Teriflunomide Promotes Oligodendroglial 8,9-Unsaturated Sterol Accumulation and CNS Remyelination

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Abstract

Background and Objectives
To test whether low concentrations of teriflunomide (TF) could promote remyelination, we investigate the effect of TF on oligodendrocyte in culture and on remyelination in vivo in 2 demyelinating models.

Methods
The effect of TF on oligodendrocyte precursor cell (OPC) proliferation and differentiation was assessed in vitro in glial cultures derived from neonatal mice and confirmed on fluorescence-activated cell sorting–sorted adult OPCs. The levels of the 8,9-unsaturated sterols lanosterol and zymosterol were quantified in TF- and sham-treated cultures. In vivo, TF was administered orally, and remyelination was assessed both in myelin basic protein–GFP-nitroreductase (Mbp: GFP-NTR) transgenic Xenopus laevis demyelinated by metronidazole and in adult mice demyelinated by lysolecithin.

Results
In cultures, low concentrations of TF down to 10 nM decreased OPC proliferation and increased their differentiation, an effect that was also detected on adult OPCs. Oligodendrocyte differentiation induced by TF was abrogated by the oxidosqualene cyclase inhibitor Ro 48-8071 and was mediated by the accumulation of zymosterol. In the demyelinated tadpole, TF enhanced the regeneration of mature oligodendrocytes up to 2.5-fold. In the mouse demyelinated spinal cord, TF promoted the differentiation of newly generated oligodendrocytes by a factor of 1.7-fold and significantly increased remyelination.

Discussion
TF enhances zymosterol accumulation in oligodendrocytes and CNS myelin repair, a beneficial off-target effect that should be investigated in patients with multiple sclerosis.
The landscape of multiple sclerosis (MS) treatment has changed dramatically over the last decades, with great therapeutic progresses achieved for the prevention of clinical relapses. However, controlling the disability worsening remains a key unsolved issue, as currently applied therapeutic strategies targeting the adaptive immune system are mostly insufficient to prevent later disability accrual and disease progression. Disability in MS is mainly a consequence of irreversible neuronal damage that could be induced jointly by chronic inflammation and persisting demyelination. Following a demyelinating insult, an endogenous spontaneous repair process may take place to prevent the deleterious consequences of demyelination and protect axons from degeneration. Whereas some remyelination may occur in MS, this regenerative process is often abortive and generally insufficient to fully repair lesion and prevent neurodegeneration. Beside immunomodulation, a major therapeutic goal aiming at promoting remyelination has therefore emerged. From in vitro and in vivo experimental models, a certain number of either repurposed or newly designed molecules have been identified as promising remyelinating compounds. Of interest, most of these molecules were shown to promote oligodendrocyte differentiation and (re)myelination independently of their canonical targets through the accumulation of 8,9-unsaturated sterols in myelinating cells. Whether some currently approved drugs targeting the adaptive and/or innate immunity could also exert a promyelinating effect through off-target mechanisms remain poorly investigated, but would open the perspective of a dual beneficial impact on MS course.

Teriflunomide (TF) is an oral immunomodulatory therapy that has been approved for relapsing-remitting forms of MS. Its primary mechanism of action is an inhibition of dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme involved in pyrimidine synthesis pathway highly expressed in proliferating lymphocytes. Beyond its impact on lymphocytes, it has been suggested that TF could promote oligodendrocyte differentiation in primary cultures from immature rat brains. However, this effect was observed only for very short pulses on immature cells, disappeared when administration was prolonged for several days, and was observed only for a single concentration of 5 μM, which corresponds approximately to the one to be found in the blood, but that could hardly be reached in the brain of human treated subjects. These results are therefore difficult to translate to human care, prompting us to explore more in depth the potential impact of this drug on myelinating cells and remyelination.

Combining a range of in vitro and in vivo models from different species, we have obtained results supporting a promyelinating effect of TF: (1) at nanomolar concentrations, TF promotes the differentiation of neonatal and adult mouse oligodendrocytes; (2) its effect on oligodendrocyte was associated with an accumulation of the 8,9-unsaturated sterol zymosterol and reverted by inhibition of the oxidosqualene cyclase; and (3) TF promoted remyelination in vivo both in Xenopus laevis and in mice.

