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Regulation of proton-coupled folate transporter in retinal Müller cells by the anti-psoriatic drug monomethylfumarate

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Abstract

Fumaric acid esters are used to treat psoriasis, an inflammatory skin disease characterized by keratinocyte proliferation. Inflammation and proliferation are hallmarks of retinal disease; hence, fumaric acid esters may have therapeutic value in retinal pathology. In diseased retinas, Müller glial cells (MCs) undergo reactive gliosis, a hyperproliferative state. MCs take up folate, a vitamin necessary for cell proliferation, via the proton-coupled folate transporter (PCFT). Here we examined the effect of monomethylfumarate (MMF), the active metabolite of fumaric acid esters, on expression and function of PCFT in MCs. Primary MCs, isolated from neonatal mouse retinas, were treated with MMF, and PCFT function was monitored by measuring uptake of radiolabeled methyltetrahydrofolate (MTF) at pH 5.5. Dose-response and time-course analyses were performed to identify optimal conditions for maximal effect. The influence of MMF treatment on kinetic parameters of PCFT was studied, and PCFT expression was analyzed at the mRNA and protein level. MTF uptake in MCs decreased by ~50% following 18 h treatment with 1 mM MMF. This effect was specific to fumaric acid esters. MMF treatment decreased the maximal velocity of the transporter without altering substrate affinity. The decrease in PCFT function following MMF treatment was accompanied by attenuated PCFT expression. This is the first report that an antipsoriatic compound can regulate folate transport in MCs and may have potential for the treatment of reactive gliosis in retinal disease.

Keywords

fumaric acid esters; vitamin transport; folic acid; Müller cell gliosis; mouse

Introduction

The major glial cell type in vertebrate retina is the radial Müller cell (MC). These cells span the neural retina forming the inner and outer limiting membrane and are in intimate apposition with every neuron type (Poitry-Yamate and Pournaras, 2011). In response to retinal pathological conditions including diabetic retinopathy, glaucoma and macular degeneration, MCs become activated. This activation, termed gliosis, is characterized by hypertrophy, proliferation, and upregulation of intermediate filament proteins (Reichenbach and Bringmann, 2010). Central to these phenomena, particularly cell proliferation, is the availability of folate, which transfers one-carbon units to appropriate metabolites in the *de novo* synthesis of DNA, RNA and some amino acids. Folates are small ($M_r \sim 500$), hydrophilic, anionic molecules that traverse the plasma membrane only by using specific uptake mechanisms (Blakely et al, 1984). Recently, we characterized folate uptake by MCs and demonstrated that two proteins, proton-coupled folate transporter (PCFT) and folate receptor alpha (FR α), are involved in the acquisition of folate (Bozard et al., 2010). PCFT, the product of the solute carrier gene *SLC46A1*, has a molecular weight of 50–65 kD, depending upon the extent of glycosylation (Unal et al., 2008). It is an integral transmembrane transporter that spans the membrane twelve times and mediates H⁺-coupled electrogenic transport of folate. PCFT operates optimally at pH 5.5 with similar affinity for oxidized and reduced forms of folate (Umapathy et al., 2007; Zhao et al., 2009). In MCs, PCFT is localized to the plasma membrane, nuclear membrane and within endosomes; it plays a major role in folate uptake by these cells (Bozard et al., 2010). PCFT functions in tandem with FR α , which is localized also to the plasma membrane and within endosomes, to enable folate entry into the cell by receptor-mediated endocytosis. In this process, FR α takes up folate with the formation of an endosome, and PCFT transports folate out of the acidic endosome into the cytoplasm (Bozard et al., 2010). FR α is anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) with a molecular mass of ~28–40 kD; it has a greater affinity for oxidized folate (folic acid) than reduced forms (e.g. methyltetrahydrofolate) (Antony, 1996; Salazar et al., 2007; Zhao et al., 2009).

