

From the Klinik für Dermatologie, Venerologie und Allergologie
(Director: Prof. Dr. med. Stephan Weidinger)
at the University Medical Center Schleswig-Holstein, Campus Kiel
at Christian-Albrechts-Universität zu Kiel - Kiel University

Validity of in vitro studies with fumaric acid esters for treatment situations in humans – a literature based analysis

Dissertation
to acquire the doctoral degree (Dr. med.)
at the Faculty of Medicine
at Christian-Albrechts-Universität zu Kiel - Kiel University

presented by
Margaret Heidelberg
from **Sofia, Bulgaria**

Kiel **2023**

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1. Introduction

1.1 Chemical structure of fumaric acid esters

Fumaric acid is an unsaturated dicarboxylic acid with the formula $C_4H_4O_4$. It is also named trans-butenedioic acid (International Union of Pure and Applied Chemistry - IUPAC) or trans-ethylenedicarboxylic acid. Fumaric acid has a characteristic double bond between C2 and C3 in a trans-position. Its cis isomer is maleic acid. Fumaric acid forms salts and esters called fumarates as a result of different chemical reactions. Depending on the number of substituents (1 or 2), it can produce mono- and diesters. The most common substituents, methyl ($-CH_3$) and ethyl ($-CH_2CH_3$) groups, further designate the fumarates synthesized as monomethyl fumarate, monoethyl fumarate, dimethyl fumarate and diethyl fumarate (1).

The first synthetic preparation of fumaric acid used succinic acid. Originally, it was synthesized through furfural oxidation with chlorate in the presence of a vanadium-based catalyst (2). Today, industrial fumaric acid production comprises isomerization of maleic acid in aqueous solutions with assistance of a catalyst. The low pH of the solution is crucial for the synthesis. The reason for this modification is that maleic acid can be easily isomerized, as it is a hydrolysis product of maleic anhydride produced by benzene or butane oxidation (3).

Fumaric acid takes part in two of the eight chemical reactions of the citric cycle. These reactions result in the release of reducing equivalents used to generate energy in the form of adenosine triphosphate (ATP) by oxidative phosphorylation. The substances entering the citric acid cycle are formed by oxidation of carbohydrates, proteins, and fats. This metabolic sequence consumes acetyl-CoA and water and reduces nicotinamide adenine dinucleotide (NAD^+) to $NADH/H^+$ and flavin adenine dinucleotide (FAD) to $FADH_2$. As a waste byproduct, carbon dioxide is produced. Fumaric acid esters (FAEs) are metabolites produced by succinate oxidation aided by the enzyme succinate dehydrogenase (SDH) and coenzyme Q10. Fumarate is then hydrated to malate with assistance of the enzyme fumarase (or fumarate hydratase) (4).

Another metabolic pathway that involves fumaric acid is the urea cycle. It is a way for the living organisms to excrete toxic ammonia produced in the amino acid catabolism by converting it to urea. The process starts when free ammonium ions interact with hydrogen carbonate forming carbamoyl phosphate, which then binds to ornithine. This is followed by a series of chemical reactions involving the bonding of aspartate and separation of fumarate as a byproduct and intended to produce and emit urea. In this case, fumarate generated in the urea cycle can be regenerated in the mitochondria to aspartate thus completing the cycle (5).

1.2 Fumaric acid esters in medicine

The first time fumaric acid esters were introduced as systemic therapeutics was in 1959. Since then, they have been a field of interest for research and further development contributing to their use for different indications. Examining the mechanism of action of FAEs, scientists have created a complex story revealing how these small molecules can influence chemical reactions in the human body.

Schweckendiek, a German chemist suffering from severe psoriasis, took the first step to the medical use of FAEs. At that time, it was believed that disturbances of the citrate cycle were the root cause of psoriasis. Therefore, he studied compounds that could be given orally in order to compensate these postulated disorders (6). Indeed, dimethylfumare (DMF) has showed itself to be efficacious in the clearance of psoriasis lesions, so Schweckendiek proposed it as an innovative treatment. Later on the idea was taken up by other researchers whose efforts resulted in developing a drug product—Fumaderm®. This oral treatment was initially licensed for use against moderate to severe plaque psoriasis in 1994 and subsequently Fumaderm® became the most frequently prescribed first-line systemic therapy for this indication in Germany with a unique market share of about 60%. DMF products have been well known as psoriasis treatments in the Netherlands as well and are still extensively used today. Although unlicensed in the UK and Ireland, Fumaderm® was used as a first-line drug to treat psoriasis by a special program of the National Health Service (NHS) (7).

The oldest and most popular FAEs based drug Fumaderm® contains a combination of DMF and three monoethylfumarate (MEF) salts. However, there is still no explanation of

medical rationales for formulating DMF within this mixture of salts as studies performed in 1990 on the oral treatment of psoriasis have shown no difference in terms of efficacy between DMF monotherapy and the combination product Fumaderm® (8). In addition to these initial studies, a multicenter phase III trial with a drug containing only DMF as an active substance in a galenic formulation which was initially developed for the treatment of psoriasis (FAG-201, later termed BG-12), showed similar clinical efficacy to that of Fumaderm®. Unfortunately, neither this phase III nor the preceding phase II trial data for BG-12 have been published in a peer-reviewed journal. Evidence for the same effect for treatment of psoriatic lesions also came from a recent clinical trial that compared Fumaderm® with a DMF-only drug (Skilarence®) having the same galenic formulation. In this trial, no difference in not only efficacy but also in safety and tolerability of the DMF/MEF-mixture Fumaderm® and Skilarence® could be found.

At the time of BG-12 development, in a small open-label pilot trial, it was discovered that Fumaderm® was efficacious in multiple sclerosis (MS) (9). Through this observation, FAEs gained popularity as novel immunomodulatory compounds able to treat more than psoriasis alone, which resulted in renewed interest in examining their mechanism of action. However, the development of BG-12 as a psoriasis treatment was stopped in 2006, after Fumapharm, the inventor, patent holder and producer of Fumaderm® and BG-12, was acquired by Biogen. Thereafter, BG-12 was marketed as Tecfidera® and now is ranked as one of the most frequently used first-line remedies for MS worldwide.

Today, the main issue regarding the use of FAEs in medicine is that, due to regulatory decisions, DMF in medicinal products indicated for the treatment for psoriasis such as Fumaderm®, Skilarence® and compounded DMF preparations in the Netherlands are considered to be different from DMF in the MS drug Tecfidera® (BG-12). In this case, the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA) have granted DMF in Tecfidera® the status of a “new chemical entity”. However, the reason for this special status remains unknown because the simple chemical compound DMF is identical in all DMF-containing drug products. Consequently, both preclinical and clinical sets of data regarding Tecfidera® become incomparable with those obtained on other DMF-containing drugs due to regulatory reasons. This phenomenon continues to exist despite the fact that, besides differences in the excipients of DMF-containing drugs (e.g.

galenic formulation), their pharmacological characteristics, mechanism of action, therapeutic effects, and adverse events are fully comparable.

Another FAE-based drug that recently gathers more importance in the treatment against severe plaque psoriasis is tepilamide fumarate, further named XP23829, which provides its effect as a pro-drug that rapidly metabolizes to MMF in vivo (10).

1.3 Aim of the study

As fumaric acid esters gather more interest in different fields of research for treatment situations in humans, the number of studies involving these substances has grown in several years. However, among all literature there is yet no actual summary showing the research status today. This study aims to provide an analysis that combines all key points of the experimental work made until today and to evaluate its significance.

2. Materials and Methods

In order to access most of the information from all published data, the form of a systematic review was chosen. Per definition, this is a review with a clearly defined question that uses analytical and precise methods to recognize, select, organize, and interpret relevant studies, as well as to extract and analyze information from included research (Definition by Cochrane Collaboration). In that manner, applying statistical methods for analyzing and summarizing the results becomes optional, as it is rather characteristic of meta-analyses. To avoid overseeing important sources that may be relevant to this form of study, all minimum stages for a literature research suggested by PRISMA were followed thoroughly. PRISMA is an acronym that stands for 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses' and aims at the improvement of data reporting in meta-analyses and systematic reviews. It uses a set of requirements that need to be met as a minimal base enabling scientists to report and evaluate correctly different types of research and appeals to authors of literature-based investigations (11). Therefore, it was considered as suitable criteria for designing this study.

