = 35; Vehicle	9–13 dpi (first peak) 18–22 dpi (relapse)	Dorsal column	Yes (here and Bechtold <i>et al.</i> , 2004)	Yes	Bechtold <i>et al.</i> , 2004	Yes	Yes	Yes	Bechtold <i>et al.</i> , 2004	Yes
rMOG1	n = 15; Saf; (90) n = 15; Vehicle	16 dpo	Dorsal, lateral, and ventral columns	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
rMOG2	n = 15; Saf; (90) n = 15; Saf; (30) n = 15; Vehicle	16 dpo	Dorsal, lateral, and ventral columns	Yes	Yes	No	N/A	Yes	Yes	Yes	Yes
rMOG3	n = 15; Saf; (90) n = 15; Saf; (30) n = 15; Vehicle	16 dpo	Dorsal, lateral, and ventral columns	Yes	Yes	No	N/A	Yes	Yes	Yes	Yes

dpo = days post onset of disease expression; dpi = days post immunization; N/A = not applicable; Saf = safinamide; SCH = spinal cord homogenate; rMOG = recombinant MOG.

10 ng/ml final concentration) for 24 h as previously described (Mead *et al.*, 2012). Safinamide or a solvent control, at the final concentrations detailed in the results section, was added at the same time as lipopolysaccharide or PMA.

Immunocytochemistry

Twenty-four hours post-treatment with lipopolysaccharide/PMA and safinamide, cells were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. Non-specific binding was blocked with PBS containing 4% normal goat serum and assessed by standard antibody isotype controls. Cultures were incubated overnight at 4°C with anti-CD68 (ED-1, Serotec, 1:250), followed by incubation at room temperature with appropriate secondary antibodies for 2 h (goat anti-mouse FITC, Abcam, 1:1000). In all studies, omission of either the primary or secondary antibody resulted in no deposition of reaction product.

Cultures were incubated with the nuclear stain DAPI, mounted with Vectashield, and examined by fluorescence microscopy.

Measurement of superoxide production and glutathione production in primary cultured microglia

Superoxide production was measured by fluorescence imaging of dihydroethidium as previously described, using Hoechst 33342 as a counterstain for total cell number (Mead *et al.*, 2012). Glutathione production was measured by fluorescence imaging of monochlorobimane as previously described, using ethacrynic acid as a control for glutathione production (ethacrynic acid inhibits glutathione-S-transferase, thereby depleting intracellular glutathione and reducing monochlorobimane fluorescence), fluorescein diacetate as a control for live cells, and propidium iodide as a control for dead cells (Keelan *et al.*, 2001; Hooper *et al.*, 2009).

All *in vivo* assessments, quantitative analyses of histological data, both *ex vivo* and *in vitro*, were conducted blindly in all studies.

Statistical analysis

In the EAE studies, all quantitative morphological data were presented as mean \pm the standard error of the mean (SEM), and Student's *t*-test was used to examine the significance of all trials where a pooled analysis using the weighted mean difference was performed. For the three independent *in vitro* studies, significant differences were established using one-way ANOVA followed by Tukey *post hoc* analysis and Student's *t*-test. In all studies, levels of statistical significance are presented as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Results

Safinamide concentration

The plasma safinamide concentrations on Days 3, 7 and 14 after subcutaneous implantation of pumps containing either 9 mg/kg/day or 90 mg/kg/day are presented in Table 2. The mean concentration over the 2 week period of pump implantation was 1.06 μ M for the three animals implanted with pumps containing the lower dose of safinamide, and 10.68 μ M for animals with the higher dose of safinamide. On the basis of the information in Table 2, the 'low dose' chosen for future experiments was 30 mg/kg/day, and the 'high dose' was 90 mg/kg/day.

Table 2 Circulating levels of safinamide achieved with osmotic pump delivery

Animal	Day 3	Day 7	Day 14
9 mg/kg/day			
15 A	0.57	0.95	0.60
15 B	1.09	2.04	1.43
15 C	0.74	1.32	0.79
Mean	0.80	1.44	0.94
90 mg/kg/day			
150 A	5.42	5.97	4.22
150 B	11.42	17.02	10.94
150 C	12.47	17.59	11.07
Mean	9.77	13.53	8.74

Data show the concentrations of safinamide circulating in the plasma 3, 7 and 14 days in micromolar, after implantation of pumps containing either 9 mg/kg/day or 90 mg/kg/day.

Safinamide therapy

Experimental autoimmune encephalomyelitis induced with spinal cord homogenate

The course of spinal cord homogenate EAE with regard to the expression of neurological deficit is shown in Fig. 1A. Two independent spinal cord homogenate trials were performed, but the findings from each trial were similar and so the results have been combined. Dark agouti rats immunized with spinal cord homogenate were implanted with osmotic mini-pumps loaded with 90 mg/kg/day safinamide or vehicle upon reaching a neurological deficit score of 2, and followed for 16 days. The mean neurological scores for the safinamide group were lower from Day 16 to Day 28, with the difference achieving statistical significance ($P < 0.05$) on Days 27 and 28.

Electrophysiological examination of all the animals at the end of trial 2 revealed a 74% increase in the area of the sensory compound action potential in the safinamide-treated group compared with the vehicle group (Fig. 2A; $P \leq 0.05$). The area of the monophasic compound action potential is proportional to the number of functional axons contributing to it, so the findings indicate that a greater number of functional axons survived in animals treated with safinamide.

Experimental autoimmune encephalomyelitis induced with recombinant myelin oligodendrocyte glycoprotein

Axonal degeneration is more pronounced in recombinant MOG-induced EAE in our experience, and so we focused on this model for our study of the efficacy of safinamide in axonal protection. Three independent trials of safinamide therapy in recombinant MOG EAE were performed, but the findings from each trial were similar and so the results have been pooled. The course of recombinant MOG EAE in the three treatment groups is shown in Fig. 1B. As with spinal cord homogenate EAE, the severity of the neurological deficit was ameliorated in animals treated with safinamide, with the difference achieving statistical significance on the last 4 days of the trial for animals treated with safinamide at either high