In the present study we investigated the role of fumaric acid esters in regulating the expression and function of PCFT in proliferating MCs. Fumaric acid esters have been used successfully in treating psoriasis, a chronic skin disease characterized by proliferation of keratinocytes (Nogales et al., 2010). Fumaric acid (or fumarate) is an intermediate of the citric acid cycle. Fumarate has poor bioavailability and induces irritation; however, esters of fumaric monomethylfumarate (MMF), dimethylfumarate (DMF), and monoethylfumarate (MEF) are well-tolerated (Rostami Yazdi and Mrowietz, 2008). Fumaric acid esters have been explored as treatments for neurodegenerative diseases such as multiple sclerosis (Moharreggh-Khiabani et al., 2009), which is characterized by glial cell proliferation (Schönrock et al., 1998; Williams et al., 2007). The only known mechanism by which fumaric acid esters attenuate cellular proliferation is through their interaction with GPR109A, a G-protein-coupled receptor (Tang et al., 2008). Since MCs proliferate in retinal disease and folate is required for proliferation, we asked whether fumaric acid esters could alter the acquisition of folate specifically by regulating expression and function of PCFT. Our data show that treatment of primary cultures of mouse MCs with the MMF decreases PCFT-mediated folate uptake.

Materials and methods

Culture of freshly isolated mouse Müller cells

Eyes were isolated from 5–7 day old C57BL/6 mice, MCs were harvested and maintained as described (Bozard et al., 2010). To assess the effects of fumaric acid esters on PCFT activity, second-passage MCs were grown in RPMI (Invitrogen, Grand Island, NY, Cat. No.

11875) containing 2.26 μM folic acid and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells were seeded at a density of $5 \times 10^4/\text{cm}^2$; 24 h later, medium was changed to folate-free RPMI (Invitrogen, Cat. No. 27016) containing 10% FBS and 1% P/S. Cells were incubated in the presence or absence of fumarate, MMD, DMF, MEF, and nicotinic acid at 0.05 – 2.5 mM concentrations over 0.5 to 18 h. Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Retinal pigment epithelial cells (RPE) were isolated as described (Martin et al., 2009). Treatment of mice conformed to policies set forth in the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and the Institutional Animal Care and Use Committee.

Immunocytochemistry

Freshly isolated MCs and RPE cells (as a positive control for GPR109A (Martin et al., 2009)) were seeded in chamber slides at a density of $7.5 \times 10^3/\text{cm}^2$, grown for 24 h, fixed in ice-cold 4% paraformaldehyde for 10 min, air dried, and washed in 0.01 M PBS (pH 7.4) containing 0.2% Triton X-100. After blocking with Power Block and cells were incubated overnight at 4°C with anti-GPR109A (1:100) (Abcam, Cambridge, MA). Cells were incubated 1 h with donkey anti-rabbit IgG coupled to Alexa Fluor 555. Chamber slides were mounted with Fluoroshield with DAPI (Sigma) and examined using a Zeiss LSM 510 confocal microscope equipped with Meta imaging software.

Functional analysis of PCFT activity in mouse Müller cells

PCFT transport function was monitored by the uptake of $[3',5',7,9\text{-}^3\text{H}]\text{-N}^5\text{-methyltetrahydrofolate}$ ($[^3\text{H}]\text{-MTF}$, specific radioactivity, 11 Ci/mmol, Moravsek Biochemicals, Inc., Brea, CA) as the tracer. Most experiments were performed using a pH 5.5 uptake buffer (25 mM Mes/Tris (pH 5.5), 140 mM NaCl, 1.8 mM CaCl_2 , 5.4 mM KCl, 0.8 mM MgSO_4 , and 5 mM D(+)-glucose). In some experiments, a pH 7.5 uptake buffer (25 mM Tris/Hepes (pH 7.5), 140 mM NaCl, 1.8 mM CaCl_2 , 5.4 mM KCl, 0.8 mM MgSO_4 , and 5 mM D(+)-glucose) was used. After cells were washed with warm Na^+ -containing uptake buffer, uptake was initiated by adding 250 μL of uptake medium containing $[^3\text{H}]\text{-MTF}$. Cells were incubated 15 min at 37°C; uptake was terminated by addition of ice-cold uptake buffer at pH 7.5. Cells were washed with ice-cold uptake buffer at pH 7.5, solubilized with 0.5 mL of 1% SDS-0.2N NaOH, and used for determination of radioactivity by liquid scintillation spectrometry.