In the first stage of the study, a research question was formulated. Following a comprehensive review of the fields of interest of recent studies on fumaric acid esters, it was established that FAEs had possessed a wide treatment capacity that is used not only in dermatology as initially. Therefore, the decision was made to investigate and compare how the effect of FAEs alters in different human tissues. Although the signaling pathway is not completely clear, the focus was set on studies involving in vitro experiments since they are crucial to the understanding of the treatment mechanisms of FAEs and their side effects. As this has been an attractive field of study for over a few decades, investigating the researches made is a very important part of finding gaps, reevaluating facts, as well as gathering useful information for future discoveries. Having a good overview might be a big step toward the future scientific development of fumaric acid esters. Therefore, it was decided to perform a literature-based analysis involving studies in diverse ranges of use. Moreover, the focus was set on in vitro studies involving fumaric acid esters in a therapeutic use because all further experiments will rely on the results of those.

The second stage comprised of a relevant data search under certain criteria. Initially, all reliable academic databases were examined in order to access the information in all published articles. As this is a systematic review in a medical, biomedical, biochemical,

biophysical and pharmacological field, only those databases representing a valid source were used. In this case, the following searching engines were included: EMBASE, PubMed, SID (Scientific Information Database), Scopus, and Google Scholar.

Next, suitable searching criteria were set. As fumaric acid and its esters are the main field of interest, all databases were searched for articles containing either of the following words and phrases: dimethyl fumarate, dimethylfumarate, monomethyl fumarate, monomethylfumarate, monoethylfumarate, fumaric acid, fumaric acid ester, and, of course, their abbreviations: DMF, MMF, MHF, MEF, FA, and FAE.

A relevant period had to be determined as well. In order to access the oldest information about fumaric acid esters and track the history of FAEs, the beginning year was set to be 1950. As long as the scientific interest in that field is growing and multiple trials are done daily, the final date as a search criterion was set on December 31, 2022.

Using the defined indicators, a detailed search was performed in every academic database listed above. As a result, over 3,000 articles were suggested starting from 1971 until December 2022.

Afterwards, more criteria were set aiming to avoid misrepresentations of the search terms. Primarily, all articles not containing information about fumaric acid esters were filtered. It became evident that many search results, in particular, those returned for the abbreviation “MMF”, did not match monomethyl fumarate, but mycophenolate mofetil (an immunosuppressive drug) instead. Then, the articles not mentioning fumaric acid esters in their header and not containing any experimental information on this substance were excluded as well.

This elimination step reduced the amount of search results considerably leaving approximately 350 potentially articles relevant for this study. As those still contained different kinds of scientific studies, more exclusion criteria were defined in order to facilitate the process of valid results recognition. Therefore, all clinical trials and patient observations were sorted out leaving the focus on the in vitro experimental research. In order to obtain outcomes that could be used for a systematic review, all kinds of biomedical interference had to be excluded since all medicinal products behave differently in living organisms and in vitro. The main factors leading to this change of behavior include multiple biochemical interactions and specific drug characteristics

involving pharmacokinetic parameters (drug absorption, distribution, metabolism, and excretion (ADME) in an organism) and pharmacodynamic ones (pharmaceutical drug action on a living organism). It was also found that many studies on fumaric acid esters examined their characteristics not only via clinical trials but also in mouse models *in vivo*. Apparently, these models were considered unsuitable for the present research. Overall, about 120 more articles were excluded proving their fail to meet the proposed criteria and finally 225 articles were considered relevant for this literature review.

In order to investigate more about the research problem of this study, all crucial information from the articles that met the inclusion criteria was extracted. As long as each of the studies described a wide range of scientific knowledge about different aspects of *in vitro* effects of fumaric acid esters for treatment situations in human cells, the data extracted needed a further classification. Thus, the third stage of the study involved the process of data extraction and data structure design in order to enable comparison and validation of the stored pieces of information. As a suitable method for this, a data table was created using Microsoft Office Excel. In that manner, a qualitative overview of the articles presented in the literature research was provided.

The data table creation was started by defining categories able to extract the most important results from the scientific article selection and set parallels between them. Initially, all articles were listed with the entire citation information including their authors, year of publication, and journal. As all studies use different chemical variations of fumaric acid and its esters, these were set as a major category thus separating researches made with DMF, MMF, MEF, and FA. In order to observe and compare the limit and effect of every active substance, it was a matter of interest to create a database describing all doses used. Therefore, it was essential to define subcategories holding various doses and drug concentrations by using the following units of measure: μM , mM, and mg/L.

Not only the dose and the chemical compound used was important for creating a good database for this research but also the drug effect and the type of cells that it had acted within. This led to designing two more categories: the first one described results achieved and the second one—the target cell line. Finding the crossing point between the type of metabolite activation and the corresponding active substance dose would quantify the intensity of the effect reached in the experiments. In order to perform the overview and

compare the measured effects achieved, a matching scale was created by using the effect level of the measured outcome according to the studies presented in the articles included. To enable simple management, the effect intensity was measured on a scale from 0 to 3, where 0 = no measured effect, 1 = slight effect, 2 = moderate effect, and 3 = strong effect. In particular, every sample that did not show a reaction different from the control sample one was marked as 0 on the scale. A score of 1 was given to the samples that showed a slight effect compared to the control samples, while the other doses in the same setting showed a better efficacy or a clearly higher significance. The samples that provided a high response in comparison to the control ones but not the highest in the experiments made, ranked 2 on the scale. The highest score = 3 was given to the most efficacious and significant doses of drugs and chemical substances.

Although the scale used enabled an efficient comparison between different kinds of research, it would be effective only when the experimental conditions match as well. Therefore, all results were organized in correlation with the specific tissues and cell cultures that had been used in the experimental data.

Recent studies confirm the hypothesis of DMF acting as a pro-drug that achieves its active form through intestinal uptake and hydrolysis with no detectable concentrations in systemic circulation and peripheral tissues (12). The active substance interacting with hydroxyl carboxylic acid receptor 2 (HCA2), a cell receptor in the periphery, is mainly MMF or adducts with glutathione (GSH), an intracellular antioxidant compound, of MMF or DMF. This results in the conclusion that only in vitro studies using MMF as an active substance obtain a reliable result because for valid DMF experiments the drug needs to pass the gastrointestinal tract for achieving the full effect. A similar impact can only be achieved by in vivo experiments based on oral treatments in animal or human models. Unfortunately, most published data still use DMF as investigational compound that has been directly applied on isolated cell cultures that are unlikely to meet and interact with this molecule in vivo. Considering this fact, it is rational to base the literature review only on the articles that report data from experiments using MMF instead of DMF as a chemical compound for their in vitro settings. Therefore several articles and research that only used DMF as an active compound in vitro were not mentioned in this study (13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65,

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3. Results

3.1 Pharmacological profile of fumaric acid esters

In order to evaluate recent studies with fumaric acid esters, solid knowledge and understanding of their pharmacological profile and chemical way of action needs to be provided. In the beginning of its use as a therapeutical drug against psoriasis and following several experiments, DMF was considered a pro-drug. These pharmaceuticals do not interact directly with their target cells, but reach their full activity after chemical reactions in the gastro-intestinal-tract or during the subsequent metabolism in the liver or peripheral tissues that lead to their active metabolite. In this case, DMF possesses ester groups, which are characteristic with their high chemical reactivity and are likely to enter a rapid spontaneous or esterase-mediated hydrolysis which leads to the formation of monomethylfumarate (MMF)–the main metabolite of DMF (7). Besides MMF, there are also other chemical forms this pro-drug can develop. When DMF is systematically administered to humans or animals, it can react rapidly with GSH as well. Upon that reaction, a stable adduct (S-(1,2-dimethoxycarbonylethyl)glutathione; GS-DMS) is formed. Further, this chemical compound metabolizes in context of biotransformation into its mercapturic acid N-acetyl-S-(1,2-dimethoxycarbonylethyl)cysteine (NAC-DMS), a degradation product which is eliminated through urine and feces (246). GSH binds not only to DMF but also to MMF, although at a much lower rate and to a smaller extent. Similarly to the biotransformation of DMF to NAC-DMS, its hydrolysis product NAC-MMS is found as an elimination compound (247). Considering the in vivo reactivity of DMF, it is likely that only irrelevant concentrations of it can be detected in the periphery. Applying high-performance liquid chromatography (HPLC)-mass spectrometry, a combined analytical technique that uses the method of physical separation of liquid chromatography and the mass analysis procedure of mass spectrography to investigate different biochemical compounds, in the field of clinical biochemistry, ex vivo data from psoriasis patients and healthy volunteers was investigated. These tests, however, have consistently shown that even in the gut's first draining portal vein, DMF is undetectable in the blood. Considering this argument, the assumption that DMF was absent in peripheral tissues including skin and central nervous system (CNS) had been strongly confirmed (247). In support of this notion, experiments using the caucasian colon adenocarcinoma (Caco-2) colonic epithelial cell line, a line derived from human colon

adenocarcinoma and characterized by homogeneity, in transwell membrane systems have shown that application of DMF to the apical compartment of the Caco-2-coated transmembrane system resulted in the detection of MMF but not of DMF on the basolateral side of the membrane (248). In fact, according to all the publications available, the only DMF-derived molecules that can be detected in the peripheral blood and later in feces, urine and exhaled air are not those of the chemical compound itself, but of its active metabolite MMF along with GS-DMS, MS-GMS, and down-stream metabolites such as fumaric acid and CO₂ (249). Different literature sources described that plasma concentrations of MMF in humans after oral administration of a single dose of 120, 240, or 360 mg DMF were below 2 µg/ml at their peak (249, 250). Food can delay t_{max} of MMF substantially, but may not affect C_{max} or area under the curve (AUC) (250). Again, in those studies DMF was always below the limit of detection in the periphery. These data together suggest that ingested DMF is rapidly metabolized before it can leave the gastrointestinal tract and that DMF as a single compound cannot interfere directly with another cell system *in vivo*.

