A novel fatty acid-binding protein 5 and 7 inhibitor ameliorates oligodendrocyte injury in multiple sclerosis mouse models

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Background: Multiple sclerosis (MS) is an autoimmune disease characterised by the demyelination of mature oligodendrocytes in the central nervous system. Recently, several studies have indicated the vital roles of fatty acid-binding proteins (FABPs) 5 and 7 in regulating the immune response.

Methods: We assessed a novel FABP5/FABP7 inhibitor, FABP ligand 6 (MF 6), as a potential therapeutic for MS therapy. In vivo, we established MOG₃₅₋₅₅-administered experimental autoimmune encephalomyelitis (EAE) mice as an MS mouse model, followed by prophylactic and symptomatic treatment with MF 6. The therapeutic effect of MF 6 was determined using behavioural and biochemical analyses. In vitro, MF 6 effects on astrocytes and oligodendrocytes were examined using both astrocyte primary culture and KG-1C cell lines.

Findings: Prophylactic and symptomatic MF 6 therapy reduced myelin loss and clinical EAE symptoms. Furthermore, oxidative stress levels and GFAP-positive and ionised calcium-binding adaptor protein-1-positive cells were reduced in the spinal cord of MF 6-treated mice. In addition, MF 6 attenuated lipopolysaccharide-stimulated interleukin-1β and tumour necrosis factor-α accumulation in primary astrocyte culture. Moreover, MF 6 indicated a powerful protective function for the mitochondria in the oligodendrocytes of EAE mice via FABP5 inhibition.

Interpretations: MF 6 is a potent inhibitor of FABP5 and FABP7; targeted inhibition of the two proteins may confer potential therapeutic effects in MS via immune inhibition and oligodendrocyte protection.

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1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease that is characterised by multifocal and temporally scattered central nervous system (CNS) damage, manifested as axonal damage [1]. Although autoimmunity plays a major role in the pathogenesis of MS, the precise underlying mechanism remains elusive [2]. The current treatment for MS is divided into three categories: treatment of exacerbations, slowing disease progression with disease-modifying therapies (DMTs), and symptomatic therapies [3]. Among them, DMTs are an integral part of the long-term management of patients with MS, and the goal of disease improvement is to reduce early clinical and subclinical disease activity, which is believed to cause long-term disability [3]. Interferons (IFNs) were the first DMTs approved for MS therapy, of which IFN-β reportedly reduces the number of exacerbations and suppresses the progression of physical disabilities. However, IFNs are associated with rare allergic reactions, including allergic reactions, seizures, and decreased peripheral blood cell counts. Another DMT available in the market, fingolimod, is a sphingosine-1-phosphate (S1P) agonist that binds and activates S1P receptors. On binding to S1P receptors, fingolimod prevents lymphocytes from exiting the lymph nodes, thus decreasing the number of lymphocytes in peripheral blood and reducing the migration of naïve T cells and memory T cells into the CNS, which in turn impedes the progression of MS [4]. However, treatment with fingolimod is associated with an increased risk of bradyarrhythmia and atrioventricular block [5]. Newer DMTs are more effective than their counterparts; however, several present uncommon but serious potential adverse effects. Thus, there is an urgent need to identify novel targets for MS therapy.

Fatty acid-binding proteins (FABPs) are a family of low molecular weight intracellular proteins with a molecular weight of 14-15 kDa and comprise up to 12 types of molecular species that exhibit distinct
Research in context

Evidence before this study
Multiple sclerosis (MS) is a potentially disabling disease of the brain and spinal cord (central nervous system) and is characterised by loss of the protective sheath (myelin) that covers nerve fibres, thus resulting in neurotransmission disorders. Several studies have indicated that fatty acid protein 5 (FABP5) and 7 (FABP7) play pivotal roles in pathological progression in an MS mouse model (experimental autoimmune encephalomyelitis [EAE] mice), suggesting novel protein targets for MS therapy.

Added value of this study
We developed a novel ligand (MF 6) exhibiting a high affinity for both FABP5 and FABP7. In EAE mice or primary cultured astrocytes, MF 6 markedly reduced astrocyte- and microglia-dependent inflammatory responses by inhibiting FABP5 and FABP7. Furthermore, in both in vivo and in vitro experiments, MF 6 rescued oligodendrocyte survival by inhibiting mitochondrial macropore formation. Finally, MF 6 reduced the severity of EAE, as assessed by neurological scores in mice. Overall, MF 6 exhibited inhibitory effects that afforded both inflammatory inhibition and oligodendrocyte protection.

Implications of all the available evidence
These findings suggest that MF 6 elicits potent prophyllactic and symptomatic effects in EAE mice by inhibiting inflammatory responses and improving oligodendrocyte survival via inhibition of both FABP5 and FABP7. Therefore, MF 6 may represent a novel therapeutic candidate for MS therapy.