Saturation kinetics was analyzed by measuring $[^3\text{H}]\text{-MTF}$ uptake with increasing concentrations of MTF. The concentration of MTF was altered as needed with appropriate concentrations of unlabeled MTF while keeping the concentration of radiolabeled $[^3\text{H}]\text{-MTF}$ constant (0.4 μM). The Michaelis constant and the maximal velocity were determined by fitting the Michaelis-Menten equation describing a single saturable transport system to the data: $v = V_{\text{max}}S/(K_t + S)$ where v is the uptake rate, S is the substrate concentration, K_t is the Michaelis constant, and V_{max} is the maximal velocity. Kinetic parameters were determined using Sigma Plot 10.0 (SPSS Inc., Chicago, IL). To determine whether the observed effects of MMF treatment on $[^3\text{H}]\text{-MTF}$ uptake were specific, the uptake of L-(carboxyl- ^{14}C)-ascorbic acid (specific radioactivity, 17 mCi/mmol, Moravsek), $[4,5\text{-}^3\text{H}]\text{-L-leucine}$ (specific radioactivity, 160 Ci/mmol, Amersham, Piscataway, NJ), and $[^3\text{H}]\text{-L-glutamate}$ (specific radioactivity, 27 Ci/mmol, Moravsek) were monitored under identical experimental conditions.

$[^3\text{H}]\text{-MTF}$ uptake was measured at pH 5.5 in the presence of NaCl, the uptake of leucine and ascorbic acid were measured at pH 7.5 in the presence of NaCl. Glutamate was used as the substrate to monitor the activity of system x_c^- (cystine-glutamate exchanger) at pH 7.5 in

the absence of NaCl. The composition of the uptake buffer was the same as described above, except that NaCl was replaced iso-osmotically with *N*-methyl-D-glucamine chloride.

Western blot analysis

Protein was extracted from MCs, subjected to SDS-PAGE and transferred to nitrocellulose membranes as described (Bozard et al., 2010). Membranes were incubated with a polyclonal antibody against PCFT (Bozard et al., 2010) or FR α (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C, followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized with ECL Western blot detection system (Thermo Scientific, Waltham, MA). β -Actin served as the loading control. Densitometry measurements were obtained by calculating the ratio of band density of PCFT to β -actin using Adobe Photoshop CS2.

Semi-quantitative Reverse Transcription–Polymerase Chain Reaction

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) was performed with total RNA samples from freshly isolated MCs and RPE cells. Samples were reverse transcribed with the Gene Amp RNA PCR kit and subjected to PCR using primers specific for mouse GPR109A (Table 1). The QuantumRNA™ Universal 18S primers (Ambion, Austin, TX) were used as internal controls. PCR was performed for 30 cycles with a denaturing phase of 1 min at 94 °C, an annealing phase of 1 min at 58°C, and an extension phase of 1 min at 72°C. PCR products were analyzed by gel electrophoresis.

Quantitative RT-PCR

RNA was isolated from MCs treated with MMF using TRIzol (Bozard et al., 2010). RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For quantitative PCR for gene expression analysis, cDNAs were amplified for 42 cycles in an iCycler (Bio-Rad Laboratories, Hercules, CA) with PCR master mix (ABsolute SYBR Green Fluorescein, ThermoScientific, Surrey, UK) and gene-specific primers (Table 1). PCFT and FR α gene expression was calculated by comparison of Ct values ($\Delta\Delta$ Ct).

Statistics

GraphPad Prism 5 (La Jolla, CA) was utilized to perform ANOVA with Bonferroni pairwise comparison or unpaired t-tests as appropriate. A *p* value of <0.05 was considered significant.

