



No evidence for interactions of dimethylfumarate (DMF) and its main metabolite monomethylfumarate (MMF) with human cytochrome P450 (CYP) enzymes and the P-glycoprotein (P-gp) drug transporter

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Abstract

Dimethylfumarate (DMF) has long been used as part of a fixed combination of fumaric acid esters (FAE) in some European countries and is now available as an oral monotherapy for psoriasis. The present investigation determined whether DMF and its main metabolite monomethylfumarate (MMF) interact with hepatic cytochrome P450 (CYP) enzymes and the P-glycoprotein (P-gp) transporter, and was performed as part of DMF's regulatory commitments. Although referred to in the available product labels/summary of product characteristics, the actual data have not yet been made publicly available. In vitro inhibition experiments using CYP-selective substrates with human liver microsomes showed 50% inhibitory concentrations (IC₅₀) of >666 µmol/L for DMF and >750 µmol/L for MMF. MMF (≤250 µmol/L; 72 hours) was not cytotoxic in cultured human hepatocyte experiments and mRNA expression data indicated no CYP induction by MMF (1-250 µmol/L). DMF (≤6.66 mmol/L) showed moderate-to-high absorption (apparent permeability [P_{app}] ≥2.3-29.7 × 10⁻⁶ cm/s) across a Caucasian colon adenocarcinoma (Caco-2) cell monolayer, while MMF (≤7.38 mmol/L) demonstrated low-to-moderate permeability (P_{app} 1.2-8.9 × 10⁻⁶ cm/s). DMF was not a substrate for P-gp (net efflux ratios ≤1.22) but was a weak inhibitor of P-gp at supra-therapeutic concentrations (estimated IC₅₀ relative to solvent control of 1.5 mmol/L; [³H]digoxin efflux in Caco-2 cells). This inhibition is unlikely to be clinically relevant. MMF was not a substrate or inhibitor of P-gp. Thus, DMF and MMF should not affect

Abbreviations: A, apical; ABC, adenosine triphosphate-binding cassette; B, basolateral; Caco-2, Caucasian colon adenocarcinoma; cDNA, complementary deoxyribonucleic acid; C_{max}, maximum plasma concentration; CYP, cytochrome P450; DDI, drug-drug interaction; DMEM, Dulbecco's Modified Eagle's Medium; DMF, dimethylfumarate; DMSO, dimethyl sulfoxide; EMA, European Medicines Agency; FAE, fumaric acid esters; FBS, fetal bovine serum; FDA, US Food and Drug Administration; GSH, glutathione; HBSS, Hanks' Balanced Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; IC₅₀, concentration of drug causing 50% inhibition of enzyme activity; ISWS, internal standard working solution; ITS, insulin, transferrin, selenium complex; JMHLW, Japanese Ministry of Health, Labour and Welfare; LC-MS/MS, liquid chromatography with tandem mass spectrometric detection; LSC, liquid scintillation counting; MDCKII, Madin-Darby Canine Kidney; MDR, multidrug resistance; MDR1, multidrug resistance protein 1; MEM, Minimum Essential Medium; MMF, monomethylfumarate; mRNA, messenger ribonucleic acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; P_{app}, apparent permeability; PASI 75, 75% improvement in Psoriasis Area and Severity Index; PES, phenazine ethosulfate; P-gp, P-glycoprotein; RT, reverse transcriptase; RT-qPCR, real-time quantitative polymerase chain reaction; TEER, transepithelial electrical resistance; WT, wild-type; β-NADPH, β-nicotinamide adenine dinucleotide phosphate.

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the absorption, distribution, metabolism or excretion of coadministered drugs that are CYP and P-gp substrates.

KEYWORDS

cytochrome P450, dimethylfumarate, drug metabolism, drug-drug interactions, fumaric acid esters, monomethylfumarate, P-glycoprotein

1 | INTRODUCTION

Dimethylfumarate (DMF) has been used for several decades for the treatment of psoriasis as part of a fixed combination of fumaric acid esters (FAE), both in Germany (Fumaderm[®]) and other European countries.¹ A novel oral formulation of DMF (Skilarence[®]) has recently been approved as a monotherapy for the treatment of patients with moderate-to-severe psoriasis.² In its pivotal phase III trial (BRIDGE), DMF demonstrated superiority over placebo and noninferiority versus the FAE combination (both $P < .001$) in the number of patients who reported a 75% improvement in Psoriasis Area and Severity Index (PASI 75) at Week 16.³ A similar oral formulation of DMF has also been approved for the treatment of patients with relapsing forms of multiple sclerosis (Tecfidera[®]).⁴

The most common adverse drug reactions associated with Skilarence[®] and Fumaderm[®] treatment are gastrointestinal disorders, including diarrhea, abdominal distension, abdominal pain, and nausea. Lymphopenia, leukopenia, and flushing are also very common ($\geq 1/10$ patients).² Adverse drug reactions reported with Tecfidera[®] in multiple sclerosis are similar: gastrointestinal disorders and flushing, as well as ketonuria, are very common ($\geq 1/10$ patients).⁴