2. Methods

2.1. Animals and EAE induction
Six-week-old female C57BMF/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and maintained in polypropylene cages (temperature: 23±2°C; humidity: 55±5%; lights on between 9 a.m. and 9 p.m.). EAE induction was performed in 8–10-week-old female mice. Briefly, mice were injected at two injection sites: the midline of the back just below the shoulders and the lower back. Each site was injected with 50 μL of emulsion, containing 50 μg of MOG35-55 (R&D systems, 149635-73-4) in complete Freund’s adjuvant (Sigma-Aldrich, F5881) supplemented with 500 μg of Mycobacterium tuberculosis H37Ra (Difco Laboratories, 231141). Additionally, each site was subcutaneously (s.c.) injected with 100 ng of pertussis toxin (List Biological Laboratories, 181) immediately after MOG35-55 injection (prophylactic treatment) or 12 days after MOG35-55 injection (symptomatic treatment); treatment was performed daily until four weeks after EAE induction (the spinal cord was fixed on the same day). The EAE group was treated with the same volume of PBS (i.g.). For the control group, mice were treated with PBS (i.g.) only. Clinical scores were designated numerically according to previous reports [26] as follows: 0, no symptoms of disease (asymptomatic); 1, limp tail or hind limb weakness; 2, both limp tail and hind limb weakness; 3, partial paralysis of the hind limbs; 4, complete hind limb paralysis; 5, moribund or dead. For each group, mice were treated and measured randomly, and there were no confounders. For anaesthesia, isoflurane was used during experiments. For euthanasia, mice were injected with overdose of pentobarbital intraperitoneally.

2.2. Primary astrocyte culture
For primary astrocyte culture, P1–2 mouse pups were decapitated, followed by isolation of the cortex from the brain and digestion in a digestion solution (13.6 mL PBS, 0.8 mL DNsase I stock solution [0.2 mg/mL], and 0.6 mL of a trypsin stock solution [0.25%]). The tissue was then sliced using a sterilised razor blade into approximately 1 mm3 chunks, centrifuged at 100 × g for 5 min, and resuspended in DMEM20S medium (DMEM, 4 mM L-glutamine, 1 mM sodium pyruvate, 20% foetal bovine serum [FBS], 50 U/mL penicillin, and 50 μg/mL streptomycin). The tissue suspension was strained using a 70 μm nylon cell strainer, seeded in poly L-lysine-coated tissue culture flask and cultured in DMEM20S medium for 2 weeks (mixed culture). The flasks were shaken for 24 h at 200 rpm at 37°C to remove microglial cells and oligodendrocytes, and the residual cells were considered crude astrocyte cultures. Finally, astrocytes were stained with GFAP.
2.3. Cell culture

KG-1C human oligodendroglial cells (RRID: CVCL_2971) were obtained from the RIKEN BRC Cell Bank (Tsukuba, Japan). Short-tandem repeat (STR) profiling of cell lines was performed by JCRB. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and penicillin/streptomycin (100 U/100 µg/mL) at 37°C under 5% CO₂. Lysophosphatidylcholine (LPC) was dissolved in methanol and stored at −30°C until use.

2.4. Protein extraction

Culture cells or isolated spinal cords were frozen in liquid nitrogen and stored at −80°C until use. Samples were homogenised with 50 µL (for each 35 mm dish) or 200 µL (for spinal cords) of Triton X-100 buffer containing 0.5% Triton X-100 at pH 7.4, 4 mM ethylene glycol, 50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 40 mM Na₄P₂O₇·10H₂O, 0.15 M sodium chloride, 50 µg/mL leupeptin, 25 µg/mL pepstatin A, 50 µg/mL trypsin inhibitor, 100 nM calyculin A, and 1 mM dithiothreitol. The concentration of the supernatant protein was normalised using the Bradford assay.

2.5. Mitochondria isolation

Mitochondria were isolated as described previously [24]. In brief, spinal cords or collected cells were suspended using mitochondrial isolation buffer (250 mM sucrose, 1 mM dithiothreitol, 10 mM KCl, 1 mM ethylenediaminetetraacetic acid, 1.5 mM MgCl₂, protease inhibitors, and 20 mM Tris-HCl, pH 7.4), homogenised with a glass homogeniser at approximately 50 strokes per pestle, and centrifuged three times at 800 g for 10 min. The supernatant proteins were collected and centrifuged at 15,000 × g for 10 min at 4°C. Supernatants were collected as cytosolic fractions (without mitochondria). The mitochondrial pellets were immediately washed three times with mitochondrial isolation buffer and homogenised with Triton X-100 buffer. The supernatant proteins were collected as mitochondrial fractions. Protein concentrations of isolated mitochondria were normalised using the Bradford assay. The number of mitochondria was estimated using VDAC-1 using western blotting (WB).

2.6. Immunoblotting analysis

Extracts from spinal cords, cells, or isolated mitochondria were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a commercially available gel (Cosmo Bio Co., Ltd.) and then transferred to poly(vinylidene fluoride) (PVDF) membranes. The membranes were incubated with primary antibodies against caveolin-1, Iba-1, 4-hydroxynonenal (4-HNE), TNF-α, IL-1β, myelin basic protein (MBP), FABP5, FABP7, VDAC-1, β-tubulin, dsDNA, and cytochrome C, followed by treatment with a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse IgG [H&L], anti-rabbit IgG [H&L], and anti-goat IgG [H&L]), and detected with an ECL detection system (Amersham Biosciences, NJ, USA) using the Image Quant LAS 4000 mini system. Intensity quantification was conducted using Image Gauge software (version 3.41; Fuji Film, Tokyo, Japan).

2.7. Immunoprecipitation analysis

For immunoprecipitation analysis, 100 µL of protein A-Sepharose CL-4B (50%, v/v) was suspended in PBS in a total volume of 1000 µL and stored at 4°C. Spinal cord extracts containing 500 µg of protein were incubated for 4 h at 4°C with 10 µg of anti-FABPP5 antibody and 5 µg of anti-VDAC-1 antibody. The mixture was incubated overnight at 4°C. The samples were then separated using SDS-PAGE using a commercially available gel (Cosmo Bio Co., Ltd.).