In order to observe whether there are detectable traces of DMF in feces, radioactive-labeled compounds were used. These experiments have shown that less than 1% of the administered DMF could be found. The elimination half-life of DMF is very short (t_{1/2} is approximately 1 hour). MMF has a low binding to serum albumin (max. 50%) and in the literature no evidence for accumulation can be found. The main part of the orally administered DMF (about 60%) is exhaled as CO₂ while urine and feces excrete the remaining 40%. Glucose, fumaric acid, and citric acid are the major MMF metabolites that can be found in plasma. *In vitro* experiments have shown that in a buffer with pH = 1 resembling the pH in the stomach, DMF and MMF remained intact (249). Investigating further how DMF behaved in other parts of the intestine system, it has been proven that at pH = 8, an environment present in deeper parts of the small intestine, DMF was rapidly hydrolyzed to MMF (249). The half-life of DMF is found to be approximately 1.5 hours. Furthermore, the terminal oxidase enzyme system cytochrome P450 was not involved in metabolism of FAEs and there was no re-esterification of MMF in the systemic circulation (Rostami and Mrowietz, unpublished data). Unfortunately, in the current studies there is no data about the kinetics of release of FAE drugs in the small intestine including local concentrations of DMF. As the DMF molecule shows a characteristic lipophilic nature, it may be absorbed into the outer mucosal layers of the small intestine. However, it was

highly likely that it would then react with intracellular GSH or be hydrolyzed by esterases before it could cross the membrane and reach other cell systems *in vivo*, as suggested by the Caco-2 data described earlier (248).

Several studies proved that DMF was not able to reach a perceptible level within peripheral tissues. That fact raised the question whether it was the systemically acting component of FAE-based drugs and suggested that it was not. On the other hand, as MMF is readily detectable in the blood, it is also a matter of interest if evidence supporting a therapeutic role for this molecule exists. Earlier studies by Lehmann et al. using a rat kidney transplant model showed that treatment with high-dose MMF-calcium salt prevented acute and chronic graft rejection alone or in combination with ciclosporin (251). A recent report compared post-ischemic recovery in mice to which DMF was orally administered by gavage and MMF was given intraperitoneally. In all performed experiments, both MMF and DMF showed similar efficacy during recovery from ischemia. The observation showed that MMF alone could have a therapeutic effect and supported the concept that orally administered DMF acted as a pro-drug in order to generate sufficient MMF levels outside of the intestine (252). However, the metabolism of DMF after intraperitoneal delivery has not been entirely investigated yet and it is likely to be different from that following oral administration. Therefore, a great caution is needed when interpreting experiments and results involving MMF administered via this specific delivery route (253).

Furthermore, the importance of MMF rather than DMF could even be supported by a clinical trial. As there is no registered medical pro-drug other than DMF, this trial examined whether oral XP23829 (now renamed to tepilamide fumarate), a molecule composed of MMF and a linker that enables improved resorption/penetration after oral administration, was likely to alleviate psoriatic lesions in patients in a phase 3 clinical trial (254). Results from this study showed efficacy comparable to that of Fumaderm®. Interestingly, the adverse event profile remained the same as the one of the traditional drug provoking gastrointestinal complaints and flush (255). Animal studies performed with oral XP23829 showed that it could ameliorate murine experimental autoimmune encephalitis (EAE) and reduce the severity of disease in the Aldara® mouse psoriasiform inflammation model in a manner similar to oral DMF (255).

Taking all published data into account, the published pharmacological and clinical data are pointing to a hypothesis that DMF is rapidly metabolized into MMF which then enters the periphery and experiences further biochemical reactions. Consequently, ascribing effects to FAE-based drugs that are based upon data derived exclusively from DMF must be undertaken with caution. The best available evidence in current literature argues strongly against the pharmacological effects and adverse events observed during FAE treatment, being caused by DMF binding directly to target proteins of cells in the blood, skin, or CNS. Instead, DMF appears to act as an oral pro-drug that is rapidly metabolized into MMF—the active in vivo compound (Figure 1).

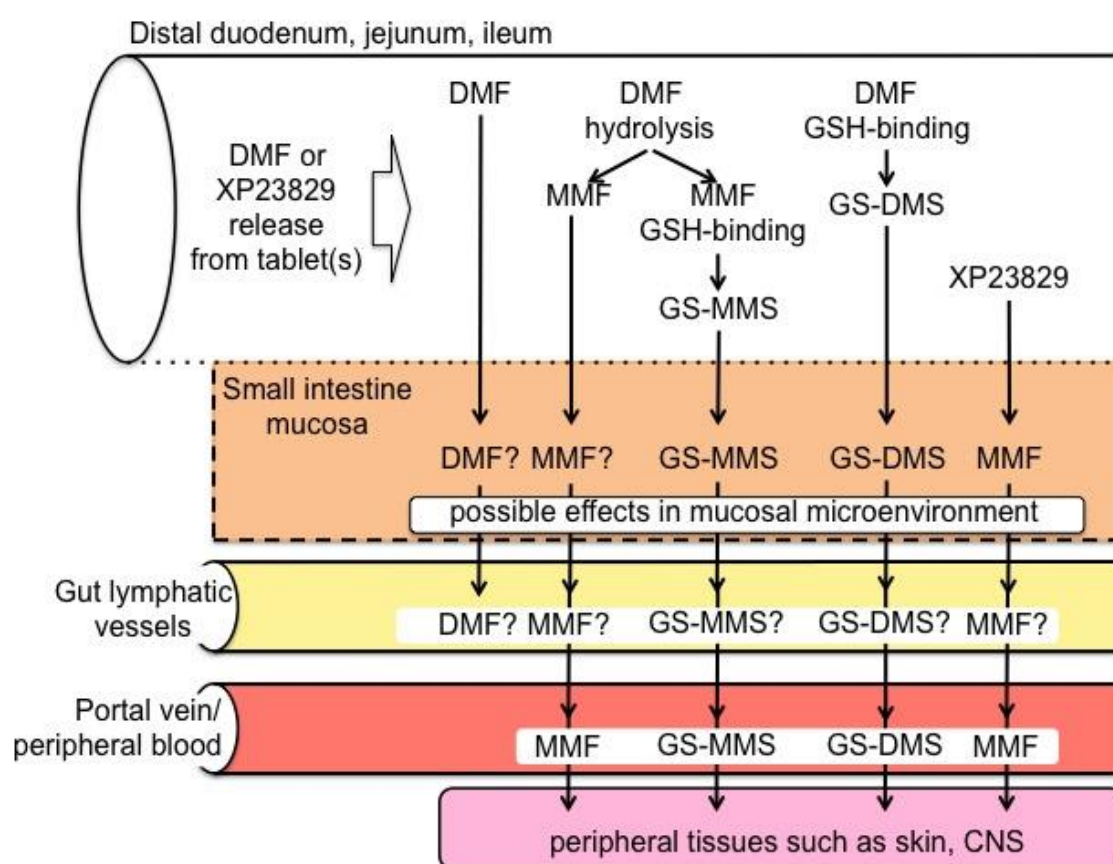


Figure 1. Pharmacological profile of oral DMF and XP23829 after being released from gastro-resistant tablets or capsules (BG-12/Tecfidera® is a capsule containing DMF in microtablets; XP23829 is a product consisting of MMF bound to a linker.)

3.2 Hydroxy-carboxylic acid receptor family

The active compound of FAE based drugs MMF does not readily diffuse across plasma membranes like its pro-drug DMF. In order to initiate its cellular effects, it must consequently interact with receptors expressed on the target cells surface. Nibbering et al. were the first to postulate the existence of an MMF receptor in 1997. In this study, it was demonstrated that MMF at a concentration of 2.6 $\mu\text{g/ml}$ (20 micromolar) could activate intracellular calcium signaling and increase the level of cyclic adenosine monophosphate (cAMP) in neutrophils (256). Another publication described that in keratinocytes MMF at a concentration of 13 $\mu\text{g/ml}$ and above evoked intracellular Ca^{2+} -mobilization and began to inhibit cellular proliferation reaching 26 $\mu\text{g/ml}$ and above (257). In subsequent experimental trials, it was shown that MMF-mediated Ca^{2+} release was sensitive to pertussis toxin (PTX) suggesting that MMF signals via a G protein-coupled receptor. Altogether, these experiments led to the conclusion that “MMF binds to specific sites on the plasma membrane of cells” (258).

