Dimethyl fumarate does not mitigate cognitive decline and β-amyloidosis in female APPPS1 mice

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A R T I C L E   I N F O

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A B S T R A C T

Introduction: Alzheimer’s disease (AD) is the leading cause of dementia and a major global health issue. Currently, only limited treatment options are available to patients. One possibility to expand the treatment repertoire is repurposing of existing drugs such as dimethyl fumarate (DMF). DMF is approved for treatment of multiple sclerosis and previous animal studies have suggested that DMF may also have a beneficial effect for the treatment of AD.

Methods: We used an APPPS1 transgenic model of senile β-amyloidosis and treated female mice orally with DMF in two treatment paradigms (pre and post onset). We quantified learning and memory parameters, β-amyloidosis, and neuroinflammation to determine the potential of DMF as AD therapeutics.

Results: Treatment with DMF had no influence on water maze performance, β-amyloid accumulation, plaque formation, microglia activation, and recruitment of immune cells to the brain. Compared to vehicle-treated animals, oral DMF treatment could not halt or retard disease progression in the mice.

Discussion: Our results do not favour the use of DMF as treatment for AD. While our results stand in contrast to previous findings in other models, they emphasize the importance of animal model selection and suggest further studies to elucidate the mechanisms leading to conflicting results.

1. Introduction

Dementia is a major global health problem with currently about 50 million cases worldwide and almost 10 million new cases per year. Alzheimer’s disease (AD) is the most common cause of dementia (Wortmann, 2012). Morphologically, AD is characterized by neurofibrillary tangles and senile plaques, consisting of hyperphosphorylated tau and β-amyloid (Aβ), respectively (Nelson et al., 2009). Eventually, these toxic aggregates cause neurodegeneration and thus, lead to AD-related symptoms including memory loss, cognitive impairment and neuropsychiatric changes (Masters et al., 2015).

The research community has experienced several major setbacks in drug development for AD as clinical trials failed after promising preclinical results (Imbimbo et al., 2021; Oxford et al., 2020). Therefore, potential drugs should be tested in a variety of models, ensuring broad applicability and thus, increasing chances for success in large clinical trials that include a variety of patients. Moreover, if there are any shortcuts on the way to phase III studies that do not compromise safety, they should be taken to avoid any more delay in the availability of treatments. One of these shortcuts is to repurpose existing drugs.

A potential candidate for repurposing is dimethyl fumarate (DMF, Tecfidera®, Skilarence®). DMF is approved for the treatment of the autoimmune, neuroinflammatory disease multiple sclerosis (MS) and the skin disease psoriasis. Recently, also monomethyl fumarate (MMF, Bafiertam®), the bioactive metabolite of DMF, has been approved as treatment for MS. DMF regulates inflammation (Longbrake et al., 2020; McGuire et al., 2016; Montes Diaz et al., 2018), halts disease progression in MS (Fox et al., 2012; Gold et al., 2012, 2017; Lanzillo et al., 2020), and affects the response to oxidative stress (Suneetha and Raja Rajeswari, 2016). Previous studies in animal models have found that DMF protects against MPTP- or Aβ mediated neurotoxicity (Campolo et al., 2017, 2018; Casili et al., 2018), improves outcome in stroke and...
intracerebral haemorrhage (Safari et al., 2017; Zhao et al., 2015), and mitigates learning and memory improvements in an induced rat model of AD (Majkutewicz et al., 2016, 2018). DMF may also improve Aβ clearance by inducing ATP binding cassette (ABC) transporters (Ji et al., 2013; Wang et al., 2014).

In the present study, we investigated the effect of treatment with DMF in a β-amyloidosis mouse model of AD. Our data show that early treatment with DMF starting before (40d) or shortly after (60d) onset of Aβ plaque deposition (from 45d), is unable to prevent disease progression in this specific AD model [APPPS1-21 featuring a combination of mutated amyloid precursor protein (APPsw)-overexpression and presenilin-1 mutations (PS1L166P)].

2. Results

We treated APPtg mice with DMF in two different early-intervention treatment paradigms. We started treatment either at 40d of age, just before first plaques become visible, or at 60d of age, right after first plaques form. We continued treatment for 40d before animals were sacrificed at 80d and 100d of age, respectively. Treatment with DMF did not result in side effects such as weight loss (Supplementary Fig. 1). To assess learning and memory performance, we conducted water maze experiments in the final week of treatment. In both treatment paradigms, DMF-treated APPtg mice had a similar average distance from the platform (Fig. 1A and B) and average latency to reach the platform (Fig. 1C and D) compared to vehicle-treated mice on all six days of training trials. After learning was concluded, we performed a probe trial on d7 and assessed the average distance to the previous platform location (Fig. 1E) and the proportion of time animals spent in the quadrant that used to contain the platform (Fig. 1F). While performance was highly individual, we could not detect significant differences between groups that could be attributed to either treatment or age.