2.8. Dot blot assay

A dot blot assay was performed to determine dsDNA levels in the mitochondrial fraction as previously described [27]. In brief, a PVDF membrane (Millipore) was placed on the top of the soaked sheets, and equal amounts of protein in a similar volume were placed in dots in specific zones. After the dots dried, the membrane was blocked with 5% nonfat milk in TTBS buffer (0.1% Tween 20 in Tris-buffered saline) for 1 h at room temperature. Membranes were subjected to WB as described above (primary antibodies against dsDNA and VDAC-1), followed by treatment with an HRP-conjugated secondary antibody (anti-mouse IgG H&L and anti-rabbit IgG H&L).

2.9. Immunofluorescence staining and confocal microscopy

Immunofluorescence staining was performed as previously described [28]. In the present study, cells were incubated with primary antibodies against 4-HNE, Iba-1, Olig2, NeuN, GFAP, FABP5, FABP7, and TOM20. Fluorescein, Alexa 405-labelled anti-mouse IgG, Alexa 488-labelled anti-goat IgG, Alexa 594-labelled anti-mouse IgG, and Alexa 594-labelled anti-rabbit IgG were used for detection. Mitotracker (CST) and Alexa Fluor™ 488 Phalloidin (Thermo Fisher Scientific) were used for mitochondria and filamentous actin staining, respectively. Immunofluorescent images were analysed using a confocal laser scanning microscope (DMIß; Leica, Wetzlar, Germany).

2.10. FABP5/7 shRNA plasmid and shRNA delivery

Human-FABP5 shRNA bacterial stock was obtained from Sigma-Aldrich, and the sequences were as follows: shRNA1 (CCCGTGGACTGTGATCATGAACAACCTCTGTTTCTACCT–CATTTTTTTT), shRNA2 (CCCGGCAACCTTACAGATGTGGCAACTCGAGGATGCACATGTTAAGCTTGTCTTTTT), and shRNA3 (CCGGTTAACCTTACATGTGGCAACTCGAGTACGTTGTTTACCTGTTT). Human-FABP7 shRNA bacterial stock was obtained from Sigma-Aldrich, and the sequences were as follows: shRNA1 (CCCGGATAAGAAATGCTGCACTGCTAGTACGTTGCTTTTTT), shRNA2 (CCG GGTTGCTGTTGCGGACATGCTGTTTAGACGCTTTTTT), and shRNA3 (CCGGGACATGGCTGATATTCAATGCGTTGAACTGCTGTTTTT). The plasmid was purified using the GenElute™ HP Plasmid Maxiprep Kit (Sigma, St. Louis, MO, USA). Primary astrocytes were transduced with an empty vector or shRNA plasmid (2 µg per 35-mm dish) using Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s protocol.

2.11. Cell death assay

Cell viability was measured using a cell counting kit (Dojindo) according to the manufacturer’s instructions. The absorbance of the viable cells was measured at a test wavelength of 400 nm and a reference wavelength of 450 nm using a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

2.12. JC-1 assay

Mitochondrial membrane potential was measured using the JC-1 Mito MP Detection Kit (Dojindo). In brief, the culture cells were washed with PBS and stained according to the manufacturer’s instructions. The relative degrees of mitochondrial polarisation were quantified by measuring the red-shifted JC-1 aggregates at 535 nm (Ex) and 585-605 nm (Em) and green-shifted JC-1 aggregates at 485
which is the maximum effective dose in brain ischemia [25] 1 week before MOG immunisation of the EAE model (Fig. 1c). MF 6 was synthesised by Shiratori Pharmaceutical Co., Ltd. Additional details are provided in the Supplementary Material section.

2.14. Statistics

Data were analysed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and are expressed as the mean ± standard error of the mean (SEM). The normality assumption was examined using the Shapiro–Wilk test, and the equal variance assumption was examined by the Brown-Forsythe test using GraphPad Prism 7. For the data that passed these assumptions, significant differences were determined using Student’s t-test for two-group comparisons (Fig. 1c, d; Fig. 5h, l) and by one-way analysis of variance (ANOVA) for other multigroup comparisons, followed by Tukey’s multiple comparisons test. For data that did not pass these assumptions, the significant differences were determined using the Kruskal-Wallis test (nonparametric version of ANOVA) (Fig. 1k, n; Fig. 2g, k; Fig. 3c, g; Fig. 5r). Statistical significance was set at \( p < 0.05 \).

2.15. Ethics statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Tohoku University Environmental and Safety Committee (2019PhLM0-021 and 2019PhHA-024). All animal experiments were performed following the animal use guidelines and ethical approval.

2.16. Role of funder

The funders were not involved in the study design, data collection, analysis, interpretation, decision to publish, or writing of the manuscript. The writing group (including the corresponding author) had full access to all the data in the study and had final responsibility for the decision to submit the manuscript for publication.