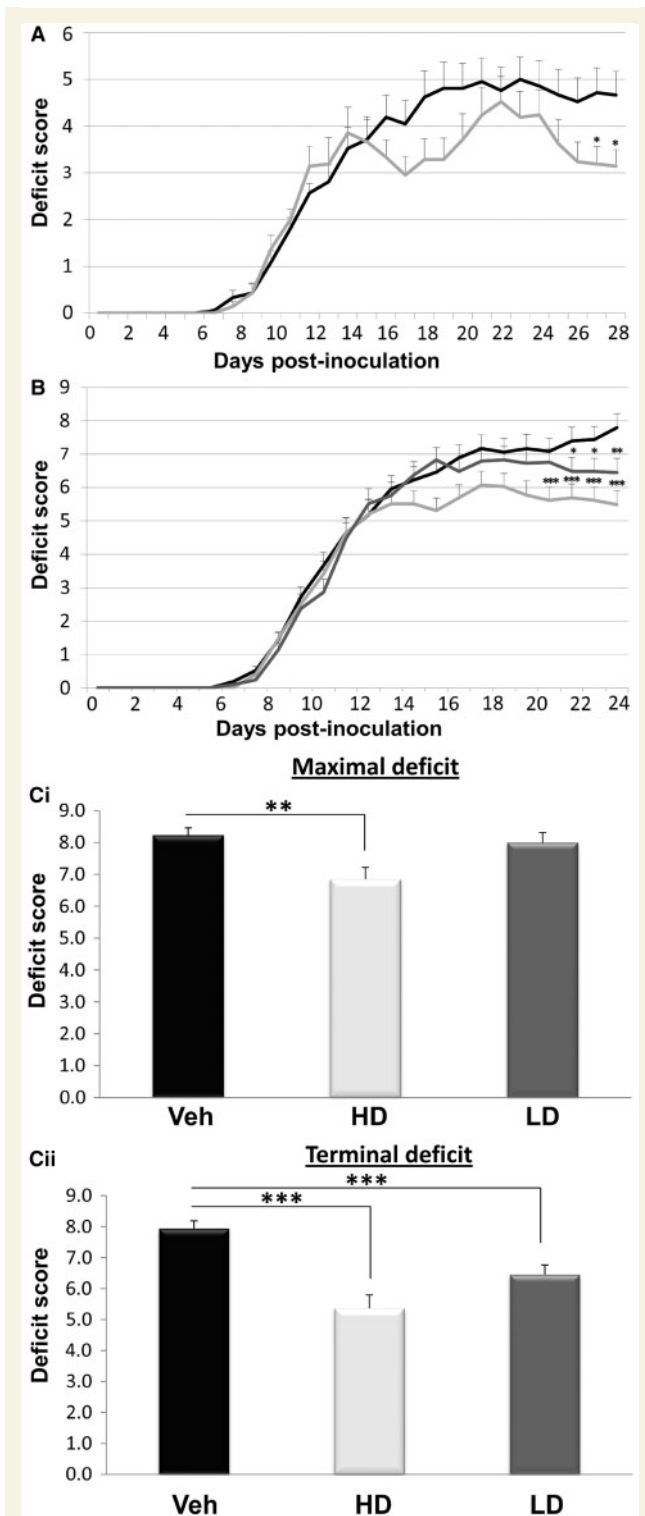


Figure 1 Graphs showing the mean scores for neurological deficit in animals with EAE induced by immunization with spinal cord homogenate (**A**; $n = 44$) or recombinant MOG (**B**; $n = 120$) treated from the onset of disease expression with either vehicle (black), or safinamide at high (light grey) or low (dark grey) dose. Treatment with safinamide reduced the magnitude of neurological deficit, achieving significance towards the end of the trials. Error bars = SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (**C**) Bar charts showing the mean maximal (**Ci**) and terminal (**Cii**) scores for neurological deficit in animals with

or low dose ($P \leq 0.05$ and 0.005). Notably, 65% of control animals terminated the trial with paralysis of both hind limbs, but this occurred in only 10% of animals treated with high dose safinamide. The maximal and terminal scores for the different treatments are shown in Fig. 1C. The protection provided by safinamide was especially pronounced with treatment at the higher dose of the drug, achieving significant protection at both peak and terminal scores ($P \leq 0.005$ and $P \leq 0.001$, respectively).

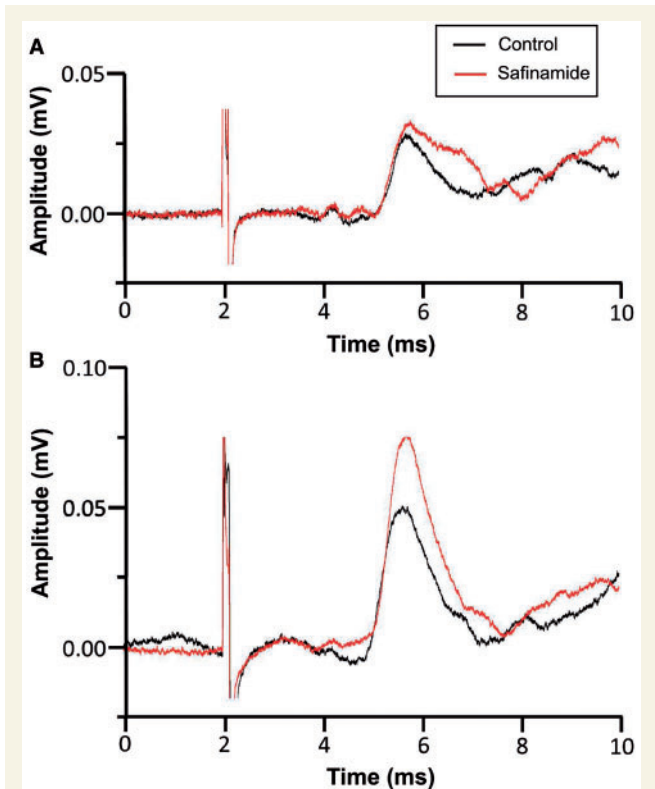


Figure 2 Sensory compound action potentials obtained from animals with (**A**) spinal cord homogenate ($n = 30$) or (**B**) recombinant MOG ($n = 30$) EAE. Each record represents the summed potentials of all the animals in the particular group. The conduction pathway includes the spinal dorsal columns, which are affected by EAE. The amplitude and area of the summed sensory compound action potentials from animals treated with safinamide (high dose) are greater than those of controls (74% and 36% increase in area under the curve in the spinal cord homogenate and recombinant MOG models, respectively), indicating an increase in the number of functioning axons within the dorsal columns in the treated animals. $P \leq 0.05$.

Figure 1 Continued

recombinant MOG EAE treated with either vehicle (Veh; black), or safinamide at high (HD; light grey) or low dose (LD; dark grey). Safinamide provided significant protection from both maximal and terminal neurological deficit, as indicated. Error bars = SEM. ** $P < 0.01$, *** $P < 0.001$.