Methods

Animals

All animal experiments were conducted with respect to the European Union regulations and approved by the ethical committee for animal use (approval number Ce5/2010/025) (APAFIS #6269 and APAFIS #5842-2016101312021965).

Mice

C57/Bl6 and transgenic PDGFRα::GFP (reference 13; RRID: IMSR_JAX:007669) mice were grown in our animal facility (agreement #A75-13-19). Experiments were conducted with respect to the European Union regulations and approved by the ethical committee for animal use (approval number Ce5/2010/025) (APAFIS #6269).

Xenopus

In the myelin basic protein–GFP-nitroreductase (Mbp::GFP-NTR) transgenic Xenopus laevis line, the expression of the green fluorescent protein GFP reporter gene and the bacterial NTR enzyme is controlled by a portion of the mouse myelin basic protein (MBP) regulatory sequence, enabling a selective expression of the transgene in myelinating oligodendrocytes and the induction of oligodendrocyte death and demyelination when metronidazole (MTZ) is added to the swimming water. In this model, remyelination (spontaneous or induced by TF) can be assessed following a recovery period of 3 days. Treatment was performed at a pre-metamorphosis stage.

Cell Cultures and Treatment

Oligodendrocyte precursor cells (OPCs) were isolated from neonatal mice (P1 or P2) using a Percoll gradient. OPCs were
isolated from 2-month-old adult platelet derived growth factor receptor-alpha/green fluorescent protein (PDGFa::GFP) hemizygous mice through GFP+/O4+ fluorescence-activated cell sorting (FACS).

At days in vitro (DIV) 3, teriflunomide (Genzyme) at concentrations ranging from 10 nM to 5 μM was added, and cells were grown for 24, 48, and 96 hours to assess the survival, proliferation, and differentiation, respectively. OPCs were either untreated or treated with 10 nM Ro 48-8071 fumarate (Tocris; an inhibitor of lanosterol synthesis), 25 μM uridine (Sigma-Aldrich), rat recombinant interferon-gamma (IFNγ) (10 ng/mL; PeproTech), and/or rat recombinant interleukin-17 (IL-17) (50 ng/mL; R&D Systems), either alone or in combination with 10 nM TF for 96 hours.

Cell Survival Assay

Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) rapid colorimetric assay (0.5 mg/mL MTT incubated at 37°C with 5% CO2 for 4 hours; Sigma-Aldrich, MS655).

Sterol Analysis

OPCs were grown on poly-L-lysine–coated 100 mm Petri dish and at DIV 3, either TF (10 nM) or and Ro 48-8071 fumarate (10 nM) or and uridine (25 μM) were added during 24 hours. Cells were dissociated using 0.5% trypsin/ethylenediaminetetraacetic acid, scraped, and centrifuged 10 minutes at 10,000 rpm, and the supernatant was frozen at −80°C.

Sterol extraction and derivatization were performed as described. Briefly, 20 μL of a mixture of deuterated sterols (Avanti Polar Lipids: Coger-Paris 15), consisting of (d5-zymosterol [1 μg/mL], d7-cholesterol [10 μg/mL], and d6-lanosterol [1 μg/mL]) in methanol as internal standards was added to 100 μL of cell lysates. Following extraction in n-hexane, the dried residue of free sterols was derivatized, dissolved in methanol, and injected into the ultra performance liquid chromatography coupled with mass spectroscopy/MS system, consisting in a Triple Quadrupole Mass Spectrometer (TQD-Waters) equipped with an electrospray ionization probe and coupled to an ultra performance liquid chromatography Acquity system (Waters). A calibration curve was obtained by a mixture of zymosterol, lanosterol (0.25 μg/mL each), and cholesterol (50 μg/mL). Sterol levels were normalized to protein concentration (bicinchoninic acid assay).