To investigate whether DMF-treatment had an effect on accumulation of β-amyloid, we quantified the concentration of soluble and aggregated Aβ in the brain by immunoassay. The difference between 80d- and 100d-old APPtg animals was highly significant for both soluble and aggregated Aβ, but treatment with DMF did not change Aβ concentration at either age (Fig. 2A and B). We further analysed Aβ plaque aggregation by immunohistological staining of brain sections against Aβ. We found an age-related increase in the number of diffuse, small and medium plaques in the cortex (Fig. 2F–H), but no difference in the total plaque number (Fig. 2C), the relative area covered by plaques (Fig. 2D) or the number of dense or large plaques (Fig. 2E and I). Similar to the immunoassay results, no differences between DMF-treated and vehicle-treated APPtg animals were detected (Fig. 2C–I).

As DMF has been reported to affect the immune system, we assessed activation of microglia, the brain resident innate immune cells. Brain sections were subjected to immunohistological staining against IBA1 for detection of microglia, CD11b for detection of astrocytes, and CD3 for detection of T cells. No changes were observed that could be attributed to either treatment or age.

To assess the influence of DMF-treatment on other populations of immune cells in the brain, we isolated mononuclear cells from fresh brain tissue, and performed flow cytometric analysis of the cell composition. Microglia cells made up >90% of mononuclear cells, whereas different recruited immune cells constituted only around 2% (Fig. 4A). Our antibody panel and gating strategy (Supplementary Fig. 2) allowed for further differentiation of the recruited immune cells. We identified neutrophils, different populations of monocytes and CD4+ and CD8+ T cells (Fig. 4B). No changes were observed that could be related to age or treatment.

3. Discussion

In this study, we investigated whether DMF, a drug approved for the treatment of MS, could be repurposed as treatment for AD. To this end, we studied its potential to mitigate cognitive impairment, β-amyloidosis and neuroinflammation in a mouse model of AD.

To assess the impact of DMF treatment at different stages of disease, we treated mice at two different time points. First, we started treatment before Aβ plaques can be detected in the brain (pre-onset treatment paradigm starting at 40d) (Radde et al., 2006). Second, we started treatment shortly after the onset of Aβ plaque formation (post-onset treatment paradigm starting at 60d).

Mice were treated with DMF at a dose of 75 mg/kg body weight daily. We chose this dose, because it results in a plasma exposure in mice similar to that in humans at the recommended human dose of 480 mg/day, and it was the highest tested dose not associated with tumours. Indeed, we found that DMF treatment at the selected dosage was safe for mice and had no side effects on general health in our model. This is in accordance with another study, where DMF was found to have fatal side effects only at higher dosages (160 and 320 mg/kg) (Hui et al., 2021).

We found that treatment with DMF compared to vehicle had no effect on learning and memory performance, β-amyloidosis and microglia activation in the brain. Surprisingly, also the small amount of immune cells recruited to the brain were unaffected by DMF treatment. These findings are in contrast to previous findings in two different rodent models of AD (Majkutewicz et al., 2016, 2018; Rojo et al., 2018).

One research group found that continuous DMF delivery via chow improved memory performance in young and aged rats that received intracerebroventricular injections of streptozotocin (STZ) to induce a phenotype that is claimed to be AD like (Majkutewicz et al., 2016, 2018). Systemic injections of STZ are thought to increase oxidative stress in form of tissue reactive oxygen species (ROS) generation (Grieb, 2016). Oxidative stress has been implicated in the pathogenesis of AD (Martin et al., 2018), but its presence and potential implications in our model are unclear. We have shown previously that early mitochondrial dysfunction restricted to microglia does not influence β-amyloidosis (Steffen et al., 2018). Considering that activation of the Nrf2 pathway by DMF to counteract oxidative stress has been well established (Rosito et al., 2020; Scuderi et al., 2020), differences in the role of oxidative stress on disease progression in the STZ model compared to the APPPS1-21 model may provide at least a partial explanation of the conflicting results.