A finding that was crucial for the understanding of MMF signaling confirmed that it indeed does bind to a Gi/Go-protein-coupled receptor (GPCR) in an agonistic manner. This corresponding cell receptor is called hydroxyl carboxylic acid receptor 2 HCA2, also known as GPR109A and NIACR1 (259). For said study, Chinese hamster ovary (CHO) cells were transiently transfected with HCA2 together with a chimeric G-protein in order to convert HCA2 to a Ca^{2+} signaling pathway. In that manner, after screening a library of 1500 carboxylic acids using an aequorin assay, MMF was found to dose-dependently increase intracellular Ca^{2+} in HCA2-transfected cells with an EC_{50} of 2.0 μM , whereas DMF was found to be inactive in this system. Furthermore, in A9 cells stably expressing HCA2, MMF achieved complete inhibition of forskolin-induced cAMP, with an IC_{50} of 70 nM. At the same time, while DMF caused a 50% inhibition at 10 μM . Taking this into account, HCA2 is considered the putative GPCR to which MMF, but not DMF binds.

HCA2 belongs to a family of 3 hydroxy-carboxylic acid receptors that includes HCA1 to 3 (260). All HCA receptor family members are activated by certain endogenous intermediates that are produced during energy metabolism and signal via Gi/Go-type G-proteins. These lead primarily to downstream inhibition of adenylate cyclase and a decreased level of intracellular cAMP. The first member of this receptor family HCA1 (also known as GPR81) is primarily expressed by adipocytes. It activates a pathway that

mediates intracellular autocrine lactate signaling which results in a reduction of lipolysis. Other than HCA1, HCA2 is not only expressed by adipocytes but also by a variety of cells including intestinal epithelial cells (IECs), keratinocytes, microglia and astrocytes. Furthermore, HCA2 is expressed by peripheral blood leukocytes including neutrophils, monocytes, macrophages, Langerhans cells, but not lymphocytes (259, 260, 261). The main endogenous ligands of HCA2 include butyrate-derivatives like 3-hydroxy-butyrate produced by β -oxidation of fatty acids and butyrate itself released by the intestinal microflora, but it is also a high affinity receptor for niacin (vitamin B3). The expression and function of the last HCA family receptor member HCA3 (also known as GPR109B) is not defined as precisely, but it seems to share a similar expression profile with HCA2 including neutrophils, monocytes, macrophages, intestinal epithelial cells, and adipocytes. Similar to HCA2, HCA3 is activated through another β -oxidation product, 3-hydroxy-octanoic acid. It is also a receptor for niacin, but with a much lower affinity compared to HCA2. In this way, it is likely that the HCA family evolved to monitor the energy state of the host and to provide negative feedback in order to limit energy consumption when food intake is interrupted.

3.3 HCA2 as a target of MMF in vivo

Given the in vitro evidence that HCA2 is the target of FAE-based drugs, it instantly raises the question whether in vivo evidence supporting this hypothesis exists. In order to establish the role of HCA2 during FAE treatment, in vivo experiments have been performed with the assistance of a generation of HCA2-knock-out mice. Experimental data in recent studies have shown that DMF was likely to ameliorate MS-like experimental autoimmune encephalitis (EAE) in mice models only in the presence of HCA2 (262). As follows, in wild-type mice DMF-treatment resulted in a significant inhibition of EAE induced by subcutaneous immunization with MOG35–55 peptide. In contrast, DMF had no effect in HCA2-knock-out mice (262). Furthermore, in the following experiments DMF treatment reduced neutrophil infiltration into the spinal cords in wild-type mice but not in HCA2-knock-out mice with EAE (262). This experimental data provided a strong argument in support of the hypothesis that HCA2 is required for the neuroprotective effect of FAE treatment.

Skin flushing is a side effect that occurs through FAE treatment. This symptom is also frequently triggered in response to niacin. A recent study has shown that HCA2 was likely to be a crucial part of the flushing mechanism. In a series of experiments mice with genetic deletions of this gene restricted to either Langerhans cell or keratinocytes showed greatly reduced flush in response to MMF (263). This leads to the conclusion that during the flush response, MMF signals via HCA2 on Langerhans cells and keratinocytes inducing the production of prostaglandins D2 and E2. These released mediators act on vascular smooth muscle cells and mast cells and lead ultimately to vasodilation and flushing. Another report of flushing as a side effect was also made in the XP23829 clinical trial. As XP23829 contains only MMF with no DMF, unlike Fumaderm®/Tecfidera®, it suggested that during FAE treatment this adverse event was likely due to MMF. As a whole, these results argue strongly that MMF binds to HCA2 in vivo to mediate both its therapeutic and adverse effects.

Current pharmacological, experimental and clinical data, as described previously, give strong support to the hypothesis that DMF indeed serves as a pro-drug. The actual active substance of DMF treatment is shown to be MMF alone or some GSH-adducts of DMF and MMF which develop their therapeutic characteristics through signaling via a specific receptor in the periphery, most likely HCA2. Nevertheless, the currently published articles contain a few studies within which MMF is used alone or in addition to DMF. On the contrary, numerous publications describe the effects of DMF on cells that, due to its rapid metabolism and binding, will never be exposed to the said compound in vivo. This leads to the conclusion that careful attention is needed when interpreting results from experiments with FAEs and, in particular, when only DMF has been investigated. In that manner, only experimental studies using MMF in vitro or DMF as oral treatment in mice or man represent a realistic model of the effects of fumaric acid esters on specific cells or tissues (Figure 2).

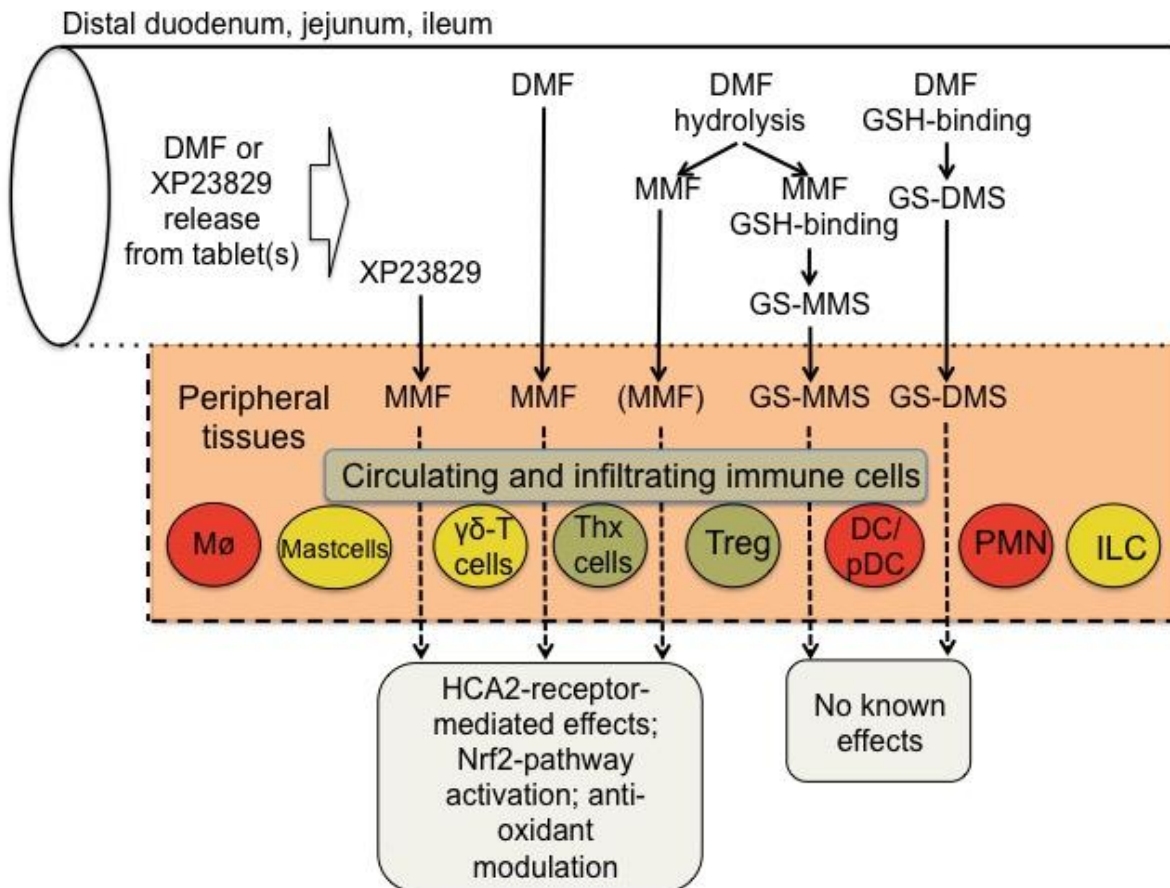


Figure 2. Effects of FAEs after intestinal uptake and metabolism in peripheral tissues and in circulating and infiltrating immune cells (M: monocytes/macrophages; Thx: Th1, Th2, Th17, and Th22 cells; DC: dendritic cells; pDC: plasmacytoid dendritic cells; PMN: neutrophils; ILC: innate lymphoid cells; red color: HCA2-expressing cells; green color: HCA2-negative cells; yellow color: unknown HCA2-receptor status)