Emopamil Binding Protein and DHODH Reverse Transcription-Quantitative Polymerase Chain Reaction

OPCs and mouse tissue (brain, spinal cord [SC], spleen, and liver) messenger RNAs were isolated with the Maxwell RSC simplyRNA Cells (Promega). Primers for emopamil binding protein (EBP) (forward primer: TGTGCGAGGAGAAGAGAT; reverse primer: GATAGGCCCACCGTTTATT) and DHODH (forward primer: TCCAATGGGATGAGCCAG; reverse primer: CAGGGCCCGTCTTCTCAG) and glyceraldehyde 3-phosphate dehydrogenase (forward primer: GGTTTCTATAATCCGGACTGC; reverse primer: CCATTTTGTCTACGGGACGA) were designed by NCBI Primer-BLAST and manufactured by Eurofins Genomics. A LightCycler 480 SYBR Green I Master (Roche Diagnostics) was used for quantitative real-time PCR. The cycle time was calculated using LightCycler 96 Software V1.1.

Antibodies

Anti-O4 antibody (mouse IgM hybridoma provided by I. Sommer) was revealed by a phycocerythrin anti-mouse IgM (BD Pharmingen). For cell immunostainings, we used a rabbit anti-Olig2 Ab (diluted 1:500, Millipore, AB15328), a mouse anti–myelin oligodendrocyte glycoprotein (MOG) Ab (diluted 1:5, hybridoma provided by Dr. C. Linington), a mouse IgG1 anti-Ki-67 Ab (diluted 1:100, BD Biosciences–Phar DIGEN), and a rat anti-bromodeoxyuridine (anti-BrdU) (diluted 1:20, Abcam).

For immunohistochemistry, we used a chicken anti-MBP Ab (diluted 1:40, Millipore), a rabbit anti-Olig2 Ab (diluted 1:500, Millipore, AB15328), and a mouse IgG2b anti–adenomatous polyposis coli (APC, clone CC1; diluted 1:300, Calbiochem). Secondary antibodies used were Alexa conjugated antibodies (Invitrogen) at a 1:1,000 dilution.

Immunofluorescence

Cells were fixed 15 minutes with 4% paraformaldehyde (PFA) and permeabilized 10 minutes by 0.1% Triton-phosphate-buffered saline (PBS). For BrdU immunocytochemistry, fixed cells were incubated 10 minutes in 1M HCl—PBS on ice and 10 minutes in 2M HCl—PBS at room temperature before applying anti-BrdU antibody overnight at +4°C.

Demyelinating Lesion Induction

Focal demyelination was induced by an injection of 0.5 mL of 1% LPC (lysophosphatidylcholine; Sigma-Aldrich, St Louis, MO) in the dorsal SC of 2-month-old anesthetized PLP-GFP transgenic mice as described.

Oral Treatment

Mice received 20 mg/kg of TF dissolved in dimethylsulfoxide and resuspended in a solution containing 0.6% carboxymethylcellulose through oral gavage daily. An equivalent volume of identical vehicle solution without TF was given to control mice. Daily administration began the day following LPC injection and was repeated for 10 days.

Perfusion and Tissue Processing for Immunohistochemistry

The spinal cords of animals perfused with 4% PFA (Sigma) were cryoprotected in 15% sucrose, frozen in 15% sucrose-7% gelatin and 14-μm serial cryostat sections were performed. Immunohistochemistry was then realized according to the already reported procedure.

Tissue Processing for Electron Microscopy

Mice were perfused with 2% PFA and 4% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), and their SCs...
were postfixed overnight. Tissue was then processed as described. One-μm-thick sections were stained with toluidine blue, and ultrathin 70-nm sections (ultramicrotome; Reichert UltraCut S) were stained with uranyl acetate and Reynold lead citrate and observed with a transmission electron microscope (Hitachi, Tokyo, Japan).

**Image Acquisition and Quantitative Analysis**

For cell immunocytochemistry, a Zeiss Axio Imager Apotome was used. Ten fields captured from each well, corresponding to approximately 80–100 Olig2+ cells per coverslip, were counted, and the percentage of myelin-forming oligodendrocyte (Olig2+/MOG+) cells was determined for at least 4 coverslips per condition.