Following oral administration, DMF is rapidly hydrolyzed by esterases of the small intestine to form monomethylfumarate (MMF), its main metabolite.⁵ DMF/MMF is believed to elicit its antipsoriatic effects via modulation of several signaling pathways including glutathione (GSH)-mediated nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) inhibition. This initiates a downstream cellular response to oxidative stress and shifts the immune response from a proinflammatory T helper 1/T helper 17 cell profile to an anti-inflammatory Th2 profile.¹ The mechanism of action of DMF may also involve the inhibition of ribosomal S6 kinases and mitogen- and stress-activated kinases, primarily via allosteric covalent binding to a conserved cysteine residue.⁶

Many drugs act as inhibitors of cytochrome P450 (CYP) enzymes in vivo and thus may affect the disposition of other coadministered drugs that are also metabolized by these enzymes.⁷ Consequently, inhibition of drug-metabolizing CYP enzymes is a major cause of clinically relevant drug-drug interactions (DDIs). Repeated administration of certain drugs may also induce CYP enzymes. Induction of this type may lead to decreased efficacy of concomitant medications and may increase formation of toxic metabolites.⁸

P-glycoprotein (P-gp) is a multidrug resistance (MDR) adenosine triphosphate-binding cassette (ABC) transporter expressed on the apical side of gut epithelia and brain endothelia that can extrude drugs from the cell via active transport and, in doing so, limit oral absorption and

brain penetration, respectively.⁹ Increased P-gp activity can therefore lead to drug resistance and may influence the kinetics of drug absorption. Conversely, inhibition of the transporter can increase absorption of P-gp substrates across the gastrointestinal tract and into other organs.

As a consequence of these findings, the European Medicines Agency (EMA),¹⁰ the US Food and Drug Administration (FDA)¹¹ and the Japanese Ministry of Health, Labour and Welfare (JMHLW)¹² recommend that interactions with CYP enzymes and the P-gp transporter are evaluated as part of the safety assessment and subsequent product labeling of new therapies.

Although, the chemical structures and physicochemical properties of DMF and MMF suggest that they are unlikely to interact with CYP enzymes or P-gp, available data on the interactions of DMF and MMF with these proteins are currently limited.¹³ The available product labels/summary of product characteristics for Fumaderm[®], Skilarence[®] (in psoriasis) or Tecfidera[®] (in multiple sclerosis) mention that DMF and MMF do not inhibit or induce CYP enzymes and are not substrates or inhibitors of P-gp.^{2,13} These DDI studies of MMF were conducted as a standard component of the Skilarence[®] regulatory submission package, while DMF DDI studies were required by the EMA as a postauthorization measure; the data from these studies have not previously been made publicly available. For transparency, we report the results of these in vitro studies investigating the potential interaction of DMF and MMF with CYP enzymes and as substrates/inhibitors of P-gp. The results show no evidence that MMF or DMF interacts with CYP enzymes or the P-gp transporter at clinically achievable concentrations. Therefore, no interactions are expected with medicinal products metabolized or transported by these systems.

2 | MATERIALS AND METHODS

2.1 | Solubility and stability of DMF and MMF

The solubility of DMF was assessed at concentrations of 6.66 to 666 μ mol/L in 100 mmol/L potassium phosphate buffer (pH 7.4) with human liver microsomes (HLM; pool of microsomes from 150 individual donors: Corning[®], Woburn, MA, USA) at 0.025 and 0.1 mg protein/mL, and cofactor (β -nicotinamide adenine dinucleotide phosphate [β -NADPH]), as applicable to a final concentration of 1 mmol/L, at 10% v/v, and at concentrations of 0.666 to 66.6 mmol/L in PreadyPort[™] buffer (Hanks' Balanced Salt Solution [HBSS] supplemented with 12.5 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], pH 7.4).

The solubility of MMF was assessed at concentrations of 0.1 to 750 $\mu\text{mol/L}$ in 100 mmol/L potassium phosphate buffer (pH 7.4) with HLM at 0.3 mg protein/mL, and cofactor, as applicable to a final concentration of 1 mmol/L, at 10% (v/v), and at concentrations of 0.1 to 750 $\mu\text{mol/L}$ in supplemented Williams' Medium E (without L-glutamine and phenol red, supplemented with a Primary Hepatocyte Maintenance Supplements Kit [Life Technologies] containing dexamethasone, a cocktail of penicillin-streptomycin, ITS+ [insulin, transferrin, selenium complex, bovine serum albumin, and linoleic acid], GlutaMAX™ [200 mmol/L L-alanyl-L-glutamine], and HEPES). Additionally, the solubility of MMF was assessed at concentrations of 0.738 to 73.8 mmol/L in CacoReady™ buffer (HBSS supplemented with 12.5 mmol/L HEPES, pH 7.4) and PreadyPort™ medium (Dulbecco's Modified Eagle's Medium [DMEM] supplemented with 20 mmol/L glucose, 25 mmol/L HEPES, 10% (v/v) fetal bovine serum [FBS], 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 1% (v/v) Minimum Essential Medium [MEM] nonessential amino acids solution [100 \times]).

DMF and MMF samples were inspected visually for any signs of insolubility. Actual concentrations of DMF and MMF were determined in solutions before and after centrifugation by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) as described in the "Analysis of DMF and MMF concentrations" section.

