



# SFX-01 reduces residual disability after experimental autoimmune encephalomyelitis

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## ABSTRACT

**Background:** Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) is a master transcriptional regulator of the protective cellular response to oxidative stress. Sulforaphane is a Nrf2 activator but is unstable at ambient temperature. SFX-01 is a novel composition comprised of synthetic sulforaphane stabilised within the pocket of an  $\alpha$ -cyclodextrin complex. Here we tested the efficacy of SFX-01 in murine relapsing experimental autoimmune encephalomyelitis (EAE), a model of relapsing-remitting MS (RRMS).

**Methods:** Relapsing EAE was induced in female SJL mice using immunization against PLP<sub>139-151</sub>. In the therapeutic experiment, the aim was to model initiation of treatment after diagnosis in RRMS, so treatment was started at day 19, one day prior to the expected relapse onset. In the prophylactic experiment, mice were treated from the time of immunization and followed for three weeks.

**Results:** SFX-01 reduced residual disability in both experiments. Most of this effect was mediated by a decrease in maximum severity of relapses and improved recovery during follow-up. Histological examination of the spinal cord was consistent with the clinical findings, with improvement in demyelination and the number of apoptotic cells, but not inflammatory cell infiltration, compared to the vehicle group.

**Conclusions:** SFX-01 is efficacious in EAE. In first-in-man and phase II clinical trials for other indications, SFX-01 was found to be well-tolerated. A trial comparing BG-12 and SFX-01 would address whether SFX-01 can offer RRMS patients a better option with respect to efficacy and tolerability.

## 1. Introduction

Oxidative stress secondary to cell-mediated inflammation plays an important role in the pathology of multiple sclerosis (MS) (Lassmann et al., 2016; Ohl et al., 2016). Oxidized lipids and nucleic acids occur in many types of cells within MS plaques, including oligodendrocytes, astrocytes and neurones, correlating with inflammation and loss of neuronal dendritic integrity (Haider et al., 2011).

Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) is a master transcriptional regulator of the protective cellular response to oxidative stress (Copple, 2012). Under physiological conditions Nrf2 is bound by Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1) which targets Nrf2 for ubiquitinylation. By virtue of multiple cysteine sulfhydryl groups, Keap1 is an intracellular redox state sensor. Under conditions of oxidative stress, Keap1 is oxidized and does not bind Nrf2, allowing Nrf2 to evade ubiquitinylation and accumulate in the nucleus where it binds to anti-oxidant response elements upstream of cytoprotective genes.

In MS lesions, nuclear Nrf2 reactivity varies by cell type (Licht-

Mayer et al., 2015). While adequate in astrocytes and oligodendrocytes, neuronal nuclear Nrf2 is very low, even in areas with extensive acute oxidative injury. Upregulation of Nrf2 protects neurones against oxidative stress (Scannevin et al., 2012). In Nrf2 knockout mice, experimental autoimmune encephalomyelitis (EAE), an animal model of MS, was worse (Johnson et al., 2010). Conversely, EAE was ameliorated by Nrf2 induction (Linker et al., 2011). Pharmacological upregulation of Nrf2 is therefore a promising therapeutic avenue in MS. A mainstay MS treatment, dimethyl fumarate, upregulates Nrf2 but it also has other mechanisms such as downregulation of aerobic glycolysis in myeloid and lymphoid cells via succination of glycolytic enzymes (Kornberg et al., 2018).

Sulforaphane is a naturally occurring isothiocyanate which stabilizes Nrf2 via an interaction with Keap1 thereby inhibiting the ubiquitinylation of Nrf2 and allowing for nuclear accumulation and activation of its transcriptional programme. The electrophilic modification of critical cysteine residues in Keap1 by sulforaphane appears to be essential (Dinkova-Kostova et al., 2017), but subsequent mechanistic steps are unclear. Sulforaphane penetrates the blood-brain barrier

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(Clarke et al., 2011; Jazwa et al., 2011) and a previous study has demonstrated sulforaphane's efficacy in EAE (Li et al., 2013). However sulforaphane is not practical to administer in clinical practice as it is highly reactive and unstable at ambient temperature. To circumvent this issue, a stable, solid form, pharmaceutical product has been developed; SFX-01 is a novel composition comprised of synthetic sulforaphane stabilised within the pocket of an  $\alpha$ -cyclodextrin complex. In this study we investigated the efficacy of SFX-01 to reduce disability in relapsing EAE. Patients with relapsing-remitting MS (RRMS) start treatment after diagnosis, so we performed a therapeutic experiment during which dosing started after EAE onset but prior to the next relapse. We also performed a prophylactic experiment, when dosing was started prior to EAE onset.