For biochemistry experiments, each sample was measured in duplicate per condition, and sterol production was normalized to protein concentration evaluated by bicinchoninic acid assay. Results were expressed as % of controls ± SEM of 3 independent experiments.

For experiments in living tadpoles, the number of cells expressing GFP cells was quantified in each optic nerve using a Macroscope Nikon AZ100 with a ×15 magnification. Data were obtained from 5 to 8 animals per condition.

For histology, we used a digital camera (AxioCam; Zeiss, Jena, Germany) attached to a microscope (Apotome; Zeiss) and performed Z-series of sections at 0.3-μm increment. Green, red, far-red, and blue fluorescence were acquired sequentially. Five to 10 sections were analyzed per animal, and for every single image, the extent of myelin loss (characterized by a lack of MBP staining) was quantified using Zen software. Inside the lesion area/lesion core, the number of Olig2+CC1+ cells was assessed.

For electron microscopy, a mean of 25 images per animal were analyzed within the lesion using a transmission electron microscope (Hitachi, Tokyo, Japan) connected to a digital camera (AMT, Danvers, MA) at ×13,000. The number of remyelinated axons was counted (ImageJ software).

**Statistical Analysis**

Data are expressed as mean ± SEM. Statistics were performed using GraphPad Prism 6 (GraphPad Software). For statistical differences between 2 groups, the unpaired 2-tailed Student t test was applied. For grouped analysis, we used a 1-way analysis of variance (ANOVA) followed by the Dunn post hoc test to compare drug treatment to control condition or Newman-Keuls test for multiple comparison. Statistical significance was defined as *p < 0.05, **p < 0.01, and ***p < 0.001.

**Data Availability**

The data that support the findings of this study are available from the corresponding author on reasonable request.

**Results**

**TF Inhibits OPC Proliferation**

In newborn glial cultures, Olig-2 and 4',6-diamidino-2-phenylindole colabeling showed an 80% enrichment in OPCs (Figure 1, A and B). Compared with untreated control, addition of TF (10 nM and 5 μM) in the culture medium for 24 hours did not affect cell viability of OPCs (Figure 1C). TF added 48 hours to the culture medium decreased OPC proliferation, measured as the incorporation of bromodeoxyuridine (BrDU), by 34% up to 63% for concentration ranging between 10 nM and 5 μM, respectively (Figure 1, D–F). This effect for the lower 10 nM concentration was further confirmed by quantifying the expression of the proliferation marker Ki67 (Figure 1, G–I).

**TF Increases Oligodendrocyte Differentiation**

We then asked whether the TF could influence OPC differentiation into mature oligodendrocytes. One of the most related markers of oligodendrocyte maturation is myelin oligodendrocyte glycoprotein (MOG).25,26 In a first set of experiments, a 96-hour treatment of cultures with TF induced an increase in the ratio of mature oligodendrocytes (Olig2+/MOG+ cells) by factors of 1.4 up to 2.1 for concentrations ranging from 10 nM to 1 μM, respectively (Figure 2, A–C). This acceleration of maturation was also efficient on adult OPC FACS isolated from the brain of 2-month-old PDGFRα::GFP mice. In these cultures of adult OPCs, the low dose of TF (10 nM) was sufficient to increase maturation (Figure 2, D–F).

**TF Promotes OPC Differentiation by Acting on the 8,9-Unsaturated Sterol Biosynthesis Pathway**

In newborn OPC cultures, 10 nM TF significantly increased OPC differentiation by a factor of 1.97-fold in comparison to control (1.97 ± 0.16; TF vs 1 ± 0.15; Ctrl). Cotreatment with Ro 48-8071, an inhibitor of lanosterol synthesis, abrogated the enhanced TF-induced oligodendrogial differentiation (1 ± 0.15 of Olig2+Mog+ cells in TF-treated OPCs; 0.94 ± 0.18 of Olig2+Mog+ cells in OPCs cotreated with TF and Ro48-8071) (Figure 3A).