In another study, DMF treatment protected older transgenic mice from motor and cognitive impairment (Rojo et al., 2018). The authors used a double-transgenic model expressing both human APP with mutations derived from familial AD and human mutated tau protein (P301L). The authors further report reduced activation of microglia and astrocytes and an increase in Nrf2 and its target genes (Rojo et al., 2018). However, results from their study cannot be compared directly to our results, as the applied models differ substantially from each other with

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1 Data described in the pharmacological review application 204063Orig1s000 to the FDA by Paul C. Brown, https://www.accessdata.fda.gov/drugsatfda_docs/nda/2013/204063Orig1s000PharmR.pdf.
with respect to plaque onset (6–9 months vs. 1–2 months) and genetic modifications (APPV717I tauP301L vs. APPKM670/671NL PS1L166P).

Other suggestions for potential positive effects of DMF in AD were taken from in vitro experiments. DMF stimulated phagocytosis by microglia (Zhao et al., 2015), and prevented activation of primary microglia and astrocytes by lipopolysaccharide (LPS) (Paraiso et al., 2018; Wilms et al., 2010). Another study described that higher doses of DMF may support myelination by increased ferritin uptake, but reduce phagocytosis (Pagani et al., 2020). However, it is important to keep in mind that in vivo, DMF is metabolized rapidly to MMF. Thus, in vitro studies using DMF instead of MMF may have limited relevance for in vivo studies (Mrowietz et al., 2018). Several publications reported differential effects of DMF and MMF on primary cell cultures. DMF but not MMF significantly altered pro-inflammatory cytokine production by primary microglia independent of Nrf2 (Peng et al., 2016). Moreover, DMF caused stronger activation of antioxidant gene expression in primary astrocytes compared to MMF (Scannevin et al., 2012). DMF also protected better from Aβ-mediated neurotoxicity than MMF (Campolo et al., 2018). With respect to phagocytosis, results for MMF are conflicting (Kronenberg et al., 2019; Parodi et al., 2015).

It has been shown that MMF reaches the intact brain after treatment with DMF, but it does so at low concentrations (Kumar et al., 2017). Indeed, both DMF and MMF are expected to have poor penetration of the intact blood–brain barrier (BBB) as both their logP values (DMF predicted logP 0.56, MMF predicted logP –0.06) are outside of the range for optimal BBB penetration (logP 1.5–2.7) (Pajouhesh and Lenz, 2005).

Consequently, availability of MMF likely depends on the integrity of the BBB with a more permeable BBB allowing for higher MMF concentrations in the brain. Differences in compound availability may provide further explanations for differences in our model compared to previous findings, where DMF was shown to have a reproducible effect on neurological conditions that feature lesions and/or loss of BBB integrity. Beneficial effects of DMF treatment have been described in experimental autoimmune encephalomyelitis (EAE) models (Chen et al., 2014; Schilling et al., 2006; Schulze-Topphoff et al., 2016), and models of stroke and traumatic brain injury (Casili et al., 2018; Kramer et al., 2017; Safari et al., 2017; Yao et al., 2016). Similarly, the above-discussed induction model using STZ injection has been associated with increased BBB permeability (Ituber et al., 2006).

In our experiments, we used APPPS1-21 mice (Radde et al., 2006) at a maximum age of 3–4 months. Other APPS1 strains [APPswe/PS1ΔE9 (Jankowsky et al., 2004) MMRC stock #34832] showed no difference of BBB integrity compared to wild-type animals at 6 months of age (Janota et al., 2015). BBB impairment in that strain was only described at old ages of 12 months and older (Minogue et al., 2014; Wang et al., 2011). Moreover, Kunze et al. (2015) described that systemic treatment with DMF actually strengthens the BBB in a stroke model. Restoring BBB integrity is beneficial in models with impaired BBB, but may be irrelevant or even counterproductive in our model considering the contribution of the BBB to Aβ clearance by active transport and passive paracellular diffusion (Lamartiniere et al., 2018; Pahinke et al., 2009; Stork et al., 2018).

Taken together, these findings suggest that cerebral MMF concentrations may be too low at the chosen time points in our model to have a significant effect on the course of disease. Accordingly, studying pharmacodynamics found that changes in gene expression in the brain only become apparent at 200 mg/kg (Brennan et al., 2016). However, high systemic doses may not be relevant for clinical application. In wild-type...
5. Material & methods

5.1. Animals

Female amyloid precursor protein (APP) and presenilin 1 (PS1) double-transgenic (APPPg) mice [APPPS1-21 (Radde et al., 2006)] were housed in groups of five to six animals at the animal care facility of the Department of Comparative Medicine (KPM Radiumhospitalen) at the University Hospital Oslo (Norway) with a 12 h/12 h light/dark cycle and free access to food and autoclaved water at a mean temperature of 22 °C. Females were housed in the same room as male mice, but experimental animals were moved to a separate room one day before behavioural testing started. All experiments were conducted in accordance with the guidelines for animal experiments of the European Union Directive and regional laws and were approved by the local animal ethics committee (approval 19/156647).