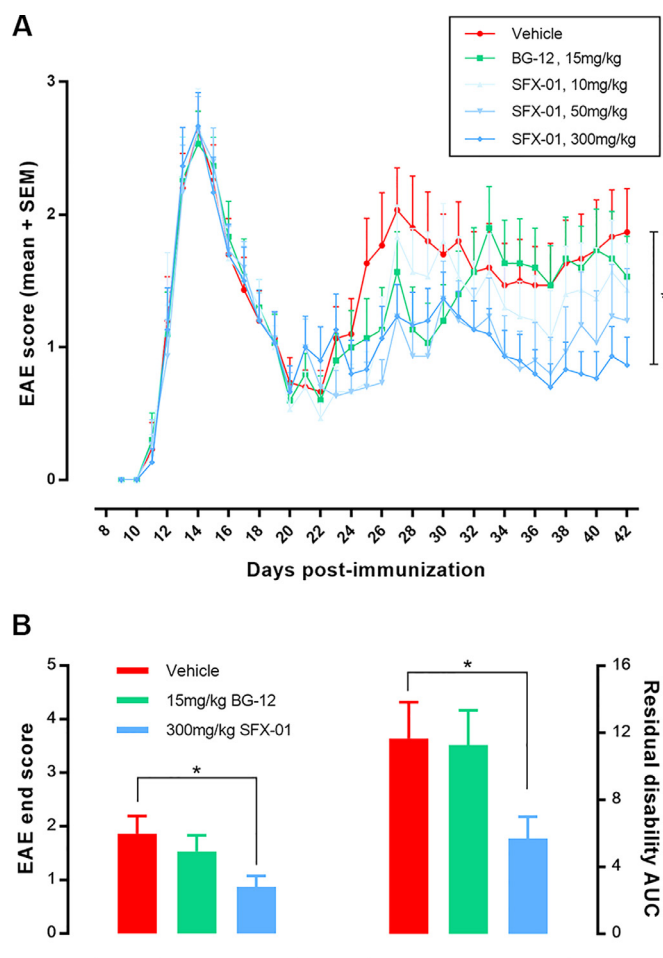
## 2. Methods

Female SJL mice, purchased from Jackson Laboratories, were acclimatized to the research facility for four weeks prior to the start of the study. Mice were kept in a temperature controlled environment, with a 12 h light/dark cycle and access to food and water *ad libitum*. Experiments were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> (McRae et al., 1992). This induces disease onset in 90–100% of animals at 11 to 15 days after immunization. After remission, which may be partial or complete, a relapse occurs in 50–80% of animals at 20–40 days after immunization. Mice were injected with: (1) 0.2 ml of an emulsion containing 0.2 mg PLP<sub>139-151</sub> in complete Freund's adjuvant (CFA) with 0.2 mg killed *Mycobacterium tuberculosis* H37Ra/mL (Hooke Kit EK-0120, Hooke Laboratories, Lawrence MA), subcutaneously in the back, distributed equally between four sites, and (2) 1  $\mu$ g of pertussis toxin (Hooke Laboratories) in 0.1 ml sterile phosphate-buffered saline (PBS) intraperitoneally. For therapeutic experiments pertussis toxin was omitted in order to increase relapse rate (Marusic et al., 2012).

EAE was scored daily, starting on day 8 or 9 after immunization, until the mice were humanely killed. The standard scoring system was used, whereby 0 = no paralysis, 1 = loss of tail tone, 2 = hindlimb weakness, 3 = hindlimb paralysis, 4 = hindlimb and forelimb paralysis, and 5 = moribund and death. Scoring was blinded to treatment and previous scores for each mouse. All mice contributed to the EAE scoring.