To investigate the mechanism involved in TF-induced OPC differentiation, we analyzed the levels of sterols produced by purified newborn OPCs. Cells were treated for 24 hours either with TF (10 nM) or Ro 48-8071 (10 nM) or cotreated with TF and Ro 48-8071. Three sterols along the cholesterol biosynthesis pathway were quantified: lanosterol (that accumulates following CYP51 inhibition), zymosterol, and cholesterol. Although TF did not significantly enhance lanosterol or cholesterol production, the zymosterol level was increased 1.9-fold in comparison to control. Addition of Ro 48-8071 effectively blocked TF-induced zymosterol production (Figure 3B). Together, these findings indicate that the accumulation of the 8,9-unsaturated sterol zymosterol in OPCs is a crucial mechanism for TF-induced oligodendrocyte differentiation.
similar accumulation of zymosterol has been previously reported with other enhancers of myelination such as clemastine and may suggest that TF, which canonical signaling pathway targets the DHODH, could also inhibit EBP, the enzyme catalyzing zymosterol in the cholesterol biosynthesis pathway.

This led us to analyze the expression of EBP and DHODH in OPCs and the brain and spinal cord from adult wild type mice.

We observed a moderate level of expression of both EBP and DHODH in OPCs relatively to the liver and spleen that were used as positive control for EBP and DHODH enzymatic gene expression, respectively, but this OPC expression was enriched compared with the brain or SC (Figure 3, C and D).

Whereas we could not demonstrate a direct interaction between TF and EBP in a specific screening assay (supplemental data, links.lww.com/NXI/A606), we showed that an excess of
uridine, that had no effect on OPC differentiation when added alone, could reverse the differentiation induced by TF treatment (Figure 3E). In addition, uridine totally blocked the accumulation of zymosterol induced by TF (Figure 3F).

Finally, adding recombinant cytokines to mimic the MS inflammatory environment, we found that a combined IFNγ/IL-17 cytokine stimulation slightly decreased the OPC differentiation in comparison to control (0.6 ± 0.1; IFNγ/IL-17 vs 1 ± 0.1; Ctrl) and that cotreatment with cytokines and TF reversed the differentiation induced by TF treatment (1.1 ± 0.2; TF + IFNγ/IL-17 vs; 3.2 ± 0.4; TF).

**TF Treatment Increased Oligodendrocyte Remyelination in Vivo**

**In Xenopus laevis**

Mbp-GFP-NTR transgenic Xenopus laevis tadpoles were demyelinated with MTZ (10 mM) for 10 days (Figure 4, A and B) and then returned to either fresh water (controls) or water containing increasing concentrations of TF. After 10 days of exposure to MTZ, the number of GFP⁺ cells per optic nerve significantly decreased from 24.75 ± 4.5 to 3.6 ± 1.1. Three days (R3) after withdrawal of MTZ, spontaneous recovery occurred, and oligodendrocytes per optic nerve reached 8.0 ± 1.1. Treatment of demyelinated tadpoles with TF improved remyelination by a factor of 1.5-, 1.8-, and 2.25-fold for concentrations of 1, 10, and 100 μM, respectively (Figure 4C). These data demonstrated a strong remyelinating efficacy of TF in live animals.

**In Mice**

Following LPC-induced demyelination in the dorsal funiculus (Figure 5, A and B), TF (20 mg/kg) was administrated through oral gavage daily, for 10 days, starting 1 day after LPC injection. The number of oligodendroglial lineage cells and postmitotic oligodendrocytes was quantified using Olig2 (Figure 5, C, D, I, and J) and APC (CC1 mAb) (Figure 5, F–J) as markers, respectively. The number of Olig2⁺ oligodendrocyte lineage cells within the lesion did not change between the groups (Figure 5, E). By contrast, TF increased the number of CC1 postmitotic oligodendrocytes in the lesion compared with animals treated with the vehicle (241.2 ± 19.8 vs 179.8 ± 20.9;
Importantly, the fraction of Olig2+ cells also positive for CC1 was significantly increased in the lesion of TF-treated mice (48.9% ± 3.1%) compared with controls (28.7% ± 2.5%) (Figure 5K). These findings demonstrated that TF promotes oligodendrocyte differentiation in vivo during remyelination following LPC-induced demyelination.