C57BL/6 mice, dosages of 160 mg/kg and 320 mg/kg have been associated with increased mortality and liver abnormality (Hui et al., 2021). Association of high dosages with tumours was also described in the prescribing information of Tecfidera® (DMF). Interestingly, nanoparticulate carriers may provide a method to increase brain availability of MMF without increasing systemic concentrations of the drug (Kumar et al., 2017).

One limitation of the present study is that we have used only female mice to study the effect of DMF treatment. Previous in vivo studies have either not specified the sex of mice used for treatment experiments (Rojo et al., 2018), or used only male rats (Majkutewicz et al., 2016, 2018), while the numerous in vitro studies inherently do not contribute to this discussion. Thus, we cannot rule out that the treatment response is influenced by sex differences. In humans, women are more likely to develop AD than men are. This observation has been attributed to oestrogen levels, higher life expectancy and socio-economic factors [reviewed by Ferretti et al. (2018)]. However, sex differences in treatment efficacy have not been studied systematically in AD patients.

Altogether, the reasons why DMF failed to have an effect on the APPPS1-21 mouse model remain unclear despite several potential explanations. This problem highlights the need for careful re-evaluation in animal models before drugs can be repurposed for other diseases in humans. Model variation e.g. due to different transgenes and, thus, different disease kinetics [reviewed by Drummond and Wisniewski (2017)] may point towards possible mechanisms and effects that can potentially benefit patients. In case of the APPPS1-21 model, we have demonstrated that with respect to potential herbal medication for AD, this model can deliver reliable results than are transferable to humans (Hofrichter et al., 2013, 2016; Pahnke et al., 2014). Moreover, our methods are sensitive enough to find age-related differences of only 20 days in age, reducing the possibility of a false-negative finding.

4. Conclusion

In the present study, we used APPtg mice to test the potential of DMF as treatment option for AD. We did not find any differences in Aβ accumulation rate, microglia activation and learning and memory performance. While our results do not favour the repurposing of DMF for AD, further studies are required to investigate which effect BBB integrity has on DMF treatment potential in different models and whether modulated targeted availability of MMF in the brain is able to overcome the problem of systemic side effects at higher doses.

5.5. Immunohistochemical analyses

Formalin-fixed hemispheres were embedded in paraffin and cut in 4-μm-thick coronal sections. Sections (approximately Bregma −2.0 mm) were stained for microglia (IBA1, 1:1000, FUHELM Film Wako Chemicals Europe GmbH, 019−19741) and Aβ (anti-human Aβ clone 4G8; 1:4000, BioLegend, USA) using a BOND-III® automated immunostaining system (Leica Biosystems GmbH, Germany) with a haematoxylin counterstain (provided with the staining kit). Sections for Aβ staining were pre-treated for 5 min with 98% formic acid before being stained.
Fig. 3. Microglia numbers are not affected by DMF treatment. (A, B, D-K). Brain sections from DMF- or vehicle-treated APPtg mice were immunohistologically stained against IBA1. To detect microglia, we used a custom deep-learning algorithm that recognizes IBA1+ somas in the cortex. Microglia analysis was performed as (A) IBA1+ somas relative to analysed cortical area and (B) average soma size. N = 11–13; data presented as mean ± standard deviation; each black circle represents one animal. (C) We assessed the activation status of microglia by flow cytometry, where we measured the mean CX3CR1 fluorescence intensity of the CD11b+CD45int population (microglia); N = 11–12; data presented as mean ± standard deviation; each black circle represents one animal. (D-K) Representative images from immunohistological stainings for each group. (D, F) 80d-old vehicle-treated and (E, G) DMF-treated, (H, J) 100d-old vehicle-treated and DMF-treated (I, K) APPtg mice. Scale bars indicate 1000 µm (overviews: D, E, H, I) or 100 µm (details: F, G, J, K). Asterisks indicate p values as follows: * p < 0.05, ** p < 0.01, *** p < 0.001. Statistical analysis performed with (A, C) Kruskal-Wallis/Dunn’s test or (B) one-way ANOVA/Bonferroni.
staining, tissue sections were digitized at 230 nm resolution using a Pannoramic MIDI II slide scanner (3DHISTECH Ltd., Hungary) (Frohlich et al., 2016; Hofrichter et al., 2016; Krohn et al., 2011, 2015; Paarmann et al., 2019; Steffen et al., 2016, 2017). Quantitative analysis was performed using deep-learning algorithms generated with DeePathology™ STUDIO (DeePathology Ltd., Israel) (Bascunana et al., 2021). We generated one algorithm to detect and categorize plaques as diffuse or dense-core plaques, and a second algorithm to identify IBA1® positive cells. Both algorithms were used separately on similar regions of interest (i.e. the cortex). Samples for immunohistological analysis were obtained during three independent experiments for each treatment paradigm, and processed and measured together. Group sizes were 11–13 animals. Recruitments were compared using two-way ANOVA without multiple comparisons. Only relevant comparisons were included, i.e. either age-matched DMF-treated vs. vehicle-treated or treatment-matched 80d-old vs. 100d-old. For repeated measures, we performed two-way ANOVA without multiple comparisons. See Supplementary Table 1 for details on which test was applied to each comparison.