DMF stability was assessed at final concentrations of 6.66 and 666 $\mu\text{mol/L}$, and MMF stability at 0.1 and 750 $\mu\text{mol/L}$. Samples were added to vials containing buffer and HLM in the presence and absence of cofactor before incubation at 37°C in a shaking water bath. At 0, 10, and 40 minutes, aliquots (25 μL for DMF or appropriate volume for MMF) were added to a 96-well plate and prepared for LC-MS/MS analysis as described in the "Analysis of DMF and MMF concentrations" section.

2.2 | Analysis of DMF and MMF concentrations

Liquid chromatography with LC-MS/MS determinations were carried out with aliquots (25 μL for DMF; appropriate volume for MMF) added to 96-well plates before and after centrifugation (Table S1). For DMF, an 8-point internal standard curve over the range 125–125 000 ng/mL was prepared in acetonitrile. For MMF, two 7-point internal standard curves, one over the range 5–2000 ng/mL and the other 1000–120 000 ng/mL of MMF, were prepared in dimethyl sulfoxide (DMSO):acetonitrile (1:1, v/v). Sample supernatants comprised similar proportions of HLM, buffer, solvent, and internal standard as present in the samples prior to analysis with LC-MS/MS.

2.3 | Assessment of DMF and MMF as inhibitors of CYP enzymes in vitro

The potential of DMF and MMF to inhibit CYP enzymes was investigated using EMA- and FDA-recommended substrates and inhibitors,^{10,11} with sample analysis by LC-MS/MS.

2.3.1 | Incubation of DMF and MMF with CYP-selective substrates

DMF and MMF were incubated at final incubation concentrations of 0, 6.66, 22.2, 66.6, 200, 400, and 666 $\mu\text{mol/L}$ and 0, 0.1, 1, 10, 100, 250, and 750 $\mu\text{mol/L}$, respectively, with HLM in the presence of selective substrates of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 (all MMF only), and CYP3A4/5 (both DMF and MMF) (Table S2). Because of instability of DMF noted in the preliminary incubation (see Results), DMF concentrations were measured over the incubation period under experimental conditions. Incubations (DMF or MMF, HLM, and buffer) were performed in triplicate in a 96-well Teflon® plate held in an oscillating plate heater (540 rpm at 37°C). Samples were pre-incubated for 0 or 30 minutes (with and without β -NADPH) prior to addition of selective CYP enzyme substrate and cofactor (if not already present in the pre-incubation) to a final volume of 200 μL . Untreated control incubations contained test compound solvent in place of the test compound. Positive control incubations contained a selective chemical inhibitor for each CYP enzyme in place of the test compound.

Reactions were terminated after a 10-minute incubation period by removal of an aliquot into methanol containing the internal standard (appropriate volume based on CYP substrate). Terminated samples were then prepared for LC-MS/MS analysis as described below.

2.3.2 | Analysis of CYP enzyme assay incubation samples

Analysis of CYP enzyme incubation samples was carried out as described in Table S3. All samples underwent specific preparation using protocols that ensured the resulting supernatant comprised the same proportions of methanol, HLM and internal standard as present in the incubation samples prior to analysis by LC-MS/MS.

2.4 | Assessment of the potential of MMF to cause cytotoxicity and to induce expression of CYP enzymes in cultured human hepatocytes

2.4.1 | Human hepatocyte thawing and assessment of cell viability

Hepatocytes obtained from BioreclamationIVT (Baltimore, USA) were resuscitated for cytotoxicity and induction experiments. To determine cell yield and viability, an aliquot of cell suspension was diluted 1:2 with 0.4% (w/v) Trypan Blue and appropriate thawing medium and loaded onto a hemocytometer. The total numbers of viable (nonstained) and dead (purple stained) cells were counted. Mean numbers of viable cells/mL and total number of cells isolated were calculated.

2.4.2 | Assessment of MMF cytotoxicity

MMF solutions of 0.2, 2, 20, 50, and 150 mmol/L and 10 mmol/L tamoxifen (positive control for cytotoxicity) were prepared in methanol on Day 1 and stored at -20°C (tamoxifen) or $2-8^{\circ}\text{C}$ (MMF) for use on Days 2 and 3. On each day of dosing, aliquots were added to supplemented Williams' Medium E incubation medium to final concentrations of 1, 10, 100, 250, and 750 $\mu\text{mol/L}$ MMF and 50 $\mu\text{mol/L}$ tamoxifen, at a final organic solvent concentration of 0.5% (v/v).

Cryopreserved plated human hepatocytes in 24-well plates, isolated from one individual (identified with lot number EBS) were used (Table S4). Following thawing, the supplemented medium was removed and replaced with 0.5 mL of fresh prewarmed supplemented medium containing the following final concentrations in appropriate (triplicate) wells: MMF 1, 10, 100, 250, and 750 $\mu\text{mol/L}$; tamoxifen 50 $\mu\text{mol/L}$; and MMF and tamoxifen solvent (methanol) at 0.5% of the final incubation volume.

Plates were incubated for 72 hours with daily media changes (plated hepatocytes therefore received three separate doses of either MMF or tamoxifen). No-cell control wells were also dosed in parallel with MMF for assessment of nonspecific binding/stability of MMF in incubation medium.