In the prophylactic experiment, the aim was to prevent or decrease severity of EAE at onset, so mice were treated from the time of immunization and followed for three weeks. In the therapeutic experiment, the aim was to decrease severity of the relapse, so treatment was started at day 19, one day prior to the expected relapse onset. Mice which had by day 19 developed clinical signs of EAE were assigned to the experimental groups. By this time, these mice had partially recovered from the first wave of EAE and had an average EAE score of approximately 1.0. Assignment was performed in a balanced manner to achieve groups with similar average time of EAE onset, similar average mean maximum score (the maximum clinical score reached, as a mean for each group) of the first wave of EAE and similar EAE scores at the time of assignment to treatment groups. Groups of 15 mice received vehicle, SFX-01 (Evgen, Cheshire, UK) at 10, 50 or 300 mg/kg (equivalent to a sulforaphane dose of 1.5, 7.7 and 46 mg/kg respectively) or dimethyl fumarate (BG-12) at 15 mg/kg, orally, twice a day. All drugs were dissolved in the vehicle, 0.5% carboxymethyl cellulose. Dosing occurred between day 0 and day 19 in the prophylactic experiment, and between day 19 and day 41 in the therapeutic experiment. In both therapeutic and prophylactic experiments, two clinical readouts were used as markers of residual disability: the EAE score at the end of the experiment, and the area-under-the-curve for EAE scores during the last six days of the experiment.



**Fig. 1.** Therapeutic experiment. EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> and CFA. Treatment was started at day 19, one day prior to the expected onset of the relapse. EAE was scored daily, starting on day 8 or 9 after immunization, blinded to treatment group. A: Whole timeline; B: End score and residual disability AUC (area-under-the-curve over the last 6 days). n = 15 per group. SEM = standard error of mean. \* p < 0.05.

In the therapeutic experiment, all mice recovered from their first relapse by day 20, six days after peak disability. Peak disability during the second relapse occurred on day 27 (Fig. 1). In order to assess residual disability, we waited for significantly longer than six days after the second relapse, specifically two weeks, to ensure that mice had recovered well from the relapse.

At the end of the experiment, mice treated with vehicle, BG-12 and 300 mg/kg SFX-01 were perfused with PBS and spinal cords were harvested in 10% buffered formalin. Spinal cord sections were developed for haematoxylin and eosin (H&E) staining and Luxol Fast Blue (LFB) histochemistry. Inflammatory foci of at least 20 mononuclear cells were counted in each whole H&E stained section. When inflammatory infiltrates consisted of more than 20 cells, an estimate was made of how many foci of 20 cells were present. Apoptotic cells, characterized by small pyknotic ( $\pm$  fragmented) nuclei and scanty hyper eosinophilic cytoplasm, were counted per section. Demyelination on LFB-stained sections was quantified as follows: 0 = less than 5% demyelinated area, 1 = 5 to 20%, 2 = 20 to 40%, 3 = 40 to 60%, 4 = 60 to 80%, 5 = 80 to 100%. Three sections from lumbar, thoracic and cervical spinal cord, from each mouse, were analysed and then averaged. All histological analysis was performed by an experienced pathologist blinded to the experimental groups and all clinical readouts. Plots were prepared in GraphPad Prism v7 and statistical analysis was performed in SPSS v23. Mann-Whitney test, Fisher exact test, and

**Table 1**  
Relapses in the therapeutic experiment. Relapse occurrence and their peak severity.

	Relapsing mice	Non-relapsing mice	Total number	<i>p</i> value <sup>b</sup>	Peak severity of relapse <sup>a</sup>	<i>p</i> value <sup>c</sup>
Vehicle	11	4	15	N/A	3.5 (1)	
BG-12 15 mg/kg	11	4	15	1	3.5 (1)	0.684
SFX-01 10 mg/kg	11	4	15	1	3.0 (1)	0.564
SFX-01 50 mg/kg	6	9	15	0.139	3.5 (0.63)	0.288
SFX-01 300 mg/kg	9	6	15	0.7	2.5 (1.25)	0.022

<sup>a</sup> (median and inter-quartile range). EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> and CFA. Treatment was started at day 19, one day prior to the expected onset of the relapse. EAE was scored daily, starting on day 8 or 9 after immunization, blinded to treatment group. *p* values.