3. Results

3.1. MF 6 attenuates EAE symptoms both prophylactic and symptomatic treatments in vivo

In previous studies, FABP5 and FABP7 deficiency conferred protective effects against EAE development, [17,20] suggesting an important role of FABP5 and FABP7 in EAE progression. Herein, we evaluated the therapeutic efficacy of MF 6 (Fig. 1a), which has a high affinity for both FABP5 and FABP7, using a mouse model of EAE (Fig. 1b) [26]. EAE mice were orally administered MF 6 (1 mg/kg), which is the maximum effective dose in brain ischemia [25] 1 week before MOG immunisation of the EAE model (Fig. 1c). MF 6 administration significantly reduced the severity of EAE, as assessed by the area under the curve (AUC) of clinical scores (EAE group: 42.21 ± 4.675; EAE + MF 6 group: 17.88 ± 4.08; \( p < 0.01 \), vs. EAE group; F (5, 5) = 3.13, n = 6) (Fig. 1d, e). As caveolin-1, lba-1, TNF-\( \alpha \), and IL-1\( \beta \) are widely used as inflammation markers, and 4-HNE is an oxidative stress marker, we analysed the levels of these factors using WB to further determine whether MF6 improves inflammation and oxidative injury in EAE mice (Fig. 1f). Our results revealed reduced inflammatory and oxidative effects, along with decreased levels of caveolin-1 (EAE group: 4.10 ± 0.39; EAE + MF 6 group: 2.28 ± 0.32; \( p < 0.01 \), vs. EAE group; F (2, 9) = 28.14, n = 4) (Fig. 1g), lba-1 (EAE group: 1.91 ± 0.11; EAE + MF 6 group: 1.39 ± 0.10; \( p < 0.01 \), vs. EAE group; F (2, 9) = 26.22, n = 4) (Fig. 1h), 4-HNE (EAE group: 3.76 ± 0.33; EAE + MF 6 group: 2.07 ± 0.3488; \( p < 0.01 \), vs. EAE group; F (2, 9) = 23.17, n = 4) (Fig. 1i), IL-1\( \beta \) (EAE group: 2.05 ± 0.23; EAE + MF 6 group: 1.11 ± 0.12; \( p < 0.01 \), vs. EAE group; F (2, 9) = 14.20, n = 4) (Fig. 1j), and TNF-\( \alpha \) (EAE group: 2.04 ± 0.20; EAE + MF 6 group: 1.018 ± 0.14; \( p = 0.0588 \), vs. EAE group; n = 4) (Fig. 1k) in MF 6-treated mice as shown by WB. In addition, the levels of MBP were upregulated in the MF6 group (EAE group: 0.34 ± 0.02; EAE + MF 6 group: 0.71 ± 0.05; \( p < 0.01 \), vs. EAE group; F (2, 9) = 57.54, n = 4) (Fig. 1l). However, WB revealed no significant changes in the levels of FABP5 and FABP7 in both the EAE models and the MF 6-treated mice when compared with the levels of \( \beta \)-tubulin (Fig. 1f). Significant changes in FABP5 levels (EAE group: 3.53 ± 0.42; EAE + MF 6 group: 1.49 ± 0.18; \( p < 0.01 \), vs. EAE group; F (2, 9) = 24.49, n = 4) (Fig. 1m) and an obvious but not significant decrease in FABP7 levels (EAE group: 3.06 ± 0.76; EAE + MF 6 group: 0.95 ± 0.18; \( p = 0.0723 \), vs. EAE group; n = 4) (Fig. 1n) were apparent, compared with the levels of MBP.

As MS therapy is typically initiated after the disease has been active and diagnosed, we further investigated whether MF 6 administration was effective for treating active EAE. Accordingly, we treated EAE mice with MF 6 along with MOG immunisation on day 12 (Fig. 2a). Consistently, MF 6 significantly reduced the severity of EAE, as assessed by the AUC of the clinical scores (EAE group: 42.72 ± 2.56; EAE + MF 6 group: 24.54 ± 1.61; \( p < 0.01 \), vs. EAE group; F (7, 6) = 2.90, n = 7) (Fig. 2c). In addition, in symptomatic treatment cases (Fig. 2d), MF 6 showed a possibly potent effect in decreasing the upregulated levels of caveolin-1 (EAE group: 4.573 ± 0.55; EAE + MF 6 group: 2.234 ± 0.25; \( p < 0.01 \), vs. EAE group; F (2, 11) = 23.03, n = 5) (Fig. 2e), lba-1 (EAE group: 3.771 ± 0.53; EAE + MF 6 group: 2.06 ± 0.26; \( p < 0.05 \), vs. EAE group; F (2, 11) = 13.72, n = 5) (Fig. 2f), 4-HNE (EAE group: 2.34 ± 0.34; EAE + MF 6 group: 1.39 ± 0.24; \( p < 0.05 \), vs. EAE group; F (2, 11) = 7.16, n = 5) (Fig. 2g), IL-1\( \beta \) (EAE group: 2.58 ± 0.31; EAE + MF 6 group: 1.72 ± 0.13; \( p < 0.05 \), vs. EAE group; F (2, 11) = 11.56, n = 5) (Fig. 2h), and TNF-\( \alpha \) (EAE group: 2.71 ± 0.12; EAE + MF 6 group: 1.36 ± 0.17; \( p < 0.05 \), vs. EAE group; F (2, 11) = 38.59, n = 5) (Fig. 2i) in EAE mice. Furthermore, MF 6 group exhibited higher MBP levels when compared with those detected in EAE mice (EAE group: 0.59 ± 0.06; EAE + MF 6 group: 1.01 ± 0.06; \( p < 0.01 \), vs. EAE group; F (2, 17) = 19.67, n = 5) (Fig. 2j). Consistent with the prophylactic treatment findings, symptomatic treatment with MF 6 significantly decreased both FABP5 (EAE group: 1.76 ± 0.11; EAE + MF 6 group: 1.17 ± 0.13; \( p < 0.05 \), vs. EAE group; n = 5) (Fig. 2k) and FABP7 (EAE group: 1.48 ± 0.12; EAE + MF 6 group: 0.90 ± 0.10; \( p < 0.05 \), vs. EAE group; F (2, 11) = 7.41, n = 5) (Fig. 2l) levels when compared with MBP levels.