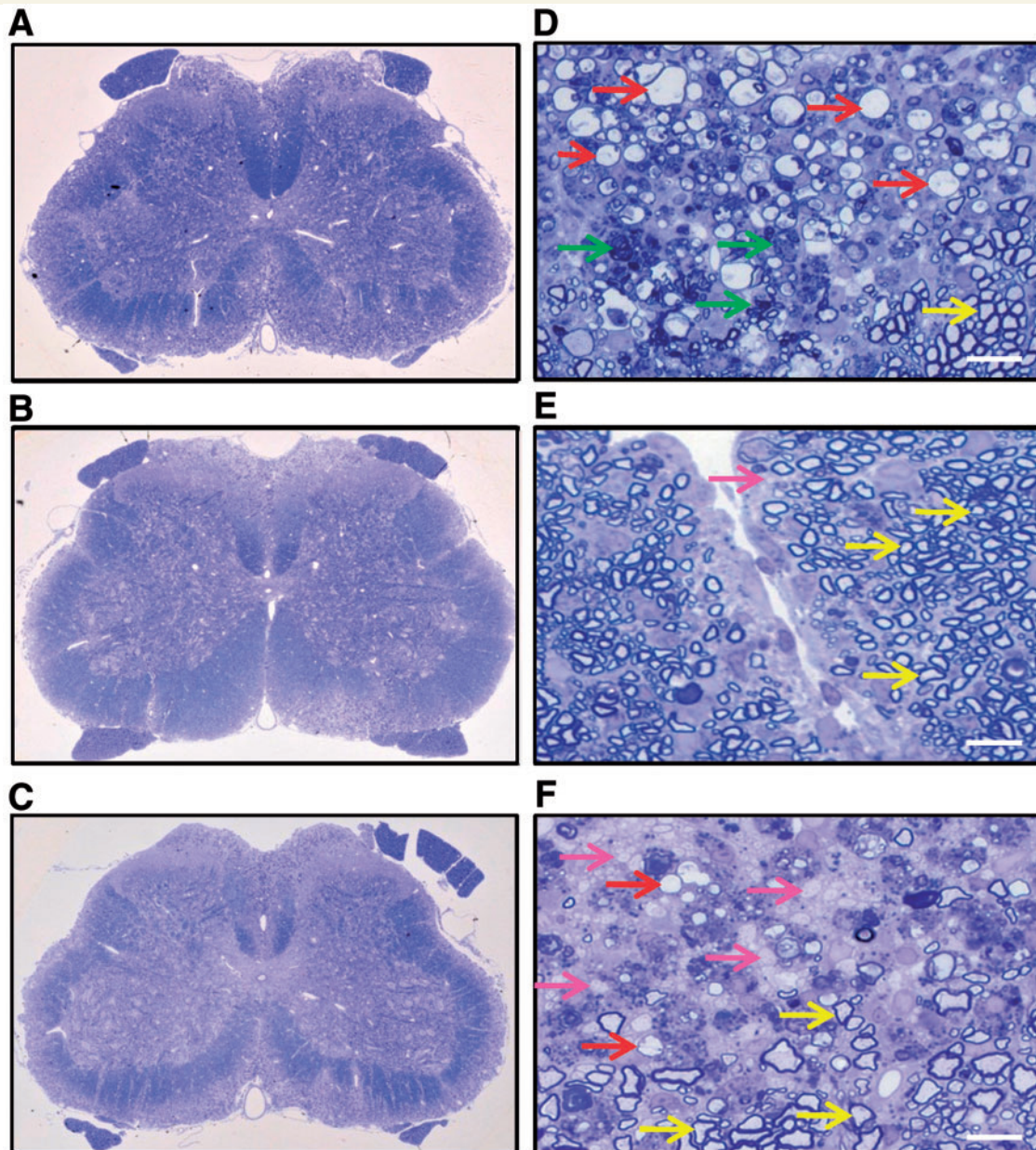


Figure 3 Photomicrographs of high resolution representative transverse sections at low (A–C) and high (D–F) magnification through the spinal cords of animals with recombinant MOG EAE treated with vehicle (A and D), or safinamide at high (B and E), or low (C and F) dose. Degenerated axons (red arrows), macrophages containing myelin debris (green arrows), demyelinated axons (magenta arrows) and myelinated axons (yellow arrows) were easily distinguished in all treatment groups, but there appeared to be more surviving axons (normal and demyelinated) in the groups treated with safinamide. Scale bar = 20 μm .

Electrophysiological assessment of all the animals in the first recombinant MOG trial ($n = 30$; comparing high dose safinamide versus vehicle) at the termination of the trial revealed that the area of the mean sensory compound action potential was 36% greater in animals treated with safinamide than in control animals treated with vehicle. As with spinal cord homogenate EAE, the greater area of the monophasic compound action potential indicates that a greater number of functional axons survived in animals treated with safinamide (Fig. 2B; $P \leq 0.05$).

Histological examination of high resolution resin sections obtained at the termination of the trial revealed the presence of many demyelinated and degenerated axons that were easily distinguished from normal axons (Fig. 3). The pathological axons tended to occur in regions segregated from the normal axons, so that it was possible to draw around areas of pathology, and thereby to express the magnitude of pathology as a percentage of the cross-sectional area of the whole section. In blindly drawn maps, the percentage of the white matter occupied by