5.7. Flow cytometry

First, mononuclear cells were isolated from brain hemispheres. To this end, we homogenized tissue in dissection buffer (Hank’s buffered salt solution containing 15 mM HEPES and 0.56 g/L glucose) and sieved it through a 70 µm strainer. The cell suspension was washed and resuspended in 3 mL 70% Percoll® (GE Healthcare, USA) before we layered 7 mL 30% Percoll® on top to form a discontinuous gradient. After centrifugation (45 min, 800g, no brake), we recovered the cells from the 70%/30% interphase. Cells were washed and transferred to 5 mL FACS tubes (Sarstedt AG & Co. KG, Germany) for surface staining. Single cell suspensions were incubated with an antibody against CD16/CD32 (S17011E, unconjugated) to block unspecific binding by Fc receptors and the fixable viability dye Zombie Aqua™ (BioLegend, USA). After a brief incubation time, we added fluorochrome conjugated antibodies in FACS buffer (PBS containing 2% FCS and 0.01% NaN₃) together with a monocyte block (True-Stain Monocyte Blocker™, BioLegend, USA) that inhibits unspecific binding of monocytes to Cy-dyes. Samples were incubated on ice for 30 min, washed once, and immediately acquired on a BD LSRFortessa™ flow cytometer. Our antibody panel consisted of CD45 (30-F11, Alexa Fluor® 488), CD11b (M1/70, APC), Ly6G (1A8, PerCP/Cy5.5), Ly6C (HK1.4, PE/Cy7), CD3 (17A2, APC/Fire™ 750), CD4 (GK1.5, Alexa Fluor® 700), CD8a (53-6.7, Brilliant Violet 605™), CX3CR1 (SA011F11, PE). All antibodies were purchased from BioLegend, USA. Data were analysed using FlowJo software (version 10.6.2, TreeStar). Flow cytometry experiments were repeated twice for each treatment paradigm and data was pooled for analysis, resulting in 11–12 animals per group (Vehicle 80d, N = 11; DMF 80d, N = 12; Vehicle 100d, N = 11; DMF 100d, N = 12).

5.8. Statistical analysis

Statistical analysis was performed with Prism version 6 (GraphPad Software, USA). All datasets were tested for normality (i.e. Gaussian distribution) using the D’Agostino-Pearson omnibus K2 normality test and for equal variances using the Brown-Forsythe test. If all groups in a comparison were normally distributed (p > 0.05) with even variances (p > 0.05), we performed one-way ANOVA with Bonferroni correction for multiple comparisons. If one or more groups failed the normality test (p < 0.05) or variances were uneven (p < 0.05), we performed Kruskal-Wallis test with Dunn’s multiple comparisons test. Only relevant comparisons were included, i.e. either age-matched DMF-treated vs. vehicle-treated or treatment-matched 80d-old vs. 100d-old. For repeated measures, we performed two-way ANOVA without multiple comparisons.

Fig. 4. Recruitment of immune cells from the periphery is not changed by DMF treatment. We investigated the composition of brain immune cells by flow cytometry according to the gating strategy shown in Supplementary Fig. 2. (A) After isolation of mononuclear cells from brain tissue, we quantified the proportions of microglia to recruited immune cells and (B) the different populations constituting the recruited immune cells. N = 11–12; data presented as mean ± standard deviation; each black circle represents one animal.
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Author contributions

L.M.: Conceived and designed the project, collected the data, performed the analysis, wrote the paper, acquired funding. M.B.: Contributed to experiments, edited the paper. P.B.: Contributed to experiments, edited the paper. J.P.: Conceived and designed the project, edited the paper, acquired funding, supervised the project.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [run on the text provided in the COI form].

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Appendix A. Supplementary data

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