<sup>b</sup> Fisher exact test *versus* vehicle.

<sup>c</sup> Mann-Whitney test *versus* vehicle. N/A: not applicable.

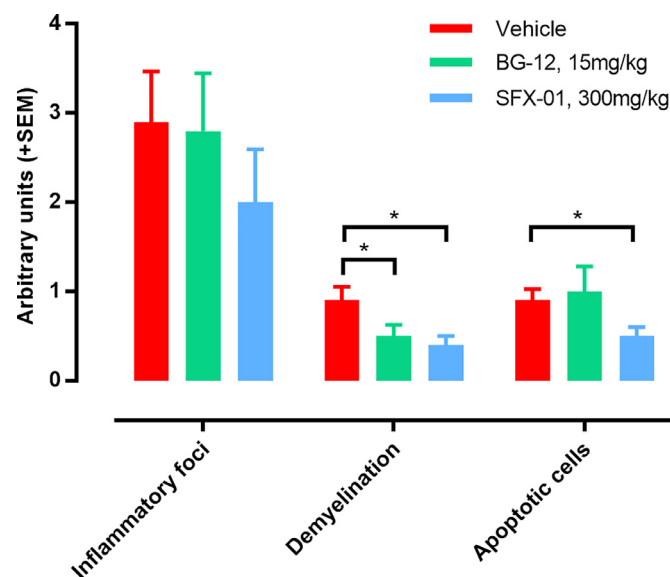
Kaplan-Meier survival analysis were used to test two-tailed hypotheses with alpha of 0.05.

### 3. Results

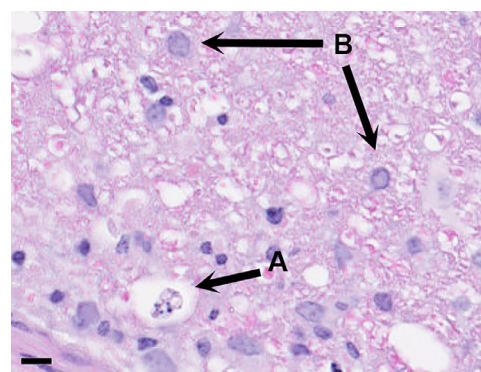
In the therapeutic experiment, the aim was to decrease relapse-related disability, so treatment was started at day 19, one day prior to the expected onset of relapse. SFX-01 caused a dose-dependent reduction in residual disability as measured by the end EAE score and the area-under-the-curve for the EAE score during the last six days, both of which were significantly different from vehicle at the 300 mg/kg dose ( $p = 0.026$  and  $p = 0.041$  respectively, Mann-Whitney test, Fig. 1A, B). 15 mg/kg BG-12 did not affect residual disability.

This decrease in residual disability could have occurred as a result of the absence of a relapse, a decrease in maximum severity of the relapse, or recovery from a relapse. Relapses occurred at a slightly lower frequency with the 50 and 300 mg/kg SFX-01 doses *versus* vehicle, but this was not significant and was not dose-dependent (Table 1). There was a lower EAE score at peak severity of the relapse with 300 mg/kg SFX-01 dose *versus* vehicle ( $p = 0.022$ , Mann-Whitney test, Table 1). In a Kaplan-Meier analysis of mice sustaining a relapse, the time taken to recover back to pre-relapse disability was significantly shorter for 300 mg/kg SFX-01 *versus* vehicle (log-rank  $p = 0.041$ , Fig. 2).