We then investigated whether TF treatment modulated myelin repair in the LPC induced focal mouse model of demyelination. In control animals, remyelinating axons were characterized by thin myelin sheaths compared with TF-treated animals (Figure 6, A and B). At 11 days post-lesion, quantification revealed 67.6% ± 3.5% of remyelinated axons in TF-treated animals compared with 56.2% ± 2.6% in control animals.

### Discussion

We report here that TF, an immune active drug approved for the treatment of relapsing MS, enhanced oligodendroglial differentiation in vitro at nanomolar concentrations, both on neonatal and adult purified OPCs in cultures, an effect mediated by an accumulation of 8,9-unsaturated sterols. We further showed that following a systemic administration, TF promoted remyelination in vivo across species in 2 noninflammatory models of demyelination. These results highlight a potential bystander effect of the drug, independent from its action on the immune system.

The classical mechanism of action of TF in MS is a reversible inhibition of dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme involved in the de novo pyrimidine synthesis pathway. This enzyme is highly expressed in proliferating lymphocytes, and its inhibition in the periphery results in a reduced proliferation of activated lymphocytes, impeding the availability of activated autoreactive immune cells to infiltrate the CNS.11 Through its action on DHODH, TF targets both T- and B-cell activation28 TF was also described to enhance, in humans, the expression of programmed

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**Figure 3 OPC Differentiation Induced by Teriflunomide Is Reversed by Ro 48-8071 and Associated With 8,9-Unsaturated Sterol Production**

(A) OPCs treated with unconditioned (Ctrl) or TF and/or Ro 48-8071 were immunostained against Olig2 and MOG at 96 hours following treatment. The percentage of Olig2+ cells coexpressing MOG was significantly increased in cultures treated with TF compared with control, an effect blocked by the Ro 48-8071 treatment. (B) Quantitation of sterol levels in OPCs treated with TF and/or Ro 48-8071. In OPC cultures treated for 24 hours with TF, the level of zymosterol was increased (results are expressed as % of controls), whereas lanosterol and cholesterol levels were unchanged. (C and D) EBP and DHODH messenger RNA levels measured by reverse transcriptase-quantitative polymerase chain reaction in OPCs, Br, and SC. The Li and Sp were used as positive control for EBP and DHODH enzymatic gene expression, respectively. Glyceraldehyde 3-phosphate dehydrogenase was used as the reference gene. (E) The percentage of MOG/Olig2+ oligodendrocytes at 96 hours was significantly increased in OPCs treated with TF compared with control, an effect reversed by the uridine treatment. (F) Uridine blocked the accumulation of zymosterol induced by teriflunomide. (G) The percentage of MOG+/Olig2+ oligodendrocytes at 96 hours following treatment with TF and/or IFNγ/IL-17 was significantly decreased in OPCs treated with TF and exposed to IFNγ/IL-17 cytokines compared with TF. Data are shown as mean ± SEM from 3 individual experiments. *p < 0.05; ***p < 0.001 (1-way ANOVA, Newman-Keuls post hoc test). Br = brain; DHODH = dihydroorotate dehydrogenase; EBP = emopamil binding protein; Li = liver; OPC = oligodendrocyte precursor cell; SC = spinal cord; Sp = spleen; TF = teriflunomide.
Dose response of remyelination potency of TF. (A and B) Detection of GFP+ oligodendroglial cells in optic nerves of stage53 Mbp:GFP-NTR tadpoles. Demyelination of stage53 Mbp:GFP-NTR tadpoles was achieved by 10 days exposure to metronidazole (10 mM) in the swimming water. Tadpoles were then returned to normal water (control) or TF for 3 days. The number of GFP+ cells in the optic nerve in control (A) is lower compared with TF-treated tadpoles (B). (C) Remyelination was assayed by counting the number of GFP+ cells per optic nerve on day 3 of the repair period. Treatment of tadpoles with TF at concentrations ranging between 1 μM and 100 μM improved remyelination up to 2.25-fold compared with spontaneous recovery (control) set as 1. Data shown are mean ± SEM, n = 5–8 tadpoles per group. *p < 0.05; **p < 0.01 (1-way ANOVA, Dunn post hoc test. Scale bar in A and B = 20 μm. MBP-GFP-NTR = myelin basic protein–GFP-nitroreductase; TF = teriflunomide.)