3.4 MMF effects on the immune system

One main target of FAE based drugs is the immune system. In this setting, FAE treatment can have dramatic effects which are often characterized by a reduction in the number of peripheral leukocytes seen in most patients. Another frequently observed effect is that 10% of FAE-treated patients are likely to develop lymphopenia. If it becomes severe

(<500 lymphocytes/ μ l), there is an increased risk for the development of opportunistic infections that include the frequently feared progressive multifocal leukoencephalopathy (PML) (264, 265). In a study from 1998, it was noted that while the number of circulating CD4 and CD8 T cells decreased in response to FAE treatment, CD8 T cells were disproportionately affected (266). Several studies with groups of DMF-treated MS patients have confirmed this effect on CD8 T cells (267, 268, 269). On the other hand, other studies have published results in which oral DMF treatment had caused general lymphopenia without subset specificity (270) and the number of some T cells subsets such as regulatory T cells (Treg) remained unaltered (271). In their report, Gross et al. described that CD4 and CD8 memory T cells were preferentially decreased by oral DMF treatment in MS patients. The CD4 memory T cell population was therefore further divided and the effect on different cell groups was examined. The results showed that in this experiment, Th1 cells decreased, Th2 cells increased and Th17 cells remained unaltered in number (272). Another study observed a similar pattern of a decrease in the proportion of IFN- γ -producing CD4 cells and a transient increase in interleukin (IL) 4-producing CD4-cells in DMF-treated psoriasis patients. Interestingly, in this case, the cytokine production pattern of CD8 cells was unaltered by treatment (273). As oral DMF treatment can cause a reduction in the number of circulating T cells, it was possible that MMF was directly inducing apoptosis. However, it has already been shown that in cultures of CD4 or CD8 T cells MMF does not increase the frequency of apoptotic cells (274).

Not only T cells are affected by DMF treatments. It has also been reported that during this treatment, the number of circulating B cells in patients with relapsing-remitting multiple sclerosis tended to decrease. This reduction in B cell number was due to losses within both the CD27^{neg} B cells and CD27⁺ memory B-cell populations. On the other hand, the frequency of blood transitional 2-marginal zone precursor (T2-MZP) B cells was increased after 4 to 6 months of FAE treatment and B1 cells were increased after one year. As a common feature, both of these populations of B cells have been shown to produce IL-10, a suppressive cytokine.

Observing the changes that have been seen within the lymphocyte compartment during FAE treatment, they have been linked to its clinical efficacy. Nevertheless, there is still no compelling evidence that T or B cells express the important cell receptor HCA2. Considering this, it is unlikely that the active compound MMF or other FAEs directly signal

to lymphocytes *in vivo*. The lymphopenia-inducing and immunosuppressive effects of oral DMF must therefore be of indirect nature. For example, FAEs might reach this effect via other HCA2-expressing cells, which can influence T and B cell homeostasis. Still, many published studies have investigated the effect of DMF on T cells *in vitro* while fewer have used the main systemic compound MMF. Additionally, interpretation of results is often complicated due to the use of peripheral blood mononuclear cell (PBMC) instead of purified cells as the cells in the PBMC fraction show high heterogeneity and some of them, including monocytes, express HCA2. Thus, an early study has shown that MMF had no effect on the proliferation of CD2/CD28-stimulated human T cells as well as on interleukine-2 (IL-2) or interferon- γ (IFN- γ) secretion and production. On the other hand, in a number of T-cell clones stimulated with CD2/CD28, MMF induced the production of IL-4 and IL-5 significantly, a common pattern also seen in PBMC and CD45RO⁺ CD4 T cells (primarily effector memory and central memory T cells), but not in CD45RA⁺ CD4 T cells (naive and effector) (275). Having in mind that T cells do not express HCA2, these experimental data could be the result of MMF binding to an unknown receptor or a non-specific physicochemical effect.

HCA2 is expressed by neutrophils and monocytes. These cell types have been found to infiltrate the skin in psoriasis and the brain in MS. The neuroprotective effect of FAEs is highly likely due to a reduction in neutrophil transmigration. Recent studies showed that MMF inhibited neutrophil adhesion to endothelial cells and Chemokine ligand 2 (CXCL2)-induced migration in a HCA2-dependent manner (262). It is possible as well that oral DMF owes its anti-psoriatic effect to this mechanism. Nevertheless, this postulation has not been confirmed yet, so further research needs to be performed. MMF is also likely to cause maturation damage by interfering with the differentiation of monocyte-derived dendritic cells. In a series of experiments, it was shown that MMF could inhibit CD1a and HLA-DR expression in human monocyte derived dendritic cells (276). Litjens et al. have demonstrated that dendritic cells were able to differentiate in response to LPS DCs and in the presence of MMF up-regulated surface CD83, co-stimulatory molecules and HLA-DR. However, this differentiation was at a lower level compared to control DCs (277). Moreover, DCs treated with MMF reduced the production of IL-12p70 and IL-10 to very low levels and decreased levels of tumor necrosis factor alpha (TNF- α). These molecules played a crucial role in the interaction between DCs and T cells. In that manner, MMF treated DCs were not as effective at inducing IFN- γ secretion from T cells in comparison

to control DCs. Nevertheless, MMF treated DCs were able to obtain the production of IL-4 and IL-10 by T cells similar to the compared control DCs (277). Consequently, the study linked to the assumption that, being able to affect the differentiation of monocyte-derived DCs, MMF could interfere indirectly with T cells. Moreover, it has actually been shown that oral DMF treatment in studies using the EAE model in vivo could interact with dendritic cell development and promote anti-inflammatory-type macrophage (M2) generation. That effect of oral DMF resulted in lower Th1/Th17 responses in the CNS and a concurrent reduction in disease severity (278, 279). Furthermore, oral DMF prevention of an EAE phenotype model was shown to be dependent on IL-4 production (279).

Altogether, the collected evidence showed that MMF could influence multiple aspects of the immune system. The immunosuppressive effect of FAE treatment was likely to be provoked by a combination of the processes occurring in HCA2-expressing cells. Still, the exact mechanism by which oral DMF and its active substance MMF released into the periphery influence the immune system is not exactly defined yet.

3.5 MMF effects on the central nervous system

Even though FAE are frequently used in the field of neurological diseases (particularly MS) as Tecfidera®, research tends rather addressed its effect on the immune rather than on the nervous system. Among these studies, only some have used MMF for their in vitro experiments. The majority of research focus on DMF, which does not interfere with these cell systems in vivo, as it is rapidly metabolized. In their report, Chen et al. have taken into account that DMF acted as a pro-drug. Therefore, MMF was used on cultured brain endothelial bEnd.3 cells. In the row of experiments, however, the main focus was anew set on MMF's immunomodulating characteristics because it was shown that it prevented neutrophil binding to TNF-pretreated bEnd.3 cells. In correlation to the hypothesis that MMF resolved its action through HCA2, it is not surprising that the active compound of DMF showed no effect on HCA2 negative neutrophils. Furthermore, it was demonstrated that MMF had an influence on the chemotactic activity of CXCL2 which regulated the migration of wild-type neutrophils (262). In this way, it is possible that the beneficial effect of FAEs in MS models was rather due to HCA2-dependent cell death induction on

immune cells and disruption of their functioning than signaling directly through binding on the neural cells.

In another study, it was investigated whether MMF had an influence on toxic actions of standard of care therapy for glioblastoma cells (280). In that manner, MMF enhanced the radiation and temozolomide (preferred chemotherapy drug for glioblastoma treatment) toxicity which was manifested through inhibition of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF κ B) p65, extracellular signal-regulated kinases (ERK) 1/2 and Akt (Protein kinase B) phosphorylation. Furthermore, it was shown that MMF could downregulate the production of IL-6, TNF α , and TGF β in activated microglia that were freshly isolated from patients' glioblastoma tumors.