Collectively, our findings indicated that MF 6 is a potent ligand that attenuates EAE clinical symptoms by decreasing inflammatory and oxidative levels in the spinal cord, as well as by protecting oligodendrocytes.

3.2. MF 6 decreases oxidative stress levels in the spinal cord

Similar to previous reports, [29,30] we investigated the relationship between oxidative stress levels and spinal cord injury in EAE mice. To verify the oxidative levels in different cell types, we co-immunostained spinal cord slices (Fig. 3a) with the oxidative marker 4-HNE and markers of microglia (lba-1), oligodendrocytes (Olig2), and neurons (NeuN). As expected, we detected an elevated number of 4-HNE-positive cells in the white matter and grey matter of the spinal cord in EAE mice when compared with that in control mice (4-HNE-positive cells in the control group: 5.20 ± 1.04; EAE group: 42.14 ± 3.35; \( p < 0.01 \), vs. control group; n = 7) (Fig. 3b, c). Surprisingly, MF 6 almost completely suppressed the upregulated oxidative stress levels and significantly decreased the number of 4-HNE-positive cells (4-HNE-positive cells in EAE + MF 6 group: 9.0 ± 1.38; \( p < 0.05 \), vs. EAE group; n = 10) (Fig. 3c). Additionally, on co-staining slices with 4-HNE and lba-1 (microglia marker), no double-positive
Fig. 1. Prophylactic treatment with MF 6 attenuates clinical symptoms of EAE. (a) Chemical structure of MF 6. (b) Schematic diagram of EAE induction; female C57BMF/6J mice were injected with MOG₃₅₋₅₅ at two injection sites. (c) Protocol for EAE induction and MF 6 prophylactic treatment. (d) Quantification of clinical scores. EAE-induced C57BMF/6J mice were treated with MF 6 (1 mg/kg) (n=6) or PBS (n=6) vehicle and were scored daily after immunisation. (e) Quantification of the area under the curve (AUC) of clinical scores (n=6). (f) Immunoblots and densitometry of the spinal cord of EAE mice treated with MF 6 or PBS vehicle and against caveolin-1, Iba-1, 4-HNE, IL-1β, TNF-α, MBP, FABP5, FABP7, and β-tubulin. (g-n) Quantification of (f) (n=4), showing that prophylactic treatment with MF 6 significantly decreases levels of caveolin-1, Iba-1, 4-HNE, IL-1β, and TNF-α and increases levels of MBP protein, as well as reduces levels of FABPs and FABP7 when compared with MBP. Data are shown as mean ± standard error of the mean (SEM) and were obtained using student’s t-test, one-way ANOVA, or Kruskal-Wallis test. *p<0.05, **p<0.01, and ***p<0.001. EAE, experimental autoimmune encephalomyelitis; PBS, phosphate-buffered saline; s.c., subcutaneously; i.g., intragastric administration; Veh., vehicle; Iba-1, ionised calcium-binding adaptor protein-1; 4-HNE, 4-hydroxynonenal; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; MBP, myelin basic protein; FABP5, fatty acid protein 5; FABP7, fatty acid protein 7.
cells were detected in the spinal cord of EAE mice, although the number of Iba-1-positive cells increased by approximately six-fold when compared with that in control mice (Iba-1-positive cells in the control group: 5.7 ± 1.24; EAE group: 26.54 ± 1.99; p < 0.01, vs. control group; n = 13) (Fig. 3d), indicating the upregulation of microglial migration. This result differs slightly from that reported in our previous study, in which the number of 4-HNE/Iba-1-positive cells significantly increased in the hippocampus of olfactory bulbectomised mice [31]. In addition, MF 6 treatment significantly decreased the number of Iba-1-positive cells (Iba-1-positive cells in EAE + MF 6 group: 16.80 ± 1.45; p < 0.01, vs. EAE group; F(2, 30) = 38.93, n = 10) (Fig. 3d), indicating attenuated inflammatory levels.