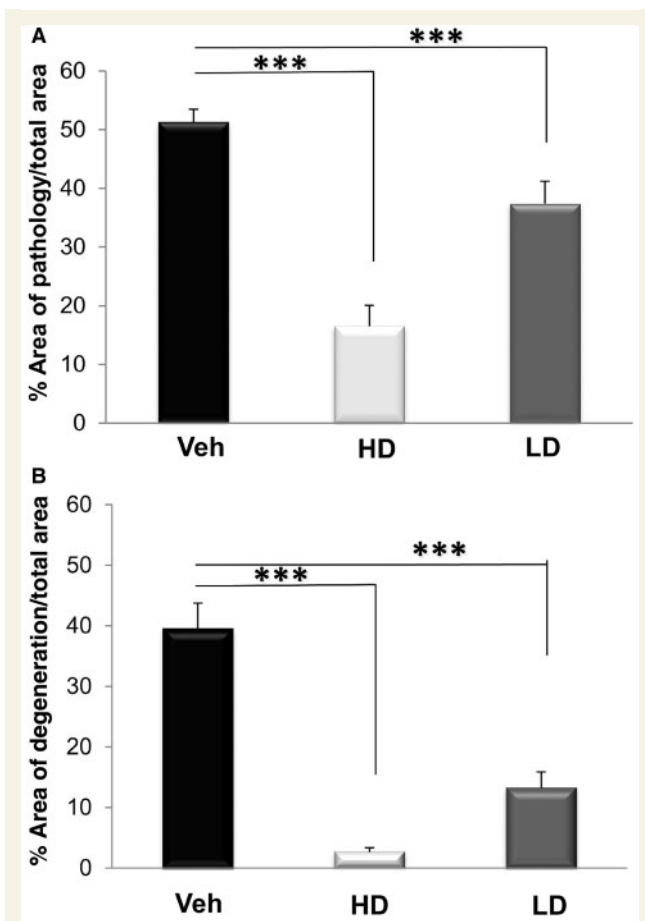


Figure 4 (A) Bar chart showing the effect of therapy with safinamide on the area of pathology (composed of demyelination and degeneration) in animals with recombinant MOG EAE treated with vehicle (Veh; black), or safinamide at high (HD, light grey), or low dose (LD, dark grey). (B) Similar bar chart showing the effect of therapy with safinamide on the area occupied specifically by degenerating axons. Safinamide treatment achieved significant protection from both overall pathology, and from axonal degeneration, which was particularly noticeable at the higher dose of the drug. Error bars = SEM. *** $P < 0.001$.

pathological axons was 52% in the vehicle-treated group, and 17% and 37% in the high and low dose safinamide groups, respectively (Fig. 4A), showing that high and low dose safinamide reduced the area of pathology by 68% ($P < 0.001$) and 28% ($P < 0.001$), respectively. Furthermore, safinamide administered at high dose was more effective in reducing the area of pathology than was the low dose ($P < 0.001$).

When the areas occupied specifically by degenerating axons were measured (blindly), the area occupied by degenerated axons was 39.5% in the vehicle-treated group, but only 2.6% and 13.2% in animals treated with safinamide at high and low doses, respectively (Fig. 4B), representing reductions of 93% ($P < 0.001$) and 67% ($P < 0.001$) in the high and low dose groups, respectively. Once again, the reduction in degenerated

axons was more pronounced in animals treated with safinamide at high dose rather than low dose ($P < 0.001$). In animals treated with vehicle, degeneration of axons was the dominant pathology.

Immunohistochemical examination revealed that both high and low doses of safinamide achieved a clear reduction in the expression of several markers of microglial/macrophage activation compared with animals treated with vehicle alone (Figs 5 and 6). The presence of activated macrophages/microglia was detected by labelling for ED-1 (CD68⁺ cells) and OX-42 (CD11b/c⁺ cells). Cells positive for ED-1 are not present in the normal spinal cord, but appear in large numbers in animals with EAE (Fig. 5). Image analysis revealed that ED-1⁺ cells occupied 65% of the white matter area of the spinal cords obtained from control animals treated with vehicle in recombinant MOG EAE, but safinamide at both high and low doses provided a significant reduction in the area of ED-1-positive cells, from 65% to 46% and 50%, respectively, namely a reduction of 29% ($P < 0.001$) and 23% ($P < 0.01$), respectively (Fig. 6A). In addition to the reduction in ED-1 expression there was a marked change in the phenotype of the ED-1⁺ cells, with the cells in safinamide-treated animals appearing much less activated (Fig. 5E and F). Safinamide treatment at high or low dose also reduced the area of cells positive for OX-42 from 42% (vehicle) to 22% and 31%, respectively, namely a reduction of 48% ($P < 0.05$) and 26% ($P < 0.05$), respectively (Fig. 6B). In animals with EAE treated with vehicle, OX-42-positivity was emphasized on rounded and amoeboid activated microglia that occurred preferentially in the perivascular regions. The number of such activated microglia in the perivascular region was significantly reduced in animals treated with safinamide, irrespective of the administered dose.

Flecainide therapy

The reduction in the area of labelled microglia/macrophages in animals treated with safinamide was clear, but safinamide has actions on both sodium channels and MAOB and so the mechanism(s) by which microglial/macrophage activation was reduced remained ambiguous. We therefore returned to an earlier protocol in spinal cord homogenate EAE in which flecainide (which has no recognized activity on MAOB) was found to provide significant axonal protection (Bechtold *et al.*, 2002, 2004b) and asked in a new trial whether this drug similarly reduced microglial/macrophage activation.

A typical course of spinal cord homogenate EAE in rats treated with flecainide is illustrated in Fig. 7, which also shows the intervals over which tissue was sampled during the first and second peaks of neurological deficit (shaded boxes). Flecainide administration reduced the severity of disability, and this beneficial effect of treatment was most notable in the relapse phase of the disease. Thus rats with EAE treated with vehicle typically experienced much greater disability during relapse (mean deficit score: 5.1 ± 0.89), than rats treated with flecainide (2.4 ± 0.62 ; $P \leq 0.01$) and, as with safinamide, the incidence of severe relapse (deficit score ≥ 6) in rats with EAE was significantly higher in rats treated with vehicle than with flecainide (53% versus 8%).

As in recombinant MOG EAE, ED-1⁺ microglia/macrophages and activated microglia were prominently expressed in the spinal