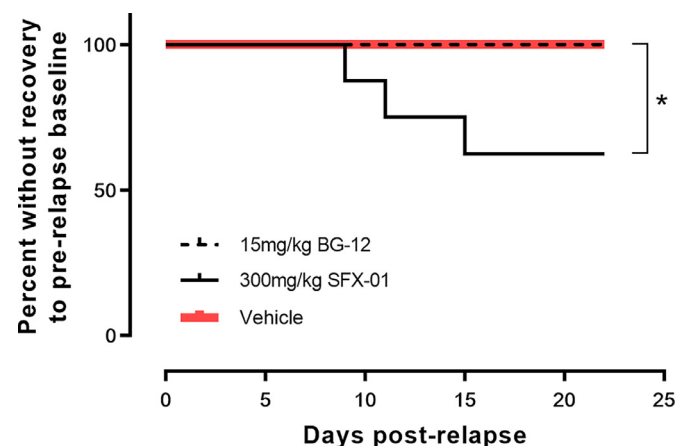
Histological examination of the lumbar, thoracic, and cervical spinal cord was consistent with the clinical findings. In animals treated with 300 mg/kg SFX-01, demyelination and the number of apoptotic cells (Figs. 3 and 4), but not inflammatory foci, were significantly improved compared to the vehicle group (Figs. 3 and 5). In animals treated with 15 mg/kg BG-12, only demyelination was significantly



**Fig. 3.** Histopathology at day 41 in the therapeutic experiment. EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> and CFA. Treatment was started at day 19, one day prior to the expected onset of the relapse. Inflammatory foci (H&E stain), demyelination (Luxol Fast Blue) and apoptotic cells (morphology) were quantified in spinal cord sections by a pathologist blinded to treatment group. SEM = standard error of mean. \*  $p < 0.05$ .



**Fig. 4.** Apoptotic cell quantification using nuclear morphology. A: Apoptotic nucleus. B: healthy nuclei. Scale bar: 10  $\mu$ m.

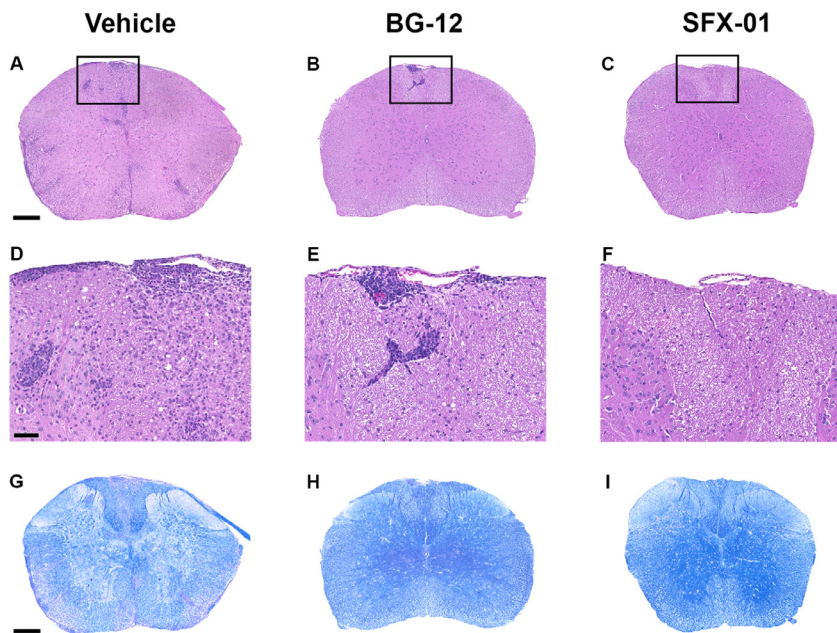


**Fig. 2.** Kaplan-Meier survival curve analysis of mice treated with vehicle, BG-12 and 300 mg/kg SFX-01 in the therapeutic experiment. EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> and CFA. Treatment was started at day 19, one day prior to the expected onset of the relapse. EAE was scored daily, starting on day 8 or 9 after immunization, blinded to treatment group. \*  $p < 0.05$ .