Results

Influence of MMF on folate uptake by Müller cells

To determine whether MMF altered transport activity of folate, radiolabeled [3 H]-MTF was used as the substrate in uptake assays. MTF is the predominant form of folate in blood and is physiologically relevant. Experiments were conducted at pH 5.5, which is optimal for PCFT. There was robust uptake of MTF by MCs. Exposure of MCs to 1 mM MMF for 18 h decreased MTF uptake by ~50% (Fig. 1). We asked whether reduced folate carrier (RFC), which functions optimally at pH 7.5 (Zhao et al., 2009), also operates in folate uptake by MCs. No uptake was observed at pH 7.5. These data are consistent with earlier data demonstrating that in retina RFC is present solely in RPE (Chancy et al., 2000). The data suggest that the effect of MMF treatment on uptake of MTF in MCs is due to decreased PCFT activity. Subsequent studies to characterize the influence of MMF treatment on MTF uptake in MCs were performed at pH 5.5.

Dose-dependence for the inhibition of PCFT activity by MMF treatment

To evaluate whether there was a dose-dependent inhibition of PCFT activity with MMF treatment, MCs were incubated in the absence or presence of increasing levels of MMF (0.05, 0.25, 1.0, and 2.5 mM) for 18 h prior to performing uptake assays. Treatment of MCs with 0.05 mM MMF did not alter MTF uptake, whereas treatment with 0.25 mM MMF inhibited MTF uptake significantly compared to control ($33 \pm 2\%$) as did treatment with 1 mM MMF ($54 \pm 5\%$). Higher MMF concentrations did not inhibit further. There was a dose-dependent decrease in PCFT activity due to MMF treatment in MCs. Subsequent studies were performed with 1 mM MMF.

Time-dependence for the inhibition of PCFT activity by MMF treatment

To assess the effect of treatment time on the inhibition of PCFT activity by MMF, MCs were exposed to 1 mM MMF for varying times (0.5, 1, 3, 6, and 18 h) after which MTF uptake was measured. Incubation of cells with MMF for up to 6 h did not alter PCFT activity whereas treatment for 18 h inhibited uptake by $\sim 60\%$ (Fig. 3). Subsequent experiments were performed with an incubation time of 18 h.

Influence of MMF treatment on kinetic parameters of PCFT

The kinetics of PCFT activity was analyzed in MCs treated with 1 mM MMF for 18 h. MTF uptake was measured with increasing concentrations of MTF (1 – 10 μM); [^3H]-MTF [0.4 μM] was present as a tracer, and the desired final concentrations of MTF were adjusted by adding unlabeled MTF. The uptake of MTF was saturable in MMF-treated and non-treated cells. The uptake was less in treated cells than in non-treated cells at all MTF concentrations (Fig. 4A). The kinetic parameters, Michaelis constant (K_t) and maximal velocity (V_{max}) for the uptake process, were calculated using the non-linear regression method (Michaelis-Menten equation) (Fig. 4A) as well as the linear regression method (linear transformation of the data using the Eadie-Hofstee equation) (Fig. 4B). Both methods yielded similar results. The values for K_t and V_{max} in control cells were $1.0 \pm 0.1 \mu\text{M}$ and $14.4 \pm 0.6 \text{ nmol/mg protein/20 min}$. The corresponding values in MMF-treated cells were $1.1 \pm 0.1 \mu\text{M}$ and $4.4 \pm 0.2 \text{ nmol/mg protein/20 min}$. These data suggested that decreased PCFT activity observed in MMF-treated cells is associated with decreased maximal velocity but no effect on substrate affinity of the transporter.

Influence of MMF treatment on PCFT mRNA and protein levels in Müller cells

To investigate whether decreased maximal velocity of MTF uptake due to MMF treatment (Fig 4) was reflected in decreased expression of PCFT, we analyzed the steady-state levels of PCFT mRNA and protein in MMF-treated and non-treated cells. Quantitative RT-PCR used primers specific for PCFT and internal controls 18S and GAPDH. Treatment of MCs 18 h with 1 mM MMF induced a modest ($\sim 20\%$), albeit non-significant, decrease in the levels of PCFT mRNA (Fig. 5A). Analysis of PCFT protein by Western blotting revealed a major band corresponding to 64 kD in both MMF-treated and non-treated cells (Fig. 5B). There were minor bands with lower molecular masses detected, which likely represent differentially glycosylated PCFT protein (Unal et al., 2008). Densitometric analysis of the ratio of band density (PCFT/ β -actin) using the predominant 64 kD PCFT band revealed $\sim 20\%$ decrease in PCFT levels in MMF-treated MCs (Fig. 5C). As with the analysis of gene expression (Fig. 5A), there was a trend toward decreased levels of PCFT in the presence of MMF (Fig. 5C), although the data did not reach statistical significance. Nonetheless, the observed decrease in PCFT mRNA and protein levels was consistent with decreased maximal velocity of the transporter.