In some studies, the in vitro effect of MMF on primary cortical and hippocampal HT22 cells against cell oxidation was compared to DMF. It was shown that, at a concentration of 10 μ M, DMF could decrease oxidative stress caused by extracellular glutamate when treated for 24h, while an effect of MMF only became evident after a treatment for 96h (281). In another study, various amounts of MMF could not protect cultured astrocytes from oxidative stress in vitro, whereas DMF had a significant effect in every used concentration (282). Another research team tested whether higher amounts of MMF had an impact on cellular GSH in OLN-93 cells, a permanent cell line derived from spontaneously transformed cells in primary rat brain glial cultures (283). For this, cells were incubated for 60min with FAEs in different concentrations. Similarly, to other reports, MMF did indeed show an effect at concentrations of 1mM and 3mM although, in the same setting, DMF was likely to reduce cellular GSH by binding at any concentration tested. Furthermore, none of these concentrations were shown to be toxic for OLN-93 cells. Knowing that DMF does not interact with neural cultures in vivo due to its rapid metabolism, these in vitro results may be most likely caused by the differences in permeability through cell membranes. As described earlier, MMF acts through a cell surface receptor (HCA2), whereas DMF is likely to diffuse directly through the cell membrane. In their report, Akay et al. proved that in a model of in vitro neurotoxicity caused by antiretroviral drugs, MMF was able to protect neuronal cultures against oxidative stress. Preincubation of primary cortical cultures with 100 μ M MMF for 30min blocked reactive oxidative species (ROS) induced by antiretroviral treatment. Additionally, it was shown that during this preincubation the protein levels of

hemeoxygenase-1 (HO-1) were increased and at the same time the microtubule-associated protein 2 (MAP-2) loss was blocked suggesting that MMF protection occurred through its augmentation through HO-1 expression (284).

In a set of different cortical cultures including primary cultures of human spinal cord astrocytes, human oligodendrocyte precursor cells (hOPC), human hippocampal neurons (hNeur), primary cultures of rat cortical neurons, and rat oligodendrocyte precursor cells (rOPCs), Scannevin et al. described further antioxidative mechanism of MMF. Using immunofluorescent microscopy and immunoblotting for Nrf2 in hOPC, rOPC, and hNeur cells treated with DMF and MMF, the research group confirmed that intracellular Nrf2 levels were likely to increase during FAE treatment (285). Protein levels of heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO1) were also proven to increase which suggests that human astrocytes are capable of responding to MMF and DMF treatment leading to induction of a prototypical phase II antioxidant response. Although DMF showed a higher potential in vitro, it was taken into account that in vivo only MMF is likely to reach and interact with human neuronal cells. Therefore, cellular redox potential during MMF treatment in astrocytes was investigated. In a row of experiments, it was reported that “MMF treatment “primes” cells and increases their capacity for dealing with oxidative stress by increasing the intrinsic pathways that result in enhanced biosynthesis of detoxification molecules such as glutathione”. Furthermore, MMF improved the viability of neuronal cell cultures after oxidative stress exposure and it was shown that Nrf2 protein was required in order to provide protection (285).

Autor	Journal	Year	MMF/MHF										Cells/System	Notes
			μM											
			0.12	1	1.1	3.3	5	10	15	30	100	500		
Albrecht	Journal of Neuroinflammation	2012						0					Hippocampal HT22 cells	
Chen	The Journal of Clinical Investigation	2014									3		cultured brain endothelial bEnd.3	wildtype neutrophils
Chen	The Journal of Clinical Investigation	2014									0		endothelial bEnd.3 cells	Hca2-/- neutrophils to TNF-stimulated bEnd.3 cells
Cross	The Journal of Immunology	2011		2			3		3	3			Rat cerebrocortical cultures	reduction of HIV/MDM-mediated neurotoxicity
Scannevin	Journal of Pharmacology and Experimental Therapeutics	2012						0					Human astrocytes	Nrf2, NQO1, HO1, GCLC mRNA expression
Scannevin	Journal of Pharmacology and Experimental Therapeutics	2012		0		0	2		3		3		Human astrocytes	GSH levels
Scannevin	Journal of Pharmacology and Experimental Therapeutics	2012		0		2	3		3		3		Human astrocytes	cellular ATP levels
Scannevin	Journal of Pharmacology and Experimental Therapeutics	2012		0		0	0		3		3		Human astrocytes	cellular redox potential
Scannevin	Journal of Pharmacology and Experimental Therapeutics	2012					3		3		3		rat cortical neurons	10 μM H ₂ O ₂
Schmidt	Neurochemistry International	2010									0		cultured astrocytes	Cellular and extracellular GSx
Schmidt	Neurochemistry International	2010									0		cultured astrocytes	Cellular and extracellular GSSG
Thiessen	Neuroscience Letters	2010									0		cultured OLN-93 cells	cellular GSx content
Thiessen	Neuroscience Letters	2010									0		Oligodendroglia-rich secondary cultures	cellular GSx content
Thiessen	Neuroscience Letters	2010									0		cultured OLN-93 cells	extracellular LDH activity
Thiessen	Neuroscience Letters	2010									0		Oligodendroglia-rich secondary cultures	extracellular LDH activity
Thiessen	Neuroscience Letters	2010									3		cultured OLN-93 cells	Concentration dependent effects on the specific GSx content
Thiessen	Neuroscience Letters	2010									2		cultured OLN-93 cells	Induction of HO-1
Akay	Journal of Neurovirology	2014									3		21 DIV rat neuroglial cultures	HO-1; untreated cell cultures
Akay	Journal of Neurovirology	2014									2		21 DIV rat neuroglial cultures	HO-1; Vehicle (DMSO) pretreated cell cultures
Akay	Journal of Neurovirology	2014									0		21 DIV rat neuroglial cultures	HO-1; Thapsigargin pretreated cell cultures
Akay	Journal of Neurovirology	2014									2		21 DIV rat neuroglial cultures	HO-1; Ritonavir/Saqinavir treated cell cultures
Akay	Journal of Neurovirology	2014									3		21 DIV rat neuroglial cultures	HO-1; AZT/Ritonavir/Saqinavir pretreated cell cultures
Booth	Cancer Biology & Therapy	2014					3						GBM5/6/ 12/14 cells	+ irradiation 4 Gy
Booth	Cancer Biology & Therapy	2014					3						GBM5/6/ 12/14 cells	+ temozolomide (3mM)
Booth	Cancer Biology & Therapy	2014					3						microglia from grade IV GBM tumors	expression of IL6 and TNFa (less)
Booth	Cancer Biology & Therapy	2014					3						microglia from grade IV GBM tumors	expression of TGFb (less)
Booth	Cancer Biology & Therapy	2014					3						activated microglia from 3 patients	cell viability determined by live / dead assay
Booth	Cancer Biology & Therapy	2014					3						GBM5, GBM6, GBM12, GBM14 cells	+ FTY720 (Fingolimod, Gilenya) 50nM

Table 1. Summary of the articles reporting the effect of MMF on neural cells (0 = no effect, 1 = slight effect, 2 = moderate effect, and 3 = strong effect)

According to the current findings, MMF possesses an antioxidative effect on neuronal cells (Table 1) even though in a lower potential than DMF does in vitro. However, it is not entirely clear whether this defines its clinical potential as an immunomodulating drug that is able to act primarily through immune cell signaling.

3.6 MMF effects on skin

Although FAEs were first used as a therapeutic drug against psoriasis, newer publications set the focus on the effect of FAEs on immune and nervous system rather than on doing further investigation on the cutaneous system. Therefore, most of the studies that examine the effect of FAEs on keratinocytes and other cutaneous cells are likely to be made in 2010 and earlier. At that point, the hypothesis that DMF acts as a pro-drug and the main active component is actually MMF had not gained enough popularity. In that manner, most experimental data is addressed to DMF as the substance of interest for in vitro research. Only a few articles investigated MMF effect as well (Table 2).

An early study investigated the effect of the three fumaric acid derivates DMF, MMF, and MEF in normal and transformed keratinocytes (SVK-14 cells). In the experiments performed, MMF increased the intracellular free calcium concentration and was shown to be the most potential stimulus among the FAEs. However, regarding cell proliferation, DMF was a more potent inhibitor than MMF (257). It was proven as well that various concentrations of the used FAEs did not increase LDH leakage and herewith suggested the absence of toxic damage in keratinocytes.

In 2007, another research team examined the effect of FAEs on the p38 MAPK signaling pathway including some of its downstream kinases in cultured normal human keratinocytes in vitro. (286). It was demonstrated that DMF, but not MHF (methylhydrogenfumarate, a synonym of MMF), specifically inhibits MSK1 and 2 activation and NF- κ B/p65 transactivation through inhibition of Ser276 phosphorylation, whereas no changes in p38, MAPK, and MK2 activation were seen. However, this finding is highly unlikely to play a crucial role for treatment situations in humans, as DMF itself does not directly affect the cutaneous system in vivo.