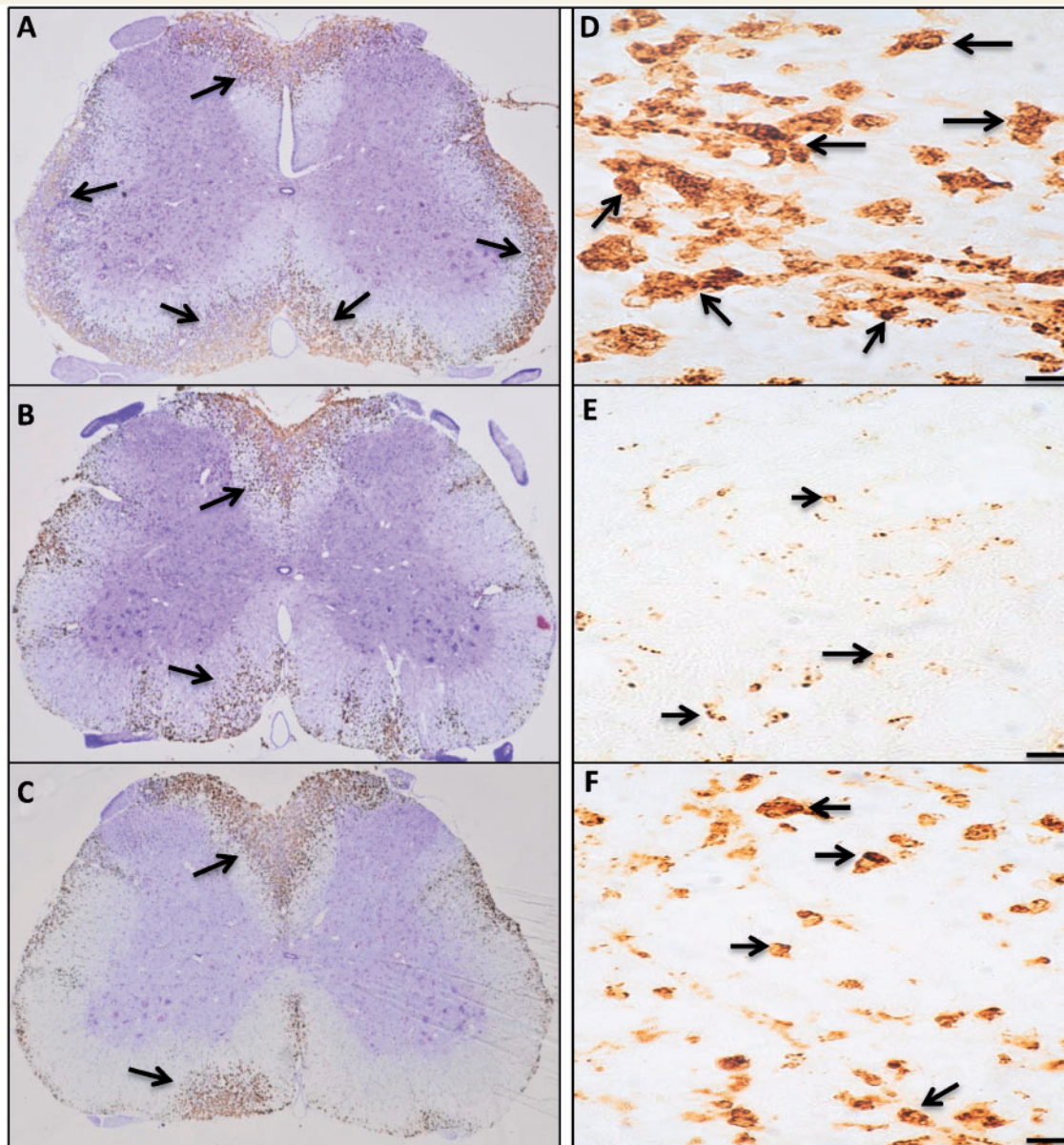


Figure 5 Photomicrographs of representative transverse sections through the spinal cords of animals with recombinant MOG glycoprotein EAE at low (A–C) and high (D–F) magnification, labelled for the expression of ED-1, and counterstained with cresyl violet. The animals were treated with vehicle (A and D), or safinamide at high (B and E), or low (C and F) dose. Activated macrophages/microglia (brown, black arrows) are present, mainly around blood vessels, in all treatment groups, but they appear less activated in animals treated with safinamide, especially when treated with high dose. Scale bar = 20 μ m.

cord during the first and, especially, the second peak of disease expression in spinal cord homogenate EAE (Fig. 8). ED-1 immunopositive cells were primarily observed in close proximity to the pial surface of the spinal cord, and surrounding blood vessels during the first peak of disease, indicative of macrophage infiltration into the CNS of these animals. During the second peak, large numbers of ED-1⁺ cells were also observed throughout the spinal cord parenchyma. In animals treated with vehicle the ED-1⁺ cells often exhibited a rounded cell body with few short processes, but in animals treated with flecainide the cells typically had a more ramified appearance. Image analysis (performed on a minimum of

four sections per animal) revealed that flecainide therapy had no significant effect on the magnitude of ED-1 expression during the first peak of neurological deficit, but it caused a significant reduction in expression during the second peak (vehicle: $11.5 \pm 1.5\%$ of spinal cord area; flecainide: $6.8 \pm 0.9\%$; $P \leq 0.05$).

Inducible nitric oxide synthase was not detected in the spinal cords of normal rats, but it was prominently expressed during the first and, especially, the second peaks of neurological deficit in spinal cord homogenate EAE (Fig. 8). Generally, iNOS⁺ cells were restricted to perivascular cuffs during the first peak of disease, but they extended further into the spinal cord parenchyma

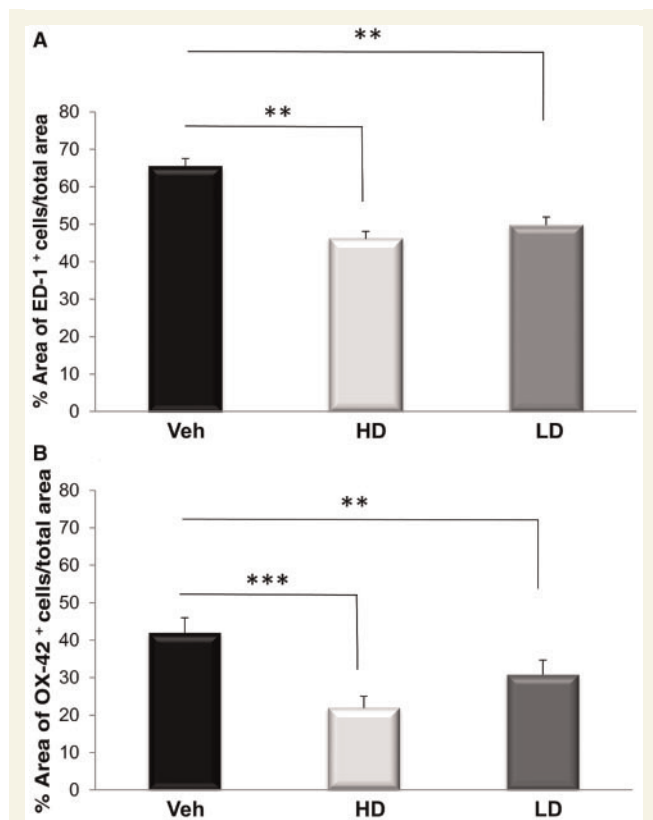


Figure 6 Bar charts showing the area occupied by whole spinal cord area occupied by activated microglia/macrophages (ED-1-positive cells, **A**) or activated microglia (OX-42-positive cells, **B**) in animals with EAE induced by immunization with recombinant MOG treated with vehicle (Veh; black), or safinamide at high dose (HD; light grey), or low dose (LD; dark grey). At both doses, safinamide significantly reduced the number of activated macrophage/microglia cells. Error bars = SEM. * $P < 0.05$, *** $P < 0.001$.

during the second peak. Dual immunolabelling revealed that the iNOS⁺ cells were routinely also ED-1⁺ (not shown). Image analysis confirmed the clear impression gained with casual observation that flecainide therapy reduced the expression of iNOS, particularly during the second peak of disease expression (first peak: vehicle: $1.4 \pm 0.4\%$ of spinal cord area, flecainide: $0.8 \pm 0.2\%$; relapse: vehicle: $2.0 \pm 0.6\%$; flecainide: $0.4 \pm 0.2\%$; $P \leq 0.05$).