Influence of other fumaric acid esters on PCFT activity

To determine whether attenuation of PCFT activity was specific to MMF or observable if cells were treated with other fumaric acid esters, MCs were incubated with 1 mM fumarate, MMF, DMF, or MEF for 18 h. Treatment with esters of fumaric acid inhibited MTF uptake by >50% compared to untreated cells, however fumarate treatment significantly increased MTF uptake (Fig. 6).

Influence of MMF treatment on other transport systems in Müller cells

To rule out the possibility that MMF treatment attenuates transporter activity generally, we examined the effects of MMF treatment on the activity of three other transport systems: [³H]-leucine uptake mediated by the amino acid transport system L, [¹⁴C]-ascorbic acid uptake mediated by the sodium-coupled vitamin C transporter (SVCT1 and/or SVCT2), and [³H]-glutamate uptake mediated by the sodium-independent amino acid transporter x_c⁻ (cystine-glutamate exchanger) (Yamamoto et al., 2010; Ganapathy et al., 2008; Mysona et al., 2009). While treatment of MCs with MMF decreased leucine uptake, it stimulated the uptake of ascorbic acid and glutamate (Fig. 7). Thus, MMF has variable effects on the activity of several transporters but does not uniformly attenuate activity.

GPR109A expression in mouse Müller cells

GPR109A is a nicotinic acid (NA) receptor known for its anti-lipolytic effect. NA is a vitamin (B₃, niacin) that has been used successfully as a lipid-lowering agent for many years (Tunaru et al., 2003). Since NA treatment is associated with discomforting side effects, research directed towards identifying alternative drugs with improved pharmacokinetic properties led to the discovery of MMF as an agonist for GPR109A (Tang et al., 2008). We investigated GPR109A expression in cultured MCs. Semi-quantitative RT-PCR using primers specific for mouse GPR109A demonstrated robust expression in MCs and RPE (Fig. 8A). LSCM detected abundant GPR109A expression in MCs and RPE cells. In MCs, it is present on the plasma membrane, the nucleus and cytoplasm (Fig. 8B–D). A similar pattern was observed in RPE cells (Fig. 8E–G).

Influence of nicotinic acid treatment on PCFT activity

To examine whether MMF inhibition of PCFT activity observed in MCs was mediated through GPR109A, cells were incubated with 1 mM NA or 1 mM MMF, following which the activity of PCFT was monitored by MTF uptake. Incubation of MCs with 1 mM NA did not alter uptake in MCs, whereas uptake was reduced significantly following MMF treatment (Fig. 9). These data suggest that MMF-induced attenuation of PCFT activity is not mediated through GPR109A.

Influence of MMF treatment on FR α mRNA and protein levels in Müller cells

PCFT works coordinately with FR α to mediate folate uptake in MCs (Bozard et al., 2010). We asked whether FR α activity and expression were altered by exposure of MCs to MMF. To analyze FR α activity, a binding assay was performed in which [³H]-folic acid was used as substrate and human placenta membrane vesicles were used as a positive control. Placenta contains abundant FR α (Prasad et al., 1994). We detected robust FR α binding activity in placental membrane vesicles, but were unable to detect FR α activity in membrane vesicles isolated from mouse MCs (data not shown). The assay was repeated for optimization; however, we were not able to isolate a sufficient quantity of MC membrane vesicles from the very small neonatal mouse retinas to perform the assays. The sheer numbers of mice needed proved to be a technical limitation. Thus, we could not determine whether MMF altered FR α activity in Müller cells. We analyzed expression of FR α mRNA and protein levels by quantitative RT-PCR and Western blotting, respectively. Treatment of

MCs with 1 mM MMF decreased FR α mRNA expression (Fig. 10A), but not FR α protein levels (Fig. 10B, C). Quantitative RT-PCR with primers specific for mouse FR α revealed ~50% decrease in FR α mRNA when MCs were treated 18 h with 1 mM MMF (Fig. 10A); Western blotting did not detect a change in FR α protein (Fig. 10B). Densitometric analysis of the ratio of FR α to β -actin band density confirmed these findings (Fig. 10C).