Cell viability was assessed using the AQ_{ueous} One Solution (Promega) colorimetric cell proliferation assay. The reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES).

The MTS-PES mixture was thawed at ambient temperature. Dosed media were removed from the hepatocyte culture and replaced with 300 μL of fresh warmed supplemented Williams' Medium E added to 60 μL of MTS-PES solution and incubated at 37°C until the cellular medium of solvent control wells turned brown. Following incubation, aliquots were removed from each well and transferred to a flat-bottomed 96-well microplate. A blank control sample containing medium and MTS-PES mixture was analyzed in triplicate aliquots. The absorbance of each well was measured at 490 nm using a plate reader (POLARStar Omega; BMG Labtech GmbH). If a concentration yielded $>10\%$ cell death, it was considered cytotoxic and was not used for the induction assessment.

On the last day of exposure, aliquots of incubation medium from cell and no-cell control wells were taken at 0, 1, 4, and 24 hours and added to equal volumes of acetonitrile-containing internal standard (250 ng/mL MMF-D₃) for analysis of MMF concentrations via LC-MS/MS (Table S1).

2.4.3 | Assessment of MMF induction potential

The potential of MMF to increase messenger ribonucleic acid (mRNA) expression of selected CYP enzymes was investigated as a means of determining the potential of MMF as an inducer of CYP activity.

Solutions of MMF (0.2, 2, 10, 20, 30, 40, and 50 mmol/L) were prepared in methanol on Day 1 and stored at $2-8^{\circ}\text{C}$ for use on Days 2 and 3. Positive (10 mmol/L omeprazole, 200 mmol/L phenobarbital, and 5 mmol/L rifampicin) and negative (5 mmol/L flumazenil)

controls for induction were prepared in methanol on Day 1 and stored at -20°C before incubation with hepatocytes.

On each day of dosing, MMF and control solutions were added to supplemented Williams' Medium E incubation medium to final concentrations of 1, 10, 50, 100, 150, 200, and 250 $\mu\text{mol/L}$ MMF, 50 $\mu\text{mol/L}$ omeprazole, 1 mmol/L phenobarbital, 25 $\mu\text{mol/L}$ rifampicin, and 25 $\mu\text{mol/L}$ flumazenil, each at a 0.5% final concentration of organic solvent by volume.

Cryopreserved plated human hepatocytes isolated from three individuals (donors XKU [donor 2], CDP [donor 3] and EJW [donor 4]; Table S4) in 24-well plates were used in this phase of the study. Following thawing, the supplemented medium was removed and replaced with fresh pre-warmed supplemented medium containing the following components in the appropriate (triplicate) wells: MMF (1 to 250 $\mu\text{mol/L}$), omeprazole (50 $\mu\text{mol/L}$), phenobarbital (1 mmol/L), rifampicin (25 $\mu\text{mol/L}$), flumazenil (25 $\mu\text{mol/L}$), and methanol, each at 0.5% of the final incubation volume.

Plates were incubated, and samples collected as described under "Assessment of MMF cytotoxicity." Plated hepatocytes were assessed for attachment and cell morphology by visual microscopic inspection. Cell viability was assessed 1 hour and 72 hours post dose on Day 1 at 0, 200, and 250 $\mu\text{mol/L}$ MMF using the AQ_{ueous} One Solution cell proliferation assay. On the last day of exposure, aliquots of incubation medium were taken at 0, 1, 4, and 24 hours and added to equal volumes of acetonitrile-containing internal standard (250 ng/mL MMF-D₃) for analysis of MMF concentrations via LC-MS/MS as described previously.

2.4.4 | Measurement of CYP mRNA levels

Samples were prepared after incubation by removing medium from the relevant wells and replacing with 500 μL RNeasy Protect (Qiagen, UK). A pipette tip was used to detach cells prior to real-time quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was extracted using the PureLink RNA Mini Kit as per manufacturer instructions (Life Technologies). On-column DNase treatment (Life Technologies) was performed on each sample and RNA samples were eluted in 30 μL RNase-free water.

Quality and quantity of RNA samples were assessed with an Experion™ Automated Electrophoresis Station (Bio-Rad Laboratories). An RNA StdSens Analysis Kit (Bio-Rad Laboratories) was used according to manufacturer's instructions. Only samples with RNA quality values ≥ 7 were analyzed by qPCR.

For complementary DNA (cDNA) synthesis, samples were prepared using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to manufacturer's instructions. Reverse transcriptase (RT) assays were performed in a volume of 20 μL with ~ 500 ng of RNA. RT conditions included 5 minutes at 25°C , 30 minutes at 42°C , and 5 minutes at 85°C . Resultant cDNA was diluted 1:5 with nuclease-free water before analysis by RT-qPCR performed in a CFX96 Touch™ real-time PCR instrument (Bio-Rad Laboratories). The 96-well plates and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) were used according to the manufacturer's instructions. Triplicate reactions were performed in 20 μL with 3 μL of cDNA. Conditions included 10 minutes

at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A melt curve was included as a final step (95°C for 0.05 seconds, 65°C to 90°C: increment 0.5°C 0.05 plate read).