Furthermore, it is crucial to determine the response of oligodendrocytes to immune inflammation, which is directly related to demyelination. In the present study, we performed co-staining with 4-HNE with Olig2. As expected, in EAE mice, the number of 4-HNE/Olig2-positive cells increased by approximately seven-fold in the white matter (4-HNE/Olig2-positive cells in the control group: 1.60 ± 0.43; EAE group: 13.75 ± 1.58; p < 0.01, vs. control group; n = 8) (Fig. 3e, g). Thus, the upregulated oxidative stress levels can be considered the main factor responsible for the death of oligodendrocytes and myelin loss in the spinal cord of EAE mice [32]. Importantly, MF 6 treatment markedly attenuated oxidative stress levels in oligodendrocytes and reduced the number of 4-HNE/Olig2 double-positive cells (4-HNE/Olig2-positive cells in the EAE + MF 6 group: 3.60 ± 0.70; p < 0.05, vs. EAE group; n = 10) (Fig. 3g). Furthermore, to understand whether immune inflammation affects neurons in the grey matter of the spinal cord, we performed co-staining with NeuN with
4-HNE. We detected an increased number of 4-HNE/NeuN double-positive cells (4-HNE/NeuN-positive cells in the control group: 3.89 ± 0.49; EAE group: 2.80 ± 2.27; p < 0.01, vs. control group; n = 10) (Fig. 3f, h), suggesting oxidative neuronal injury in EAE mice. However, MF 6 had a lower impact on the reduction of oxidative stress in neurons than in oligodendrocytes (4-HNE/NeuN-positive cells in the EAE + MF 6 group: 14.40 ± 1.93; p < 0.05, vs. EAE group; F (2, 27) = 24.34, n = 10) (Fig. 3h); this finding could be explained by the fact that FABP5 and FABP7 are not endogenously expressed in neurons, and MF 6, which targets FABP5 and FABP7, cannot function directly in neurons.

Overall, oxidative stress was significantly elevated, especially in the oligodendrocytes and neurons in the spinal cord of EAE mice, and MF 6 treatment almost completely blocked oxidative stress accumulation, especially in oligodendrocytes, but had a less effect on neurons.

3.3. MF 6 inhibits astrocyte activation

It has been previously established that EAE is mediated by the myelin-reactive T cell response against mature oligodendrocytes and possesses an inflammatory signature [33,34]. In the CNS, microglia and astrocytes are also involved in the EAE inflammatory response, [19,35] in addition to IL-β and TNF accumulation [36]. Previous studies have shown that FABP5 inhibition mediated by inhibitors significantly suppresses lymphocyte migration and pathogenic functions...
via T cell regulation [17]. Herein, we mainly focused on assessing whether inhibition of FABP5 or FABP7 also plays a role in regulating astrocyte-dependent inflammatory responses. The number of GFAP-positive cells increased by approximately two-fold in the spinal cord of EAE mice (GFAP-positive cells in control group: 5.25 ± 0.65; EAE group: 10.67 ± 1.38; p < 0.01, vs. control group; n = 6) (Fig. 4a, b). MF 6 significantly suppressed the abnormal increase in GFAP-positive cells (GFAP-positive cells in EAE + MF 6 group: 4.57 ± 0.69; p < 0.01, vs. EAE group; F (2, 18) = 12.76, n = 7) (Fig. 4b). Furthermore, to determine whether MF 6 also impacts the levels of proinflammatory cytokines such as TNF-α and IL-β, we cultured astrocytes (Fig. 4c), and the purity of astrocytes was approximately 94% (94% ± 3.29%, n=4) (Fig. 4d). Meanwhile, we observed that primary astrocytes endogenously expressed both FABP5 and FABP7 (Fig. 4e). To trigger the cellular inflammatory response, we treated astrocytes with lipopolysaccharide (LPS; 10 μg/mL) for 48 h and observed significant accumulation of both IL-1β (control group: 1.00 ± 0.11; EAE group: 1.59 ± 0.08; p < 0.01, vs. control group; n = 4) (Fig. 4f, g) and TNF-α (control group: 1.00 ± 0.03; EAE group: 1.46 ± 0.07; p < 0.01, vs. control group; n = 4) (Fig. 4f, h) in LPS-treated cells. As expected, in the MF 6-treated astrocytes, we observed significantly attenuated IL-1β (EAE + MF 6 group: 1.18 ± 0.08; p < 0.05, vs. EAE group; F (2, 9) = 10.73, n = 4) (Fig. 4g) and TNF-α (EAE + MF 6 group: 0.93 ± 0.07; p < 0.01, vs. EAE group; F (2, 9) = 21.93, n = 4) (Fig. 4h) levels, indicating a suppressed inflammatory response. However, the FABP5 inhibitor MF 6 has a high affinity for both FABP5 and FABP7. This binding and regulation mediated by MF 6 attenuated the inflammatory response in activated astrocytes. To bridge this gap, we knocked down FABP5 and FABP7 by shRNA (Fig. 4i) and observed a significant decrease in expression levels of IL-1β (LPS group: 2.39 ± 0.27; LPS/F5i 3 group: 1.32 ± 0.06; p < 0.01, vs. LPS group; n=4; LPS/F5i 3 group: 1.68 ± 0.16; p < 0.05, vs. LPS group; F (3, 12) = 12.21, n=4) (Fig. 4j, k) and TNF-α (LPS group: 2.15 ± 0.14; LPS/F7i 2 group: 1.37 ± 0.07; p < 0.01, vs. LPS group; n=4; LPS/F5i 3 group: 1.19 ± 0.16; p < 0.01, vs. LPS group; F (3, 12) = 19.02, n=4) (Fig. 4j, l), in both FABP5- and FABP7-knockdown astrocytes.

Collectively, MF 6 treatment markedly decreased the number of astrocytes in the spinal cord following EAE induction, significantly inhibited proinflammatory cytokine accumulation, and reduced the astrocyte-dependent inflammatory response by inhibiting both FABP5 and FABP7.