Discussion

This is the first study of the regulation of PCFT in retinal MCs. Specifically, we analyzed the effect of MMF, a fumaric acid ester, on PCFT activity. Four important findings emerged from these studies.

First, MMF inhibited PCFT activity. MCs were incubated with MMF, and the transport function of PCFT was monitored by uptake of radiolabeled MTF at pH 5.5. The assays were conducted at an acidic pH to mimic the optimal conditions used by the transporter. MC plasma membrane expresses FR α and PCFT (Bozard et al, 2010). When the GPI-anchored FR α binds folate, it internalizes folate along with PCFT. Within the acidic endocytic compartment, PCFT functions optimally to transport folate into the cytoplasm. Thus, to analyze PCFT activity independent of FR α requires the use of an acidic pH. PCFT activity was attenuated with increasing doses of MMF (~50% decrease following 18 h MMF treatment). The decreased PCFT activity was associated with a slight decrease in PCFT mRNA and protein levels. Repeated attempts to analyze FR α binding activity as a function of MMF exposure in MCs were not successful due to technical limitations. We were able to measure FR α mRNA levels and observed ~50% decreased expression in the presence of MMF, however this was not accompanied by altered FR α protein expression.

Second, kinetic analysis of PCFT activity following MMF treatment in MCs revealed that PCFT inhibition by MMF was due to a decrease in the maximal velocity of the transporter with no change in its binding affinity for [3 H]-MTF. The $V_{\max}$ decreased by ~70% in control versus treated cells. The decline in $V_{\max}$ is accompanied by a slight decrease in PCFT mRNA and protein levels.

Third, the inhibition of PCFT activity was specific to fumaric acid esters. DMF and MEF along with MMF attenuated [3 H]-MTF uptake and hence PCFT activity, whereas fumarate did not inhibit PCFT activity. This is noteworthy as controversy exists regarding which fumaric acid ester(s) is/are the active metabolite(s) of fumaric acid ester therapy (Rostami-Yazdi et al., 2010). Interestingly, incubation of cells with fumarate increased the activity of PCFT although the effect was modest, which is consistent with its low bioavailability as a polar compound (Rostami-Yazdi and Mrowietz, 2008).

The effect of MMF in attenuating PCFT was not unique to this transporter as it had a similar, but dampened, effect on system L. The effect was not nonspecific however because MMF increased the activity of transporters for vitamin C (SVCT1/2) and glutamate (system x_c^-). Vitamin C is present at 15-fold higher concentration in neural retina compared to plasma, acting as a free radical scavenger to provide protection from the high level of light-induced oxidative damage (Ganapathy et al., 2008). Increased vitamin C transporter activity following MMF treatment suggests that MMF may augment vitamin C levels in MCs and therefore their antioxidant potential. System x_c^- also plays an important role in antioxidant defense through modulation of cellular glutathione levels (Lo et al., 2008). An increase in system x_c^- activity following MMF treatment suggests that MMF may increase glutathione production. MCs synthesize glutathione from cysteine, glycine, and glutamate, and provide it to neurons (Pow and Crook, 1995). DMF alters intracellular thiol levels by increasing glutathione levels (Mrowietz and Asadullah, 2005), but it is not known whether MMF exerts

this same effect. The data suggest that MMF, like DMF, may enhance the antioxidant capacity of cells. Whether this is the case would be a fruitful area of future study.

Fourth, attenuation of PCFT activity by MMF in MCs was not mediated through the receptor GPR109A. MMF binds this NA receptor (Tang et al., 2008; Hanson et al., 2010; Dunbar and Gelfand, 2010). Our studies showed that incubation of cells with NA did not alter [³H]-MTF uptake and therefore PCFT activity, suggesting that the effect of MMF treatment on PCFT activity was not mediated through GPR109A. Although GPR109A is an established therapeutic target for MMF, it is uncertain whether the antidyslipidemic effect of NA occurs solely via GPR109A (Ahmed et al., 2009). The present data suggest downregulation of PCFT as a new mechanism by which MMF may mediate its effects.