The following primers were used for RT-qPCR (final primer concentrations): CYP1A2 Forward (300 nmol/L); CYP1A2 Reverse (50 nmol/L); CYP2B6 Forward (300 nmol/L); CYP2B6 Reverse (300 nmol/L); CYP3A4 Forward (300 nmol/L); CYP3A4 Reverse (300 nmol/L); β -Actin Forward (50 nmol/L); and β -Actin Reverse (300 nmol/L).

Changes in mRNA levels were assessed and compared to control values. Data were reported as a fold-change in expression of CYP mRNA as compared to control values. β -Actin was used as an endogenous reference.

2.5 | Assessment of DMF and MMF as substrates and inhibitors of P-gp in vitro

2.5.1 | Test systems

Both human Caucasian colon adenocarcinoma (Caco-2) and transfected Madin Darby Canine Kidney (MDCKII) cell lines were used in these investigations. Caco-2 cells were sourced as CacoReady™ Kits (ReadyCell S.L., Spain). Each kit contained a 24-well plate of Caco-2 cells seeded onto Transwell inserts and expressing endogenous levels of ABC transporters. Plates were cultured under sterile conditions as per manufacturer instructions until use (approximately 21 days old). On Day 18, shipping medium was liquefied in a humidified CO₂ incubator at 37°C for 3-4 hours before replacing with culture medium (DMEM supplemented with 1 g/L glucose, 10% (v/v) FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin [CacoReady™ medium]). CacoReady™ buffer was used for incubation.

MDCKII cells were sourced as PreadyPort™ Kits (ReadyCell S.L.). Each kit contained a 24-well plate of transfected MDCKII cells containing singly expressed P-gp (multidrug resistance protein 1; MDR1) ABC transporter and wild-type (WT) MDCKII cells seeded on Transwell inserts. Plates were cultured under sterile conditions as per manufacturer instructions until use (approximately 11 days old). PreadyPort™ medium was used on Days 8-12 and PreadyPort™ buffer used for incubation.

Both cell systems were stored as per manufacturer instructions. The transepithelial electrical resistance (TEER) of each well was recorded prior to use. TEER values had to be >1000 Ω .cm² for Caco-2 cells and >83 Ω .cm² for WT and transfected MDCKII-MDR1 cells to meet acceptance criteria.

2.5.2 | Permeability assessment of DMF and MMF

The apparent cell permeability (permeability coefficient, P_{app}) for apical to basolateral (A-B) and basolateral to apical (B-A) transport of DMF and MMF was assessed in triplicate over a range of concentrations (DMF: 0.333-6.66 mmol/L; MMF: 0.0738-7.38 mmol/L) using the Caco-2 cells.

Plates were initially rinsed three times with CacoReady™ buffer and pre-incubated for 30 minutes at 37°C and 5% CO₂. DMF and

MMF were administered to apical or basolateral well compartments. Triplicate samples from the corresponding solutions were also sampled as a measure of the time zero concentration. The transport experiment was established via reconstruction of the receiver and donor compartments into one plate following dosing.

Samples were taken from the donor compartment at 120 minutes and from the receiver compartment at 0 and 120 minutes. The permeability experiment was terminated after 120 minutes by separation of the receiver and donor compartments. Bidirectional transport of DMF and MMF was measured, as was bidirectional transport of the high and low permeability markers propranolol and atenolol. Samples were prepared for analysis by LC-MS/MS as described in Table S1 (DMF and MMF) and Table S5 (atenolol and propranolol).

2.5.3 | P-gp substrate assessment

DMF and MMF were assessed as potential substrates of P-gp in the MDCKII cell line transfected with the human P-gp gene (MDCKII-MDR1). Monolayers were cultured and evaluated on permeable filter supports in a 24-well plate, according to manufacturer's instructions. DMF and MMF were incubated in triplicate with both transfected (MDR1) and WT MDCKII cells at concentrations of 0.333, 0.666, 3.33, and 6.66 mmol/L and 0.0738, 0.246, 0.738, and 7.38 mmol/L for DMF and MMF, respectively. Plates were initially rinsed three times with PreadyPort™ buffer and pre-incubated for 30 minutes at 37°C and 5% CO₂ in either PreadyPort™ buffer or in PreadyPort™ buffer with verapamil (a reference inhibitor of P-gp; 50 or 100 μ mol/L).

DMF or MMF was placed in apical or basolateral well compartments in the presence or absence of verapamil. Samples were taken from the donor and receiver compartments at 120 minutes; a 0-minute sample was taken from the dosing solution. Bidirectional transport of DMF and MMF in transfected MDCKII-MDR1 cells was measured in triplicate and compared with WT cells. Samples were analyzed by LC-MS/MS as described in Table S1. Bidirectional transport of the probe substrate [³H]digoxin with and without verapamil was also assessed in triplicate after 120 minutes.

2.5.4 | P-gp inhibition assessment

DMF and MMF were assessed as potential inhibitors of P-gp in both Caco-2 and MDCKII-MDR1 and MDCKII-WT cells. Monolayers were cultured and incubated in triplicate as described above. [³H]Digoxin (10 μ mol/L) in the presence of DMF or MMF at concentrations of 0, 0.333, 0.666, 3.33, and 6.66 mmol/L or 0, 0.0738, 0.246, 0.738, and 7.38 mmol/L, respectively, were placed in apical and basolateral well compartments as described earlier under "Permeability assessment of DMF and MMF." [³H]Digoxin was also incubated with and without verapamil (50 μ mol/L) in triplicate for 120 minutes.