Of note, in both treatment groups (flecainide and vehicle) ED-1 and iNOS expression showed a significant correlation with neurological deficit score during disease relapse in spinal cord homogenate EAE rats (Table 1), suggesting that the magnitude of neuroinflammation has a direct impact on neurological function in the animals.

Effects of safinamide on microglia *in vitro*

The results of the experiments with EAE induced by spinal cord homogenate and recombinant MOG indicate that therapy with

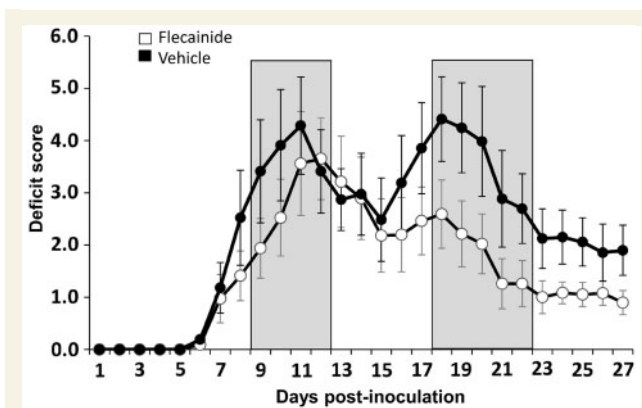


Figure 7 Graph showing the mean scores for neurological deficit in animals with EAE induced by immunization with spinal cord homogenate ($n = 68$) treated with flecainide or vehicle every 12 h from 7 days post-inoculation until termination. Treatment with flecainide reduced the magnitude of neurological deficit, most notably in the relapse phase of the disease. Tissues were collected for assessment of neuroinflammation during the first peak (9–13 days post-inoculation) and disease relapse (18–22 days post-inoculation) as indicated by the shaded areas.

safranamide and flecainide has prominent effects on microglia/macrophages, raising the possibility that the axonal protection observed may be achieved, at least in part, by the direct suppression of microglial activation, or the transformation of microglia to a less activated phenotype. To explore this possibility, the effects of safranamide were examined on cultured microglia *in vitro*, exposed to lipopolysaccharide or PMA and safranamide, for 24 h. As expected, exposure of microglia to PMA (10 ng/ml) or lipopolysaccharide (1 $\mu\text{g/ml}$) for 24 h resulted in a marked increase in superoxide production, but safranamide (0.375 μM) entirely prevented this increase (Fig. 9A). Exposure to safranamide (0.375 μM) for 24 h increased the microglial concentration of the antioxidant glutathione (Fig. 9B); the fluorescence of monochlorobimane was sensitive to ethacrynic acid (1 mM final concentration), indicating that the fluorescence reflected the concentration of glutathione (Keelan *et al.*, 2001).

Discussion

The findings show that safranamide treatment provides significant protection against axonal degeneration and demyelination, and against persisting loss of function, even if its administration is delayed until after the onset of neurological deficit. The beneficial effects of safranamide on axonal and neurological function are achieved despite the fact that safranamide is a sodium channel blocking agent and so may be expected to reduce these functions. Notably, these protective effects occurred on a background of reduced activation of ED-1⁺ and OX-42⁺ microglia/macrophages. Experiments with the classical sodium channel blocking agent flecainide in EAE revealed that the axonal protection achieved by this agent (Bechtold *et al.*, 2002, 2004b) was also achieved on a