3.4. MF 6 protects oligodendrocytes by inhibiting mitochondrial macropore formation

Previously, we have reported that FABP5 is involved in VDAC-1- and BAX-dependent macropore formation under psychosine exposure. Additionally, the inhibition of FABP5 by FABP5 inhibitors (such as MF 6) suggested potent effects on blocking mitochondrial macropore formation, thereby rescuing oligodendrocytes [24]. In the
present study, DNA microarray analysis revealed that mitochondria-related genes, such as mitochondrial fusion proteins 1 and 2 (MFN1, 2) and ATP6AP2, were downregulated in the spinal cord of EAE mice (Fig. 5a) (original data are from Gene Expression Omnibus [GSE60847]). Furthermore, using a spinal cord slice, FABPS was double-stained with the mitochondrial outer membrane protein TOM20. We observed that FABPS co-localised with TOM20 in oligodendrocytes of EAE mice (Fig. 5b). To further determine whetherVDAC-1-dependent macropore formation is stimulated in EAE mice, we blotted mitochondrial fractions with VDAC-1 antibody and detected elevated VDAC-1 oligomer levels in the spinal cord of EAE mice (VDAC-1 oligomer levels in the control group: 1 ± 0.03; EAE group: 1.59 ± 0.07; p < 0.01, vs. control group; n = 4) (Fig. 5c, d), suggesting accelerated mitochondrial macropore formation. On blotting the same membrane with FABP5 antibody after stripping, we detected increased levels of FABP5 in both oligomers (FABP5 oligomer levels in the control group: 1 ± 0.09; EAE group: 1.88 ± 0.15; p < 0.05, vs. control group; n = 4) (Fig. 5c, e) and monomers (FABP5 monomer levels in the control group: 1 ± 0.07; EAE group: 2.68 ± 0.11; p < 0.01, vs. control group; n = 4) (Fig. 5c, f), suggesting that FABP5 is also associated with VDAC-1 oligomerisation in EAE mice. In MF6-treated mice, we observed reduced levels of both VDAC-1 (VDAC-1 oligomer levels in EAE+MF6 group: 0.96 ± 0.08; p < 0.01, vs. EAE group; F(2, 9) = 30.08, n = 4) (Fig. 5d) and FABP5 oligomers (FABP5 oligomer levels in EAE+MF6 group: 0.99 ± 0.28; p < 0.05, vs. EAE group; F(2, 9) = 6.99, n = 4) (Fig. 5e) and monomers (FABP5 monomer levels in EAE+MF6 group: 1.15 ± 0.15; p < 0.01, vs. EAE group; F(2, 9) = 64.45, n = 4) (Fig. 5f) in the mitochondria; consistently, the
Overall, MF 6 is one potential therapeutic approach for MS therapy via the two pathways. EAE, experimental autoimmune encephalomyelitis; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; ROS, reactive oxygen species.

**Fig. 6.** Schematic representation of pathways underlying MF 6-mediated therapy in EAE. MF 6 inhibits inflammatory response by inhibiting TNF-α and IL-1β accumulation and release in astrocytes and microglia. On the other hand, MF 6 also reduces mitochondria macropore formation, thereby reducing ROS levels, and improves oligodendrocyte survival.

4. Discussion

MS is an autoimmune disease that targets the myelin of the central and peripheral nervous systems. MS is potentially associated with auto-reactive CD4+ T cells, which are activated in the periphery and cross the blood-brain barrier (BBB) to reach the CNS. This migration triggers an inflammatory reaction, including the recruitment of other leukocytes, such as B cells and macrophages [38]. Additionally, recent studies have indicated an important role of local microglia [39,40] and astrocytes, [41,42] which promote inflammation, demyelination, and neurodegeneration and critically contribute to the
development of MS injuries [43,44]. Microglial activation in the early stages of MS results in increased microglial proliferation in response to neuronal insults. In the present study, we detected an increased number of Iba-1-positive cells in the spinal cord of EAE mice. Moreover, abnormal accumulation of activated microglia clusters can be observed in the white matter of patients with MS and are associated with degenerating axons, stressed oligodendrocytes, and deposits of activated products in the complement pathway [45,46]. However, in addition to leucocytes, including T cells, B cells, and macrophages, stimulated microglia and astrocytes also secrete inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [41] known to be involved in the secondary oxidative stress targeting oligodendrocytes as an internal mechanism for MS pathology.

Table 1

| Designation  | Source                      | Identiﬁers | Dilution ratio | RRID       |
|--------------|-----------------------------|------------|----------------|------------|
| Caveolin-1   | BD Transduction Laboratories | 610406     | 1:500          | AB_214110  |
| Iba-1        | Fujifilm                    | 016-20001  | 1:1000         | AB_839506  |
| 4-HNE        | SfCa                        | MHN-100P   | 1:1000         | AB_1106813 |
| TNF-α        | Abcam                       | ab92324    | 1:500          | AB_10561788|
| IL-1β        | Abcam                       | ab9722     | 1:1000         | AB_308765  |
| MBP          | Abcam                       | ab7349     | 1:500          | AB_305869  |
| FABP5        | R&D Systems                 | AF5077     | 1:200          | AB_2100340 |
| FABP7        | R&D Systems                 | AF1166     | 1:200          | AB_2100475 |
| VDAC-1       | CST                         | 4866       | 1:500          | AB_2272627 |