Samples were taken from the receiver and donor compartments at 120 minutes. Samples containing [³H]digoxin were

reconstituted in 2 mL Ultima Gold™ XR Scintillation cocktail and quantified by liquid scintillation counting (LSC). A Packard Scintillation Counter instrument was used with a limit of quantification taken as twice the background value in dpm (disintegrations per minute) for samples of the same type. The concentration of radioactivity in each sample together with the specific activity of the dosed material was used to calculate the concentration of [³H] digoxin in the sample.

Bidirectional transport of [³H]digoxin across transfected MDCKII-MDR1 cells was compared with that across WT cells (net efflux ratio). IC₅₀ (concentration of drug causing 50% inhibition of enzyme activity) values for the inhibition of P-gp were derived using GraFit v7.0.2 (Erithacus Software Ltd.).

Bidirectional transport of the high and low permeability markers propranolol and atenolol was also measured; these samples were analyzed by LC-MS/MS (Table S5).

3 | RESULTS

3.1 | Assessment of DMF and MMF as inhibitors of CYP enzymes in vitro

3.1.1 | Preliminary solubility experiment

Preliminary solubility experiments showed that DMF and MMF were soluble up to 666 and 750 μmol/L, respectively. Calculated recoveries of DMF before and after centrifugation were generally low, most likely a consequence of its instability in the presence of HLM (see "Preliminary metabolic stability experiment"). Concentrations within 20% of nominal were achieved upon spiking MMF into solutions of HLM at 0.025 and 0.3 mg/mL. MMF concentration did not change upon centrifugation (mean recovery >92.2%).

3.1.2 | Preliminary metabolic stability experiment

The assessment of metabolic stability indicated that DMF was unlikely to be stable under the incubation conditions (target concentrations were achieved with 666 μmol/L DMF in 0.1 mg/mL HLM only). In contrast, 750 μmol/L MMF was stable in the preliminary investigations. To ensure that inhibition could be ascribed to DMF in the CYP assays, DMF concentrations were measured over the incubation period under experimental conditions (Table S6). Concentration checks performed within the assays confirmed the presence of DMF (albeit at much reduced concentrations) at the end of the incubation period.

3.1.3 | Inhibitory potential of DMF and MMF for CYP enzymes

DMF and MMF were investigated for their potential as both a direct competitive inhibitor and time-dependent inhibitor of selected

CYP enzymes. This was achieved by assessing the potency of inhibition following no pre-incubation (0 minute) and a pre-incubation of 30 minutes with or without β-NADPH, prior to addition of the CYP substrate. For this study, an IC₅₀ shift of >2-fold following a 30-minute pre-incubation indicated time-dependent (and therefore possibly irreversible and mechanism-based) inhibition. No differences in inhibition between the two assays were interpreted as direct (and therefore reversible) inhibition.

DMF at concentrations up to 666 μmol/L did not directly inhibit CYP3A4/5. Following a 0-minute pre-incubation, the highest concentration of DMF demonstrated 0% inhibition. At 666 μmol/L DMF, with testosterone as a substrate, CYP3A4/5 showed 35.5% inhibition following a 30-minute pre-incubation in the presence of cofactor. In the absence of this cofactor, no inhibition was seen (Table 1). With midazolam as a substrate, 8.9% inhibition was reported (Table 1). In all scenarios tested, IC₅₀ values for DMF could not be calculated, as insufficient inhibition (<50%) was achieved. By inference, the IC₅₀ was >666 μmol/L.

Positive control inhibitor assays showed inhibition of at least 95.0% and at least 70.6% with ketoconazole and azamulin, respectively, in the presence of both testosterone and midazolam (data not shown). These data confirmed that the experimental system was capable of monitoring inhibitory activity.

MMF at concentrations up to 750 μmol/L did not inhibit any of the CYP enzymes investigated (maximum reversible inhibition was 30% of CYP2D6 and maximum time-dependent inhibition was 28% of CYP2C19) (Figure 1). In all scenarios tested, IC₅₀ values could not be calculated as insufficient inhibition (<50%) was reported. Thus, by inference, IC₅₀ values were >750 μmol/L. Positive control inhibitor data confirmed that the experimental system was capable of monitoring inhibitory activity (data not shown).

Together, these data indicate that DMF and MMF are not reversible or time-dependent inhibitors of any of the CYP enzymes examined.

3.2 | Cytotoxic potential of MMF and its ability to induce CYP enzymes in cultured human hepatocytes

3.2.1 | MMF solubility in incubation medium

On spiking the prepared MMF solution into warm, supplemented Williams' Medium E, no precipitation or insoluble material was observed. Post-centrifugation recoveries of MMF up to 750 μmol/L were ≥86.5% and therefore no solubility issues were noted.