Taken collectively, the data suggest that MMF attenuates folate uptake in retinal MCs and may have potential therapeutic value in retinal disease. MCs undergo reactive gliosis in retinopathies and their excessive proliferation leads to glial scar formation and periretinal fibrocellular membranes (Bringmann et al., 2006, 2009a,b). These features of diseases such as retinal detachment, diabetic retinopathy, proliferative retinopathies, and glaucoma (Lewis et al., 1999; Fletcher et al., 2005; Pannicke et al., 2004; Fisher and Anderson, 1994; Nork et al., 1987; Tezel et al., 2003), may be abrogated by compounds such as fumaric acid esters that have proven useful in the treatment of psoriasis and multiple sclerosis (Mrowietz and Asadullah, 2005; Moharreh-Khiabani et al., 2009). This is the first report that a drug used in treatment of proliferative skin disorders can regulate folate uptake in MCs.

The present work lays the foundation to explore MMF as an antiproliferative agent against reactive gliosis in retinal disease. A comprehensive assessment will be required in future studies to determine whether MMF inhibits MC proliferation, utilizing markers of proliferation (e.g. PCNA) and proliferation assays (e.g. thymidine incorporation). If the data from these future *in vitro* studies suggest that MMF can inhibit proliferation of MCs, it will then be critical to assess the effects of MMF in models of retinal disease in which gliosis is pronounced. In retinal disease there are two phases of gliosis, conservative and massive. Preventing conservative gliosis may prove problematic since this phase is considered to be beneficial to retinal health. Thus, carefully planned studies to determine appropriate timing and dosage of this agent will be needed to assess potential therapeutic value.

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Figure 1. Influence of MMF on MTF uptake in MCs

Early passage mouse MCs were cultured in the presence of 1 mM MMF for 18 h, following which uptake of [^3H]-MTF (200 nM) was measured at pH 5.5 and pH 7.5. Results are means $\pm$ SE (n = 5). (***) Significant: $p < 0.001$ determined by t-test.)

Figure 2. Dose-response of MMF treatment on PCFT activity in MCs
MCs were treated for 18 h with MMF (0, 0.05, 0.25, 1.0, and 2.5 mM); [^3H]-MTF (200 nM) uptake was measured at pH 5.5. Results are means $\pm$ SE (n = 6). (***)Significant $p < 0.0001$ determined by ANOVA and Bonferroni post-hoc test.)

Figure 3. Influence of treatment time on MMF-induced inhibition of PCFT activity
MCs were exposed to 1 mM MMF for 30 min, 1 h, 3 h, 6 h, and 18 h. [^3H]-MTF (200 nM) uptake was measured at pH 5.5 as described. Results are means $\pm$ SE (n = 6).
(***Significant $p < 0.0001$ determined by ANOVA and Bonferroni post-hoc test.)

Figure 4. Influence of MMF treatment on kinetic parameters of PCFT activity

(A) MCs were treated with 1 mM MMF for 18 h following which activity of PCFT was monitored at pH 5.5 with increasing concentrations of MTF (20 min incubation). [^3H]-MTF (400 nM) was present in all uptake measurements as the tracer, and the concentration of MTF was adjusted by the addition of unlabeled MTF. (B) Eadie-Hofstee plot (v/S versus v ; v = uptake in pmol/mg protein/20 min; S = MTF concentration in μM). Results represent means $\pm$ SE ($n = 4$).

Figure 5. Quantitative RT-PCR and Western blot analysis of PCFT in MCs exposed to MMF RNA and protein were isolated from MCs cultured in the absence or presence of 1 mM MMF. (A) qRT-PCR was employed to detect PCFT mRNA levels normalized to GAPDH and 18S. Results are means $\pm$ SE (n = 6), t-test: p = 0.19. (B) Cell lysates were subjected to Western blotting to detect PCFT (~64 kD) and β -actin (~45 kD), a loading control. (C) Densitometry measurements analyzed the ratio of PCFT to β -actin band density. Results are means $\pm$ SE (n=3), t-test: p = 0.50.