β-tubulin  Sigma-Aldrich  T0198  1:4000  AB_477536
OLG2  Sigma-Aldrich  MBNS0  1:500  AB_1090710
dsDNA  Abcam  ab27156  1:1000  AB_470907
Cytochrome C  CST  4272  1:1000  AB_2090454
TOM20  Santa Cruz  sc-11415  1:500  AB_2207533
Anti-mouse IgG (H&L)  SouthernBiotech  1031-05  1:5000  AB_2794307
Anti-rabbit IgG (H&L)  SouthernBiotech  4000-05  1:5000  AB_2795053
Anti-goat IgG (H&L)  Rockland Immunocchemicals  605-4302  1:5000  AB_219485
Alexa 405-labelled anti-mouse IgG  Thermo Fisher Scientific  A-31553  1:500  AB_221604
Alexa 488-labelled anti-goat IgG  Thermo Fisher Scientific  A-11055  1:500  AB_2534102
Alexa 594-labelled anti-mouse IgG  Thermo Fisher Scientific  A-21203  1:500  AB_141633
Alexa 594-labelled anti-rabbit IgG  Thermo Fisher Scientific  A-21207  1:500  AB_141637

Iba-1, ionised calcium-binding adaptor protein-1; 4-HNE, 4-hydroxynonenal; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; MBP, myelin basic protein; FABP5, fatty acid protein 5; FABP7, fatty acid protein 7. This is not a pattern of ‘rrid:software’ external object linking.

We have recently reported that FABP5 forms complexes with VDAC-1 protein and induces VDAC-1 oligomer-dependent mitochondrial macropore formation. Furthermore, inhibition of FABP5 by MF 6 improved mitochondrial function and rescued oligodendrocytes from psychosine-mediated toxicity [24]. Thus, we postulated that FABP5 also mediates mitochondrial dysfunction in EAE mice. MF 6 protected oligodendrocytes from this pathway via FABP5 inhibition. Moreover, we observed impaired mitochondrial functions in both EAE-and LPC-stimulated KG-1C cells. As expected, MF 6 significantly disrupted FABP5 and VDAC-1 complexes, thereby blocking mitochondrial macropore formation. This finding might explain the inhibition of mtDNA and cytochrome c release and the rescue of mitochondria.

Previous studies have revealed that FABP5 upregulation exacerbates the development of EAE, a mouse model of human MS [16]. Additionally, FABP5, which is expressed in dendritic cells and macrophages, promotes the generation of inflammatory cytokines such as TNFα and IL-1β [16]. These elevated levels were attenuated following MF6 administration. Furthermore, during early MS progression, astrocytes exhibit morphological and biochemical alterations, leading to astrogliosis [53]. Notably, FABP7 expressed in the astrocyte lineage was considerably upregulated by activated astrocytes during the early progression of MS [54-56]. In the present study, MF 6 improved neurological behaviours and reduced the expression of inflammatory factors following both prophylactic and symptomatic treatment regimens. This may indicate a potential effect of MF 6 in treating relapsing-remitting MS. In a previous study, MF 6 administration did not induce toxicity at high doses [17]. However, the secondary target of FABP5 inhibitors remains unclear. PPARγ may also exert protective effects in ameliorating MS [17]. Moreover, in our previous study, MF 6 was shown to reduce cell proliferation in U251 cells, [22] which may be associated with the FABP7 inhibition. Currently, FABP inhibition therapy remains in the preclinical stage, and potential adverse reactions following clinical application remain uncertain. However, we have initiated a toxicological evaluation of FABP7 inhibitors, including MF 6. We hope that MF 6 and other efficient ligands can be employed for MS therapy in the future.
In the present study, FABP5 and FABP7 inhibitor, MF 6, improved the severity of EAE and attenuated oxidative levels and the inflammatory response, indicating close associations between these factors and EAE progression. However, it is difficult to confirm whether these factors induce or result in MS progression based on the present findings. We observed that the inhibition of FABP5 and FABP7 by MF 6 decreased both the immune response and oxidative stress, thereby rescuing oligodendrocytes and improving EAE progression. Although we were unable to determine which cytokines are released from T cells, dendritic cells, astrocytes, and microglia and whether the cytokines stimulate oxidative stress in the acceptor cells such as neurons and oligodendrocytes, it will be of considerable interest to address these issues in future investigations.

As the therapeutic effect of FABP5 inhibitors has been previously defined by other groups,[17] in the present study, we focused on the local activation of astrocytes, microglia, and oligodendrocytes in the spinal cord. In summary, MF 6 attenuated the inflammatory response of activated microglia and astrocytes by inhibiting FABP5 and FABP7. Furthermore, MF 6 improved the survival of oligodendrocytes by disrupting FABP5/VDAC-1-dependent mitochondrial macropore formation, thus rescuing mitochondria. Importantly, MF 6 reduced clinical symptoms of EAE following both the prophylactic and symptomatic treatments. Thus, MF 6 is a novel and potential therapeutic approach for MS.

Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

Contributors

A. Cheng: Data acquisition and original draft writing
W. Jia: Data acquisition and design of the methodology
I. Kawahata: Data acquisition
K. Fukunaga: Supervision, project administration, funding, and reviewing and editing the manuscript.

All authors critically reviewed the manuscript and approved the final version of the manuscript. A. Cheng and K. Fukunaga verified the data and had final responsibility for the decision to submit for publication.

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Data Sharing Statement

The data supporting the findings of this study are available in the article and/or supplementary materials. The data in Fig. 5a were based on publicly available data from the Gene Expression Omnibus (GEO): [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60847]. Readers are welcome to contact the corresponding author for the raw data used in this work.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103582.

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