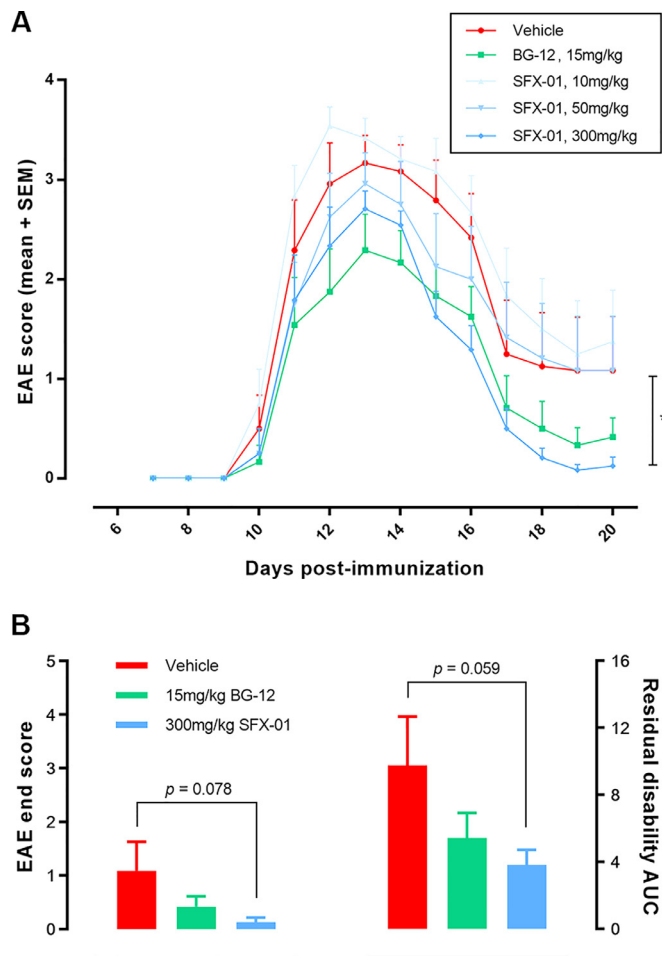
improved.

The therapeutic experiment suggested that SFX-01's mechanism of action was mainly by reducing peak and residual disability from relapses, more so than by preventing relapses. We sought corroboration of these findings in a prophylactic experiment during which treatment was administered prior to EAE onset, from day 0. All animals developed EAE. Once again, SFX-01 caused a dose-dependent reduction in residual





**Fig. 5.** Histopathology at day 41 in the therapeutic experiment. EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> and CFA. Treatment was started at day 19, one day prior to the expected onset of the relapse. Inflammatory foci (H&E stain: A, B, C and corresponding inset in D, E, F) and demyelination (Luxol Fast Blue: G, H, I) were quantified in spinal cord sections by a pathologist blinded to treatment groups (A, D, G: vehicle; B, E, H: 15 mg/kg BG-12; C, F, I: 300 mg/kg SFX-01). Scale bar = 250  $\mu$ m (A-C, G-I), 50  $\mu$ m (D-F).



**Fig. 6.** Prophylactic experiment. EAE was induced at 10 weeks of age by immunization against PLP<sub>139-151</sub> with CFA and pertussis toxin. Treatment was started from the time of immunization. EAE was scored daily, blinded to treatment group. A: Whole timeline; B: End score and residual disability AUC (area-under-the-curve over the last 6 days).  $n = 12$  per group. SEM = standard error of mean. \*  $p < 0.05$ .

disability, compared to vehicle, as measured by the end EAE score and the AUC for the EAE score during the last six days, both of which approached significance at the 300 mg/kg dose ( $p = 0.078$  and  $p = 0.059$  respectively, Mann-Whitney test, Fig. 6A, B). BG-12 did not affect residual disability ( $p = 0.567$  and  $p = 0.271$  respectively, Mann-Whitney test).

#### 4. Discussion

In this study, we investigated the therapeutic potential of SFX-01, a stable pharmaceutical form of sulforaphane currently in phase II clinical trials for other indications, in a model of RRMS in mice. SFX-01 reduces residual disability when administered after EAE onset. Most of this effect was mediated by a decrease in maximum severity of relapses and improved recovery during follow-up. Although there was some evidence of reduction in relapse frequency with 50 and 300 mg/kg of SFX-01, this was not significant at the sample size used in this study, and it was not dose-dependent. When SFX-01 was administered prior to EAE onset, a similar pattern was observed with no reduction in EAE onset, but peak EAE severity and residual disability were lower with 300 mg/kg of SFX-01, *versus* vehicle. EAE onset is triggered after myelin-specific lymphocytes in the systemic compartment are primed and then traffic into the central nervous system. Since EAE onset and relapses were not significantly decreased by SFX-01, it appears that SFX-01's effect on residual disability was mediated by other mechanisms such as an increased resilience of oligodendrocytes to oxidative damage associated with inflammation. *In vitro*, sulforaphane protected an oligodendrocyte cell line from oxidative toxicity after exposure to tert-butyl hydrogen peroxide by upregulating Nrf2-inducible anti-oxidant proteins (Lim et al., 2016). Despite low neuronal expression of Nrf2, sulforaphane also protected neurones in primary cortical cultures from hydrogen peroxide oxidative toxicity, an effect that was dependent on Nrf2 activity in astrocytes (Kraft et al., 2004). In line with this interpretation, 300 mg/kg SFX-01 decreased demyelination and apoptotic cells bodies, but not inflammatory foci.