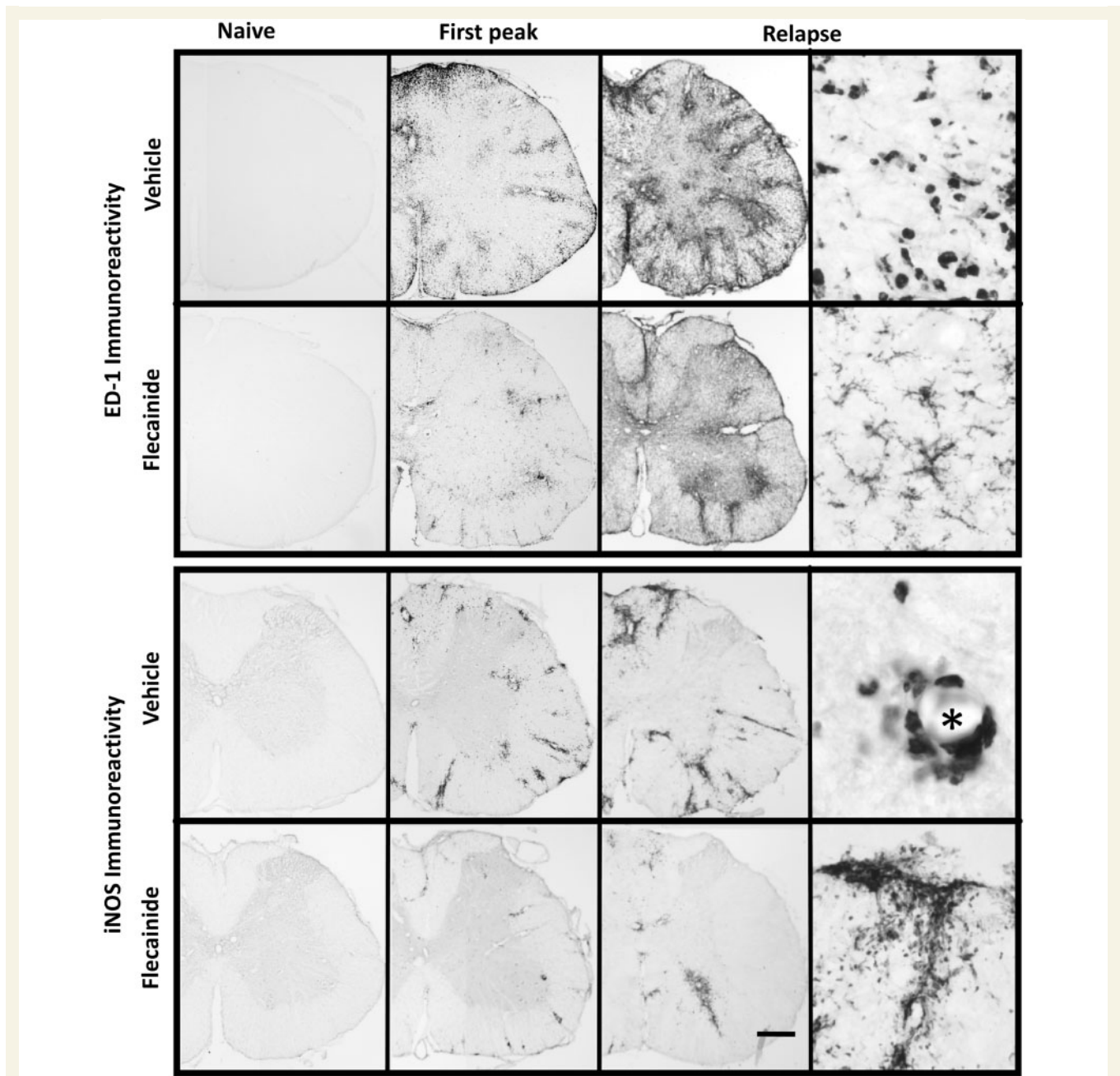


Figure 8 Photomicrographs of representative transverse sections through the spinal cords of animals with spinal cord homogenate EAE at low and high magnification, labelled for the expression of ED-1, or iNOS. The animals were treated with vehicle, or flecainide and sacrificed during the first peak or relapse phase of EAE. Activated macrophages/microglia and iNOS expression are present in all treatment groups, especially during relapse. Flecainide treatment resulted in reduced activation of macrophages/ microglia and reduced expression of iNOS both at first peak and relapse of EAE. Scale bar = 250 μm in low magnification images, 40 μm in the higher magnification images, and 7 μm in the picture of the perivascular cuff (*).

background of reduced microglial/macrophage activation, as indicated by reduced ED-1 and iNOS expression. The possibility that the reduction in innate activation may be instrumental in the axonal protection was supported by our observations *in vitro*, which revealed that safinamide could suppress the formation of superoxide by microglia. Furthermore, safinamide promoted an increased production of glutathione in primary microglial cultures,

indicating that safinamide may induce a more anti-inflammatory microglial phenotype. The beneficial effects of safinamide were achieved when the drug was administered over 2 weeks by mini-pump delivery to provide continuous drug exposure in the range of 4–10 μM . Given that these values are similar to drug levels reported in patients taking 100 mg safinamide on a daily basis in clinical testing (1–8 μM), the findings indicate a potential

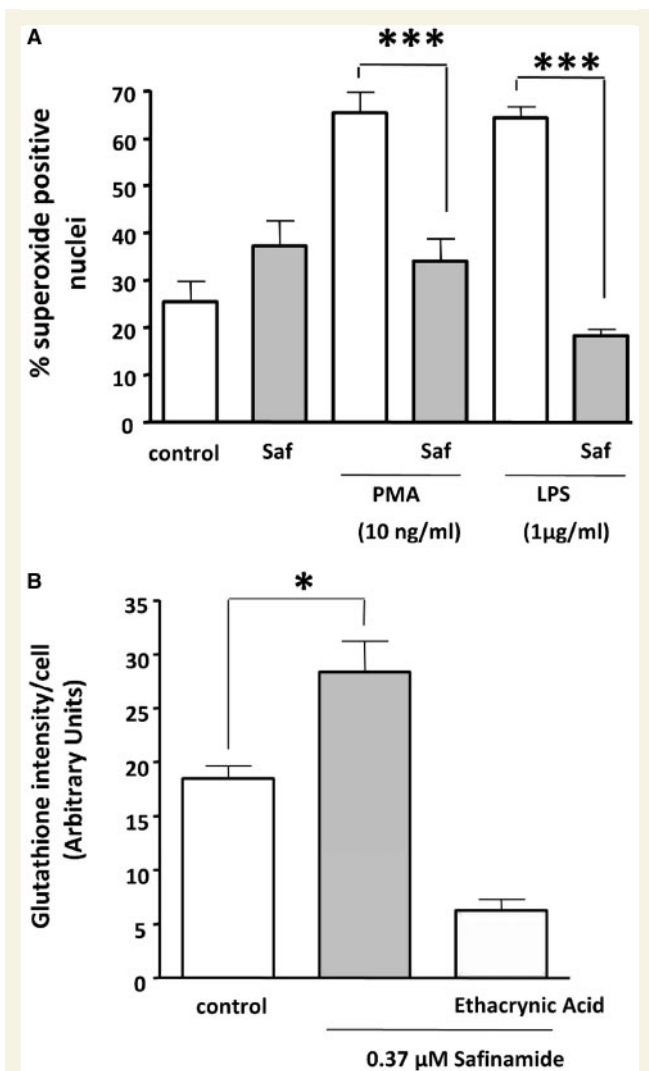


Figure 9 Bar charts showing the effects of safinamide (Saf) on superoxide production (A) and glutathione content (B) in cultured microglial cells exposed to either PMA (10 ng/ml) or lipopolysaccharide (LPS; 1 µg/ml). (A) Safinamide (0.375 µM) prevents superoxide production in PMA- or lipopolysaccharide-activated microglia. (B) In primary microglial culture, exposure to safinamide (0.375 µM; 24 h), enhanced the production of the antioxidant, glutathione. In the presence of ethacrynic acid the monochlorobimane fluorescence was reduced, confirming that it reflected the glutathione concentration. Error bars = SEM. * $P < 0.05$, *** $P < 0.001$.

clinical value for safinamide in axonal protection in multiple sclerosis.

Mechanisms of action

Although it is clear that safinamide is an effective agent for axonal protection, the mechanism(s) by which it achieves this protection remains uncertain, not least because the drug is not only an inhibitor of sodium channels, but also a reversible inhibitor of MAOB (Caccia *et al.*, 2006). However, it seems unlikely that its effects on MAOB will play a dominant role in the current observations, as

EAE is primarily manifest in the spinal cord where there is little expression of MAOB [although some labelling of reactive astrocytes has been described in the spinal cord of patients with amyotrophic lateral sclerosis (Eklom *et al.*, 1994)]. The dose dependence of the reductions in ED1 and OX-42 staining also argues against MAOB inhibition being an important underlying mechanism, since at the brain safinamide levels achieved in these studies even with the low dose, MAOB is predicted to be fully inhibited. Moreover, the non-selective irreversible monoamine oxidase inhibitor phenelzine has been reported not to reduce microglial activation or lymphocyte infiltration in the mouse MOG35-55 variant of the EAE model (despite improving some other aspects of EAE, effects tentatively attributed to the normalization of ventral horn 5-HT levels), at a dose where changes in brain neurochemistry and behaviour attested to effective inhibition of brain enzyme activity (Musgrave *et al.*, 2011). In contrast, the beneficial effects of safinamide in EAE are shared by several other sodium channel blocking agents, as noted above, indicating that this property of the drug is most likely to be important in achieving white matter protection.