3.2.2 | Assessment of the cytotoxic potential of MMF

No significant cytotoxic effects on human hepatocytes were observed with MMF (≤250 μmol/L) following a 72-hour incubation (AQ_{ueous} One Solution assay [Promega]). At 750 μmol/L, cell viability

TABLE 1 The effect of DMF on testosterone 6 β -hydroxylase and midazolam 1'-hydroxylase (CYP3A4/5) activities

DMF concentration (μ mol/L)	Rate of 6 β -(testosterone) or 1'-(midazolam) hydroxylation (pmol/min/mg) ^a						Inhibition of formation of 6 β -hydroxytes- tosterone or 1'-hydroxymidazolam (%) ^a		
	0 min PI		30 min PI + cofactor		30 min PI - cofactor		0 min PI	30 min PI + cofactor	30 min PI - cofactor
	Mean	SD	Mean	SD	Mean	SD	Mean	Mean	Mean
Testosterone									
0	2280	33	3250	430	1740	174	NA	NA	NA
6.66	2290	57	3180	392	2430	166	0	2.16	0
22.2	2330	78	3160	92	2360	85	0	2.80	0
66.6	2440	301	2870	90	2400	67	0	11.6	0
200	2270	60	2280	120	2140	52	0.288	29.9	0
400	2310	141	2140	224	2030	84	0	34.2	0
666	2300	92	2090	151	2200	112	0	35.5	0
Midazolam									
0	618	47	559	39	439	25	NA	NA	NA
6.66	736	57	597	18	472	54	0	0	0
22.2	691	19	536	21	528	51	0	4.18	0
66.6	689	11	565	35	439	NC	0	0	0
200	698	50	555	22	465	66	0	0.767	0
400	685	40	487	12	318	NC	0	13.0	27.7
666	696	39	510	29	A	A	0	8.93	A

Note: A, no two values within 20% deviation of mean, so all values excluded; DMF, dimethylfumarate; NA, not applicable; NC, not calculable (mean of two replicates only, third replicate outside acceptance criteria); PI, pre-incubation; SD, standard deviation.

^aAt least two (duplicate) determinations.

was reduced to 71.6%, indicating possible toxicity at this concentration (Figure 2).

3.2.3 | Assessment of CYP induction potential by MMF

No concentration-dependent induction effects were demonstrated overall in the CYP1A2 (Figure 3) and CYP2B6 (Figure 4) mRNA expression experiments. In donor 4 (EJW), at 250 μ mol/L MMF, 4.78- and 17.6-fold increases were noted for CYP1A2 and CYP2B6 mRNA, respectively. At 1 μ mol/L MMF, donor 2 (XKU) demonstrated a >2-fold increase in CYP3A4 mRNA (Figure 5), but this was not concentration dependent and the effect was not replicated in donors 3 (CDP) or 4 (EJW). Overall, the results suggest that MMF 1-250 μ mol/L does not induce CYP.

The concentrations of MMF at 0, 1, 4, and 24 hours on the last day of dosing to human hepatocytes were reduced in media over the incubation period, although levels remaining after 24 hours were still quantifiable. MMF concentrations were also slightly reduced over time in the no-cell control plate over the 72-hour incubation period, although not to the same extent as in the presence of hepatocytes (over 65% of MMF was still present after 24 hours' incubation).

3.3 | DMF and MMF as substrates and inhibitors of P-gp in vitro

3.3.1 | Solubility of DMF and MMF in incubation media

DMF solutions prepared at 19.98 and 66.6 mmol/L in PreadyPort™ buffer showed precipitate, indicating insolubility. When concentrations were reduced to 6.66 and 0.666 mmol/L, DMF appeared to be soluble. Concentrations of 0.333, 0.666, 3.33, and 6.66 mmol/L were therefore used for assessment of DMF interactions with P-gp. MMF was soluble at all concentrations up to 7.38 mmol/L.

3.3.2 | Permeability of DMF and MMF across Caco-2 cell monolayers

The permeability of DMF was concentration-dependent at all of the timepoints investigated. Moderate-to-high P_{app} was seen in both A-B and B-A directions ($P_{app} \geq 2.3-29.7 \times 10^{-6}$ cm/s). At 120 minutes, recovery of DMF was poor in the A-B direction (11.4%-22.6%) but moderate in the B-A direction (67.1%-71.5%). Poor A-B recovery may have been a consequence of nonspecific binding or degradation of DMF when in contact with the Caco-2 cell line.