During these experiments, 15 mg/kg of BG-12 was administered as a positive control, since this dose of BG-12 was previously found to be effective in EAE (Schilling et al., 2006). BG-12 is a Nrf2-inducer in clinical use as Tecfidera (Brennan et al., 2015). Sulforaphane exhibits an order of magnitude greater potency towards Nrf2 compared to BG-12 (Copple et al., 2014). In another study, Nrf2 induction in neurones

was similar between sulforaphane and BG-12, even though sulforaphane was used at a six fold lower concentration than BG-12 (Petrillo et al., 2017). BG-12 prevents relapses in MS at a dose of 240 mg twice daily (Xu et al., 2015), which would equate to a dose of 42 mg/kg in mice using established guidelines for allometric dose conversion between mice and man (Reagan-Shaw et al., 2008). SFX-01 is currently in clinical trials at a dose of 300 mg twice daily and, using the same guidelines, this equates to 52.5 mg/kg in mice. Hence no inferences can be made at the moment regarding relative efficacy of BG-12 and SFX-01 in man.

This study has a number of other limitations. Although EAE may recapitulate some aspects of MS, it does not model the disease entirely (Lassmann and Bradl, 2017). The mechanism of action of SFX-01 was not explored in detail. Future studies are needed to confirm the hypothesis that SFX-01's main effect is via upregulation of Nrf2-mediated anti-oxidant proteins, as opposed to lymphocyte activation and trafficking into the central nervous system. Sulforaphane crosses the blood-brain barrier (Clarke et al., 2011; Jazwa et al., 2011), and since SFX-01 releases sulforaphane in the gastrointestinal tract and from there into the systemic circulation, one would expect the same blood-brain barrier permeability, although this requires confirmation by measurement of levels of SFX-01 in the brain and spinal cord. SFX-01 holds promise as a disease-modifying drug in progressive MS and future studies could explore its therapeutic utility in the Biozzi model of secondary progressive MS (Hampton et al., 2008).

Currently BG-12 is the most commonly prescribed oral disease-modifying agent for RRMS, yet it causes troublesome flushing and gastrointestinal side effects which impact considerably on adherence to treatment. In a real-life setting, 80% of patients on BG-12 reported AEs, and 28% discontinued the drug (Sejbaek et al., 2018). Of those patients stopping BG-12, 77% stopped because of side effects, half of which were gastrointestinal in origin. BG-12 may also cause a lymphopenia which when severe has been associated with progressive multifocal leukoencephalopathy (Lehmann-Horn et al., 2016), so patients need regular blood test monitoring and the drug is discontinued if lymphopenia severity reaches Grade III. Therefore, it is important to identify more tolerable therapeutic agents that retain efficacy in RRMS patients.

SFX-01 has undergone first-in-man phase I clinical trials (NCT01948362 and NCT02055716) and is currently in phase II studies for subarachnoid haemorrhage (NCT02614742) and metastatic breast cancer (NCT02970682). In all of these clinical trials, SFX-01 has been reported to be well tolerated with no patients discontinuing the drugs and with no drug-related adverse events, such as lymphopenia. Based on data presented here and previous studies of sulforaphane in EAE (Li et al., 2013), a phase II trial of SFX-01 in RRMS is warranted. However, given the proven efficacy of early treatment in MS, it is unethical to conduct placebo-controlled trials, so a trial comparing BG-12 and SFX-01 would address whether SFX-01 can offer RRMS patients a better option with respect to efficacy and tolerability.

## Declaration of interest

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