Autor	Journal	Year	MMF/MHF										Cells/System	Notes
			μM											
			0.8	1	10	12	50	100	140	200	400	800		
Gesser	Investigative Dermatology	2007							0				cultured normal human keratinocytes	IL-1b-induced p38 MAPK, ERK p44/p42, and MK2
Gesser	Investigative Dermatology	2007							0				cultured normal human keratinocytes	IL-1b-induced MSK1 and 2
Gesser	Journal of Investigative	2007							0				cultured normal human keratinocytes	IL-1b-induced phosphorylation of CREB and the CREB-related
Gesser	Journal of Investigative	2007							0				cultured normal human keratinocytes	IL-1b-induced NF- κ B binding to the IL-8 β Band the IL-20 β B-
Gesser	Journal of Investigative	2007											cultured normal human keratinocytes	IL-1b-induced IL-8 and IL-20 mRNA expression
Thio	British Journal of Dermatology	1994								3			human keratinocytes	intracellular free calcium concentration
Thio	British Journal of Dermatology	1994								2			SVK-14 cells	intracellular free calcium concentration
Thio	British Journal of Dermatology	1994		0	0		2	2		3	3		human keratinocytes	intracellular free calcium concentration
Thio	British Journal of Dermatology	1994		0	0		0	2		3	3		SVK-14 cells	intracellular free calcium concentration
Thio	British Journal of Dermatology	1994	0		2			2		3	3	3	human keratinocytes	proliferation
Thio	British Journal of Dermatology	1994		0	0			2		2	3	3	SVK-14 cells	proliferation

Table 2. Summary of the articles reporting the effect of MMF on cutaneous cells (0 = no effect, 1 = slight effect, 2 = moderate effect, and 3 = strong effect)

3.7 MMF effects on stem cells

A novel field of interest over the last decades was the effect of drugs and other chemical substances on stem cells. In that manner, many scientific researches followed this trend to investigate the influence of fumaric acid and its esters on pluripotent cells. Main target in all published reports have been human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC). As DMF shows a significantly higher effect than MMF in experimental settings, most studies set their focus on DMF. Only in very few articles, a comparison with MMF and other FAEs has been presented.

Already in 2001, the question about the molecular mechanisms of different FAEs that inhibit endothelial cell activation has been raised (287). For this purpose, HUVEC cells treated with TNF- α were exposed to different concentrations of DMF and MMF in vitro and the expression of CD62E, a TNF α -induced E-selectin, was measured. DMF was able to suppress the expression of CD62E in a dose-dependent manner, while MMF showed no significant effect. More recent studies, taking up this question, investigated further the

inhibition potential of FAEs on CD62E expression including not only HUVEC, but human placental microvascular endothelial cells (HPEC-A2) as well (288). Similarly to the results obtained by Loewe et al., DMF, but not MMF, suppressed TNF α -induced CD62E expression in both cell lines. Furthermore, DMF was able to inhibit the expression of the surface proteins VCAM-1 and ICAM-1 in HUVEC. Eventually, MMF showed no suppression of TNF α -induced transcription of activation-associated endothelial proteins.

An area of interest, however, is not only the suppression of TNF α -induced protein expression but also the FAE-induced inhibition of angiogenesis as such. Thus, proliferation, migration, and tubulogenesis of stem cells have been investigated (289). Analogously to previous experiments, it was not surprising that MMF had no impact on proliferation and migration in neither HUVECs nor BAECs. However, at 200 μ M it showed a slight inhibition of tubulogenesis in HUVEC.

Autor	Journal	Year	MMF/MHF								Cells/System	Notes
			μM									
			5	15	25	40	50	100	140	200		
García-Caballero	Journal of Investigative Dermatology	2011								0	BAEC	inhibition of proliferation
García-Caballero	Journal of Investigative Dermatology	2011								0	HUVEC	inhibition of proliferation
García-Caballero	Journal of Investigative Dermatology	2011								0	BAEC	inhibition of tubulogenesis
García-Caballero	Journal of Investigative Dermatology	2011								1	HUVEC	inhibition of tubulogenesis
García-Caballero	Journal of Investigative Dermatology	2011								0	BAEC	inhibition of migration
Loewe	Journal of Investigative Dermatology	2001			0	0			0		HUVEC	supression of CD62E expression
Wallbrecht	Experimental Dermatology	2011	0	0	0		0			0	HUVEC	supression of TNFα-induced transcription of activation-associated endothelial proteins
Wallbrecht	Experimental Dermatology	2011	0	0	0		0	0		0	HUVEC	inhibition of surface protein expression VCAM-1 and ICAM-1
Wallbrecht	Experimental Dermatology	2011	0	0	0		0	0		0	HUVEC; HPEC-A2	expression of E-selectin (CD62E)

Table 3. Summary of the articles reporting the effect of MMF on stem cells (0 = no effect, 1 = slight effect, 2 = moderate effect, and 3 = strong effect)

3.8 MMF effects on other cell systems

As FAEs are used as immunomodulating drugs in the field of skin diseases (psoriasis) as Fumaderm®/Skilarence® and neurological diseases (MS) as Tecfidera®, studies have been designed primarily for treatment situations in the human immune, nervous, and cutaneous systems. Some experiments were made with stem cells investigating possible interactions of FAEs with pluripotent cells. In the field of oncology, there are still research groups that compare DMF treatment in vitro to common guideline set treatment of e.g. breast cancer (280, 290, 291, 292, 293), colon cancer (291, 294, 295), and hepatoma (296, 297). Seidel et al. experimented with primary human asthmatic and nonasthmatic airway smooth muscle cells (298, 299, 300, 301) whereas MacDonald et al. examined pancreatic

islet cells (302). However, all these studies described only the effect of DMF on different cell lines in vitro. In vivo, it is likely that DMF would act differently from the obtained results as it is rapidly metabolized to MMF. In all current studies, there is almost no information about the impact of MMF on cell systems other than the mentioned above (Table 4).

In their study, Meili-Butz et al. demonstrated that 10 μ M of MMF slightly blocked NF- κ B translocation and its reduction to the nucleus in rat heart endothelial cells whereas, in the same setting, DMF achieved highly significant results in vitro (303). MMF was also able to maintain cell viability against toxicants in rat hepatocytes but only at a high dose of the treatment administered (304).

As DMF shows a higher reactivity than MMF in vitro, most articles address only DMF itself. Therefore, no precise conclusion can be drawn on the actual impact in humans, knowing that DMF, as a single compound, cannot interfere directly with these cell systems in vivo.

Autor	Journal	Year	MMF/MHF						Cells/System	Notes
			μM		mg/l					
			10	1000	10	100	1000			
Meili-Butz	European Journal of Pharmacology	2008	1						rat heart endothelial cells (RHEC)	block of NF-κB translocation
Meili-Butz	European Journal of Pharmacology	2008	1						rat heart endothelial cells (RHEC)	reduction of NF-κB translocation to the nucleus
Rao	Journal of Ethno Pharmacology	1998			0	0	3		rat hepatocytes	Galactosamine; cell viability against toxicants
Rao	Journal of Ethno Pharmacology	1998			0	0	3		rat hepatocytes	Thioacetaamid; cell viability against toxicants

Table 4. Summary of the articles reporting the effect of MMF on heart endothelial and hepatic cells (0 = no effect, 1 = slight effect, 2 = moderate effect, and 3 = strong effect)

4. Discussion

4.1 Potential novel in vivo functions of FAEs

A recent study has published very interesting data that have been generated in an in vitro model of HIV-mediated neurotoxicity using HIV-infected monocyte-derived macrophages (305). It has been shown that HIV infection can cause cognitive impairment and neurodegeneration. This is due to chronic inflammation and neurotoxin production induced by infected macrophages and microglia. In fact, both of these cell types express HCA2 thus showing that MMF is able to suppress HIV replication and neurotoxin release by inhibiting NF- κ B nuclear translocation and signaling. Concentrations of MMF equal to those that can be obtained by oral DMF treatment significantly enhanced neuronal survival. At the same time, MMF was also shown to increase heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO1) expression, but had no effect on NRF2. This indicates that MMF can alleviate the abnormal redox state that is induced by HIV infection (305). All together, these described effects of MMF on HIV infected cells could be used in the future to limit neurodegeneration in patients.