Figure 6. Specificity of fumaric acid esters for inhibition of PCFT activity

MCs were treated for 18 h with fumarate (F) and its esters: monomethylfumarate (MMF), dimethylfumarate (DMF), and monoethylfumarate (MEF). Untreated cells were used as control (con). [^3H]-MTF (200 nM) uptake was measured at pH 5.5. Results are means $\pm$ SE ($n = 3$). (*Significant $p < 0.01$; *** $p < 0.0001$ determined by ANOVA and Bonferroni post-hoc test.)

Figure 7. Specificity of MMF-induced inhibition of PCFT activity

MCs were incubated in the absence or presence of 1 mM MMF for 18 h prior to measuring the uptake of [^3H]-MTF (200 nM), [^3H]-L-leucine (Leu) (12.5 nM), [^{14}C]-L-ascorbic acid (AA) (117.6 μM), and [^3H]-L-glutamate (Glu) (33.4 nM) for 15 min. Results are means $\pm$ SE (n = 6). (***) Significant: $p < 0.001$ determined by t-test.)

Figure 8. GPR109A expression in mouse MCs and RPE cells

(A) Total RNA was isolated from early passage MCs (1°MC) and retinal pigment epithelial (1°RPE) cell cultures. RT-PCR analysis was performed using primers specific for GPR109A (expected product size: 443 bp). 18S (315 bp) served as an internal standard. (B–G) MCs and RPE cells were subjected to LSCM to detect GRP109A (red B, E), cells were counterstained with DAPI (blue, C, F) to visualize nuclei. Merged images are shown in panel D and G. (Scale bar = 10 μ m)

Figure 9. Influence of nicotinic acid treatment on PCFT activity

MCs were cultured in the presence of 1 mM MMF or 1 mM NA for 18 h, following which the uptake of [^3H]-MTF (200 nM) was measured. Results are means $\pm$ SE (n = 6).

(***Significant $p < 0.0001$ determined by ANOVA and Bonferroni post-hoc test.)

Figure 10. Quantitative RT-PCR and Western blot analysis of FRα in MCs exposed to MMF
Total RNA and protein lysates were isolated from MCs cultured in the absence or presence of 1 mM MMF. (A) To detect FRα mRNA levels, qRT-PCR was employed, and FRα expression was normalized to GAPDH and 18S. Results are means $\pm$ SE (n = 10), t-test: * p < 0.05 (B) Cell lysates were subjected to Western blotting as described, following which the blot was incubated with antibodies against FRα (~50 kD) and β-actin (~45 kD), serving as a loading control. (C) Densitometry measurements analyzed the ratio of FRα to β-actin band density. Results are means $\pm$ SE (n=4), t-test: p = 0.88.

Table 1

Sequences of mouse primers used in semi-quantitative and quantitative RT-PCR.

Gene	NCBI Accession No.	Primer Sequence	Predicted band size
PCFT	NM_026740	FORWARD:5'-GAATGGTGGTCTTTGCGTTT-3' REVERSE:5'-TCCGTACCCTGTGAACATGA-3'	56bp
FR α	NM_008034	FORWARD:5'-AGCTGGCGCAAAGAGCGGATC-3' REVERSE:5'-GGACACTCGTTATGCCCCGAGGA-3'	143bp
GPR109A	NM_030701	FORWARD:5'-GTTACAACTTCAGGTGGCACGAT-3' REVERSE:5'-CTCCACACTAGTGCTTCGGTTATT-3'	443bp
GAPDH	NM_008084	FORWARD:5'-CATGGCCTCCAAGGAGTAAGA-3' REVERSE: 5'-GAGGGAGATGCTCAGTGTGG-3'	105bp
18S	NR_003278	FORWARD: 5'-AGTGCGGGTCATAAGCTTGC -3' REVERSE: 5'-GGGCCTCACTAAACCATCCA-3'	90 bp