Two major pathways may be involved in the protection of axons by sodium channel blockade: (i) a direct effect on axons; and (ii) a direct effect on microglia/macrophages. First, evidence suggests that axons may succumb in EAE due to energy insufficiency resulting in part from nitric oxide-mediated mitochondrial inhibition (Bechtold and Smith, 2005; Trapp and Stys, 2009). If so, axons, and particularly demyelinated axons, may accumulate sodium ions by virtue of a lack of ATP to power the Na^+/K^+ ATPase (i.e. the sodium pump), resulting in a lethal series of complications including calcium entry by reverse action of the $\text{Na}^+-\text{Ca}^{++}$ exchanger (Stys, 2005; Trapp and Stys, 2009). Partial sodium channel blockade would alleviate the accumulation of sodium ions, thereby reducing the likelihood of lethal complications. The validity of this rationale was supported in experiments where flecainide was shown to protect axons from degeneration resulting from sustained impulse activity in the presence of nitric oxide (Kapoor *et al.*, 2003). An additional effect of sodium channel blockade on immune cells has been suggested by observations that neuroinflammation is reduced in white matter spinal cord of animals with EAE treated with flecainide (Bechtold *et al.*, 2004a), phenytoin (Craner *et al.*, 2005; Black *et al.*, 2007) or carbamazepine (Black *et al.*, 2007). It remains unclear from these studies whether the reduction in inflammation was a consequence of the reduction in degeneration, which may have been driving the inflammation, or whether the reduction in inflammation was responsible for the reduction in degeneration. However, evidence for a direct effect on microglia was provided by the demonstration that tetrodotoxin (a specific sodium channel blocking agent) and phenytoin were effective in reducing several aspects of microglial and macrophage activation *in vitro*, and the later demonstration of $\text{Na}_v1.1$, $\text{Na}_v1.5$ and $\text{Na}_v1.6$ sodium channels in monocytes and microglia provided a pathway by which the effects may be achieved (Craner *et al.*, 2005; Carrithers *et al.*, 2007; reviewed in Black *et al.*, 2009; Black and Waxman, 2012). In the current study safinamide was shown to act directly on microglia in culture, diminishing the formation of superoxide and increasing glutathione levels, supporting an interpretation that direct effects on

microglia are an important component of the axonal protection achieved. The safinamide appeared to suppress superoxide generated intracellularly by NADPH oxidase activity, as the drug blocked superoxide production evoked by PMA exposure (Fig. 9A) (Mead *et al.*, 2012). Oxidative damage is known to promote axonal degeneration and it has recently been implicated in active demyelination, and in neuronal and axonal injury, in lesions in multiple sclerosis (Haider *et al.*, 2011; Smith, 2011). Microglia are a major source of oxidative free radicals, so the current findings in this regard encourage confidence that therapy based on the blockade of sodium channels may be beneficial in multiple sclerosis (and possibly in a range of neurological disorders in which the innate immune system plays a prominent role).

The increase in glutathione levels raises the new possibility that safinamide may not only diminish damaging aspects of microglial activation, but also promote the expression of a more protective microglial phenotype, with increased expression of an antioxidant. Increased glutathione in microglia may not only reduce stress responses in these cells, which unbridled can trigger death in neighbouring cells, but may also contribute to the glial pool of glutathione required for neuronal protection (Fernandez-Fernandez *et al.*, 2012).

Clinical

The need for an effective neuroprotective therapy in multiple sclerosis is emphasized by the fact that axonal degeneration is an important cause of permanent neurological deficits in the disease (Tallantyre *et al.*, 2010). As noted above, several studies have described very beneficial effects of a range of sodium channel blocking agents in different models of multiple sclerosis, and these observations, coupled with the good safety record of such drugs, recommend sodium channel blockade as a potential strategy for neuroprotection in multiple sclerosis. The initial optimism for this approach was however dampened by the results from a trial of lamotrigine in secondary progressive multiple sclerosis (Kapoor *et al.*, 2010), because treated patients showed an initial, unexpected loss of brain volume, and the drug was not as well tolerated as expected, due to a dose-related suppression of function that was most apparent in more severely affected patients. It may, however, be premature to dismiss sodium channel blockade as a potential therapeutic strategy.

In retrospect, and now armed with current knowledge about the presence of pronounced inflammation in secondary progressive multiple sclerosis (Frischer *et al.*, 2009), the loss of brain volume can be interpreted as a positive sign, indicating drug efficacy in suppressing inflammation and swelling. Indeed, this view corresponds with the improved ambulatory performance and reduced concentration of circulating neurofilament (a marker of neurodegeneration) in patients with multiple sclerosis treated with lamotrigine. In fact, active demyelination and degeneration are only seen in patients with pronounced inflammation (Frischer *et al.*, 2009), suggesting that suppression of inflammation and swelling may be a necessary component of effective neuroprotection. If so, an initial loss of brain volume, termed pseudoatrophy, may be an inevitable consequence of effective therapy, and indeed it has been observed during the first few months of

therapy with some immunomodulatory drugs (Frank *et al.*, 2004; Hardmeier *et al.*, 2005; Miller *et al.*, 2007). The occurrence of pseudoatrophy can also explain why brain volume partly rebounded upon the phased removal of lamotrigine at the end of the clinical trial (Kapoor *et al.*, 2010), an observation reminiscent of the resumption of CNS inflammation upon the withdrawal of sodium channel therapy in a mouse model of EAE (Black *et al.*, 2007).

It is predictable that the balance of beneficial and deleterious effects of therapy will be different for different drugs, and perhaps partly dependent on actions on targets and tissues not necessarily within the nervous system. Differences in the relative selectivity for the various sub-types of sodium channels will also play a role, especially given that the deleterious effects regarding tolerance will probably be mainly mediated by effects on axons and their complement of sodium channels, and the beneficial effects partly mediated by actions on microglia and macrophages, which will probably have a different balance of channels (Nicholson and Randall, 2009; Black and Waxman, 2012). In this case it is notable that safinamide is proving to be well tolerated in patients with Parkinson's disease, at a similar plasma concentration as was effective in achieving axonal protection in the current study. It is not unreasonable to suspect that at least some of the mechanisms responsible for loss of function in Parkinson's disease may be similar to those in multiple sclerosis, in which case safinamide may be well tolerated by patients with multiple sclerosis.

With respect to tolerability, it is also worth considering that to date, the dose chosen for sodium channel blocking agents has been influenced by the dose demonstrated to be effective in other neurological diseases, such as epilepsy, but if the primary target is actually the innate immune system rather than the axons, it may be possible to reduce the dose (thereby avoiding issues of tolerability), but still retain efficacy in neuroprotection.

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