Another area of interest for novel treatment situations in vivo is the influence of FAEs on immune homeostasis. Recently, it has become clear that the interaction between the host and microbiota plays a pivotal role confirming that the tissues which interface with the external environment such as skin, airways and gut are constantly exposed to microorganisms and dietary components. In order to prevent inappropriate immune responses to innocuous antigens while maintaining an effective defense against pathogens, the immune system is tightly regulated in these tissues. The priming of T cells by innate immune cells such as macrophages and DCs is critical. This ability is crucial for the maintenance of mucosal homeostasis. For example, specialized CD103⁺ DCs in the gut mucosa are responsible for directing T cell immune responses into different lineages depending on activating stimuli and microenvironmental factors (306). Herewith, CD103⁺ DCs are particularly important for regulatory T cell (Treg) homeostasis which, on the other hand, is critical to the maintenance of immune tolerance and is involved in multiple autoimmune diseases (307). Regulation of Tregs in the small intestine by CD103⁺ DCs is dependent upon metabolites and nutrients derived from the diet and released by commensal microbes. Such compounds include short-chain fatty acids (SCFAs) such as butyric acid and vitamins A, B3 (niacin), and D3 (308). Actually, both

niacin and butyric acid are known HCA2 agonists. It has also been shown that in an HCA2-deficient mouse model, the number of Tregs was decreased in favor of increased colonic inflammation. Butyric acid can signal via HCA2 that is expressed by IECs leading to the production of IL-10 and the induction of Treg differentiation (309). Similarly, in psoriasis, Tregs are often decreased and therefore treatments improving skin lesions are based on restoration in cell number (310).

Expression of HCA2 also plays an essential role for butyric acid mediated induction of IL-18 in colonic epithelium. In a HCA2-deficient mouse model, an increased development of colonic inflammation and colon cancer was observed. This phenomenon could be decreased by niacin suppressed in a HCA2-dependent manner (309). In this case, a part of anti-inflammatory effects of FAE treatment may be explained by modulation of the gut immune system. However, further research needs to be done to confirm this postulation.

4.2 Conclusions

According to the latest available data, special caution is needed when exploring the mechanism of action of FAEs. In this case, it is essential to work within the hypothesis that in vivo only MMF is responsible for the drug pharmacological effects on the tissues outside of the intestinal mucosa. In all registered oral DMF-based drugs, DMF acts as a pro-drug which, due to its rapid metabolism, cannot reach any other cells of the body besides the initial layer of IECs. Therefore, in vitro studies using DMF only while often showing dramatic effects, do not reflect the in vivo reality. The same relates to the studies that use abnormally high concentrations of MMF. Furthermore, the published pharmacological and biochemical data as well as the clinical studies with the MMF-linked compound (XP23829) provide a sound basis for the assumption that the only active in vivo compound of FAE drugs is MMF.

The HCA2 cell receptor is crucial for FAE-based medications. Both the beneficial therapeutic effects and adverse events such as flushing and lymphopenia observed during FAE treatment depend upon HCA2-MMF interaction and signaling. In the literature, there is strong evidence that cells of the myeloid lineage such as neutrophils, monocytes, macrophages, DCs, and microglia as well as tissue-resident cells such as adipocytes, keratinocytes, astrocytes, and IECs express HCA2. On the other hand, there is no evidence

that the receptor is expressed by lymphoid cells. This may explain the fact that, at pharmacological concentrations, MMF appears to have no direct effect on lymphocytes. However, the exact mechanism for FAE-induced lymphopenia has still not been fully elucidated. Furthermore, there has been no published data yet examining whether cells of the intestinal mucosa respond to DMF or MMF released from the enteric-coated tablets/capsules. Therefore, it will be interesting to determine whether these cells contribute to the modulation of immunologic pathways that are responsible for the subsequent disease-beneficial effects seen in psoriasis and MS. Animal models in which DMF is given orally (e.g. gavage, drinking water) may be helpful in this area. However, the expression of the HCA2 receptor should be verified in order to interpret results correctly. On the other hand, DMF delivery by intraperitoneal route of administration should be considered with caution due to the unknown pharmacokinetics and pharmacodynamics of this kind of drug administration and remembering that the route mentioned is not used in the treatment of humans with FAEs.

Altogether, much remains unknown regarding the countless biological effects of FAEs. A better understanding of the mechanism of action of MMF *in vivo* may not only provide a basis for other indications apart from psoriasis and MS but also for the improvement of these medications in order to reduce side effects and maximize therapeutic benefit.

5. Abstract

Besides methotrexate, ciclosporin, hydroxycarbamide and retinoids, fumaric acid esters based drugs are used since 1971 as an immunosuppressive, respectively immunomodulatory, medication in the therapy of severe psoriasis. The latter substances are currently subject of great interest as an oral treatment for several autoimmune diseases including multiple sclerosis. During the past years, several pharmaceutical products containing dimethylfumarate as main active component of all licensed drugs have been released. In all in vivo studies, dimethylfumarate showed the most significant effects. Despite this, the exact mechanism of action of fumaric acid esters remains an open problem.

Recent studies demonstrate that dimethylfumarate have also a significant biological effect in vitro. However, the pharmacokinetics and chemistry of dimethylfumarate and other fumaric acid esters are often not fully considered in the design and interpretation of the most experiments, which questions the validity of the results achieved in vitro.

In this literature-based analysis, all minimum stages for a literature research have been followed in order to compare and validate experimental setup and results of in vitro studies for treatment situations in humans. Using several search engines in the field of science, the content from more than 300 articles dating from 1971 to 2022 has been used to create a database to facilitate comparison of significant results obtained for different fumaric acid esters in vitro. During this process, a data table was formed, in which different cells and tissues that were used in the experimental settings were organized in categories and linked with their interaction with different concentrations of fumaric acid esters. For ease of comparison of results, a scoring system dependent on effect intensity has been applied.

Based on this data it was discovered that monomethylfumarate has a higher biochemical impact than dimethylfumarate. This observation is most likely due to the fact that dimethylfumarate is a plasma-membrane permeable prodrug that in vivo rapidly converts to monomethylfumarate. This makes monomethylfumarate the main active compound of dimethylfumarate -based drugs and means that monomethylfumarate is the only substance to directly interact with the intracellular signaling pathways in vivo.

Recent works are often concentrated on the direct effects of dimethylfumarate on cells in vitro, which, having the metabolism of dimethylfumarate in mind will never be exposed to this particular fumaric acid ester in an actual human organism. This shows that, unfortunately, most of the performed in vitro studies are not valid for treatment situations in vivo. On the other hand, only some research groups have used monomethylfumarate in their in vitro experiments.

In order to evaluate the biological effects of dimethylfumarate -based drugs correctly, monomethylfumarate should be addressed as the molecule of interest in in vitro experiments. In future studies, it is important to consider the biological effects of monomethylfumarate as this is an indispensable part of taking science one step closer to understanding its mechanism of action.

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7. Appendix

7.1. Abbreviations

Akt - RAC-alpha serine/threonine-protein kinase (Akt/PKB)

ATP – adenosine triphosphate

AUC - area under the curve

BAEC – Bovine Aortic Endothelial Cells

CaCo2 - heterogeneous human epithelial colorectal adenocarcinoma cells

cAMP - Cyclic adenosine monophosphate

CD62E - E-Selectin

CHO – chinese hamster ovary cells

CNS – central nervous system

CXCL2 - C-X-C motif chemokine 2

DC – dendritic cell

DMF – dimethyl fumarate

EAE – Experimental autoimmune encephalomyelitis

EMA - European Medicines Agency

ERK - extracellular signal-regulated kinases

FA – fumaric acid

FAD - flavin adenine dinucleotide

FAE – fumaric acid ester

FDA - U.S. Food and Drug Administration

GPCR - G protein-coupled receptors

GS-DMS - S-(1,2-dimethoxycarbonylethyl)glutathione

GSH - Glutathion

HCA2 - Hydroxycarboxylic acid receptor 2

HO-1 - Heme oxygenase 1

hOPC - High Osmotic Pressure Chromatography

HPEC-A2 - SV40-transformed placental venous endothelial cell line

HPLC - high performance liquid chromatography

HUVEC - human umbilical vein endothelial cells

IECs - Intestinal epithelial cells

IFN - Interferon

IL - Interleukin

MAP-2 - Microtubule-associated protein 2

MEF - monoethyl fumarate

MHF - methyl hydrogen fumarate

MMF – monomethyl fumarate

MS – multiple sclerosis

NAC-DMS - N-acetyl-S-(1,2-dimethoxycarbonyl)ethyl)cysteine

NAD⁺ - nicotinamide adenine dinucleotide

NfκB - nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NHS – National Health Service

NQO1 - NAD(P)H Quinone Dehydrogenase 1

OLN-93 - permanent oligodendroglia cell line

PBMC - Peripheral Blood Mononuclear Cell

PML - Progressive multifocal leukoencephalopathy

PTX - pertussis toxin

rOPC - rat oligodendrocyte precursor cells

ROS - reactive oxidative species

SDH - Succinate dehydrogenase

T2-MZP - immature transitional-2 B cells

TNF α – tumor necrosis factor α

Treg - regulatory T cells

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