A key finding of our study was the identification of the 8,9-unsaturated sterol pathway as a potential mechanism involved in the promyelinating effect of TF. Those intermediate metabolites in the cholesterol synthesis cascade have been identified as able to promote the formation of new mature oligodendrocytes and their accumulation was proposed as a shared mechanism by many small molecule enhancers of remyelination independently of their canonical target.9 Inhibition of a narrow range of cholesterol biosynthesis enzymes between CYP51 (a target of imidazole antifungal drugs) and EBP, a potential target for the promyelinating drugs clemastine, tamoxifen, and bazedoxifene,9,37 has therefore emerged as a promising pharmacologic pathway for remyelinating strategies. Inhibition with Ro 48-8071 of the oxidosqualene cyclase, an enzyme that catalyzes the step just before the synthesis of lanosterol, the first 8,9-unsaturated sterol, reverted the oligodendroglial differentiation induced by TF in culture, demonstrating the involvement of this cascade.
Furthermore, we showed that TF significantly enhanced the accumulation of zymosterol, which has been identified as the most potent sterol in oligodendroglial differentiation assays.\(^9\) We therefore hypothesized that TF could inhibit EBP, the enzyme catalyzing zymosterol in the cholesterol biosynthesis pathway. We found that OPCs indeed express moderate levels of messenger RNAs for EBP, but subsequently failed to show a direct effect of TF on EBP. Conversely, oligodendrocyte
diff'rentiation and zymosterol accumulation induced by TF were both abrogated in the presence of an excess of uridine, suggesting an involvement of the DHODH canonical pathway. Whether inhibiting DHODH in oligodendrocyte could result in an indirect effect on cholesterol biosynthesis cascade is a burning question that should be addressed in further research.

One limitation of our study is that it focused on noninflammatory animal models, questioning about its relevance in the human context. MS is an inflammatory demyelinating disease in which immune cells could inhibit oligodendroglial differentiation and remyelination. It was reported that the blocked oligodendroglial differentiation induced by proinflammatory cytokines was not restored by the application of oligodendroglial differentiation promoting drugs. According to this, we found that the direct effect of TF on oligodendrocyte was not maintained in the presence of interleukin-17 and interferon-γ. However, adding TF to peripheral blood mononuclear cells was also shown to induce oligodendroglial differentiation, attesting that the dual effect of this drug, on inflammatory and remyelination cells, could indeed favor regeneration in the human context. In clinical phase 3 trials, TF reduced the annualized relapse rate in relapsing MS (around 30% reduction compared with placebo) and clinically isolated syndrome. However, despite this moderate effect on relapses, TF consistently improved several outcomes linked to persistent tissue damage such as relapses with sequelae, brain atrophy, and hypointense lesions on MRI. It also significantly reduced disability progression in the 2 placebo-controlled phase 3 studies in relapsing MS. Although not demonstrating a regenerative or neuroprotective effect of the drug, these results have raised the possibility of a protective action beyond immunomodulation. How the impact on remyelination could be explored in clinical trials is a tricky question, as it would imply to differentiate the anti-inflammatory and the remyelinating properties. A reanalysis of previously acquired imaging data from pivotal trials focusing on markers more sensitive to myelin content such as T1/T2 ratios or more advanced quantitative sequences when available would be of great added value to achieve this goal. An alternative would be to design novel and focused trials aiming at investigating remyelination using the most specific tools available, for instance electrophysiology, quantitative MRI, or positron emission tomography.

In conclusion, we have shown that TF decreases OPC proliferation, induces OPC differentiation, and promotes remyelination in living animals. Importantly, our study provides a novel mechanism of action of TF on remyelination in vivo through the accumulation of 8,9-unsaturated sterols, a crucial cellular mechanism involved in oligodendrocyte physiology. These findings reinforce the idea that modulation of the cholesterol biosynthesis pathway represents a promising therapeutic target for the development of remyelinating strategies and open the perspective of an additional benefit of TF treatment for patients with MS.

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Appendix

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