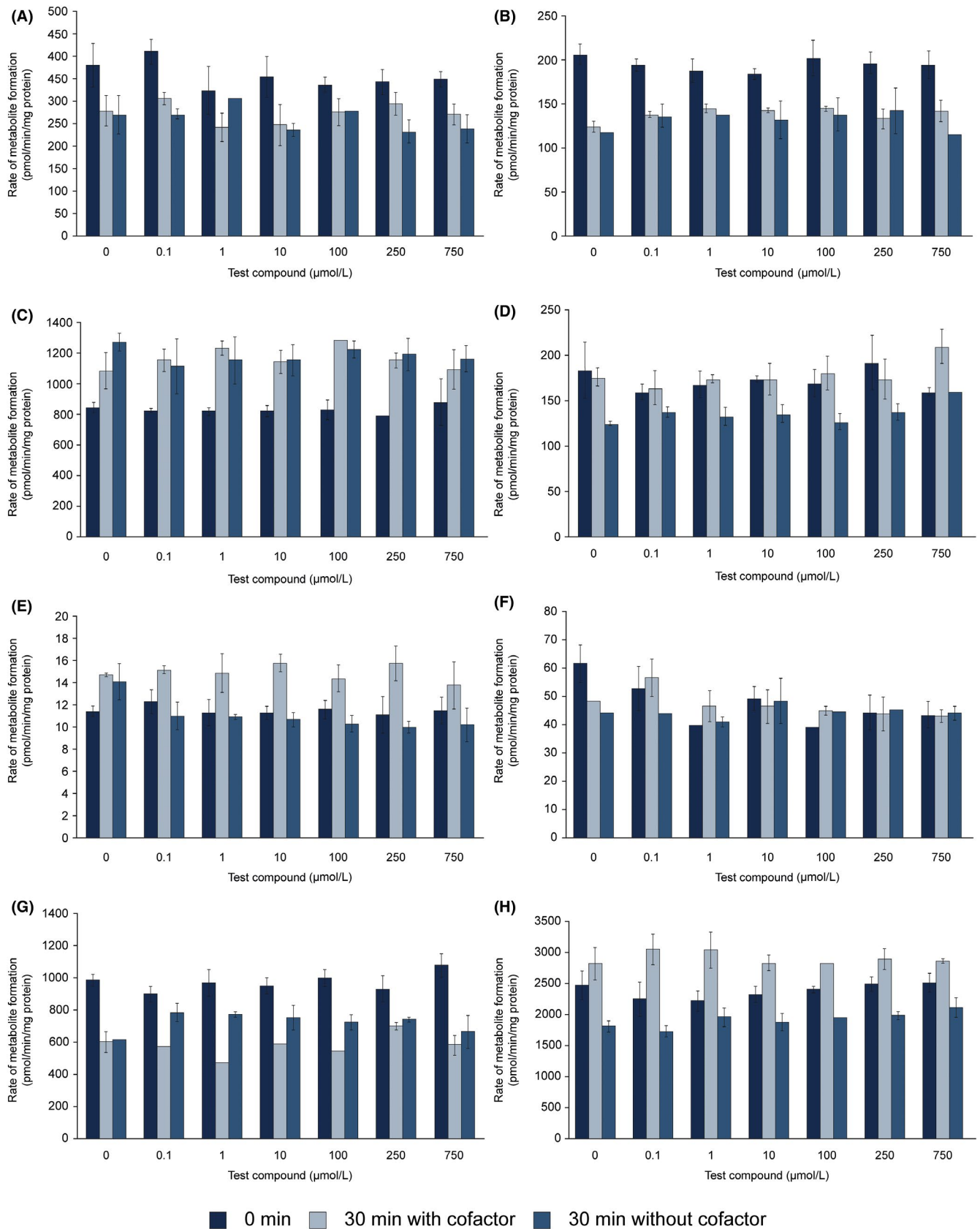
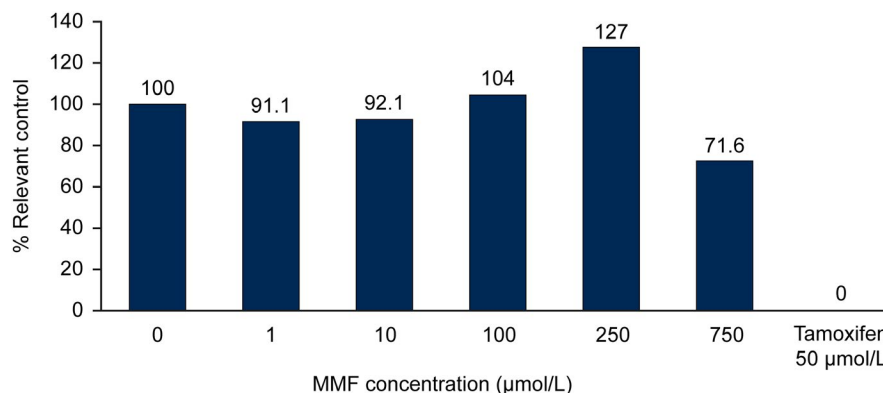


FIGURE 1 The effects of MMF on (A) phenacetin O-deethylation (CYP1A2 activity); (B) bupropion hydroxylation (CYP2B6 activity); (C) amodiaquine N-deethylation (CYP2C8 activity); (D) tolbutamide 4'-hydroxylation (CYP2C9 activity); (E) 5-mephenytoin 4'-hydroxylation (CYP2C19 activity); (F) bufuralol 1'-hydroxylation (CYP2D6 activity); (G) midazolam 1'-hydroxylation (CYP3A4/5 activity); and (H) testosterone 6 β -hydroxylation (CYP3A4/5 activity). CYP, cytochrome P450; MMF, monomethylfumarate

FIGURE 2 Assessment of the cytotoxic potential of MMF on human hepatocytes (donor 1: EBS) in culture, using the AQ_{UEOUS} One Solution Assay. Data presented as mean of triplicate results. MMF, monomethylfumurate



Permeability of MMF in the Caco-2 system was low-to-moderate ($P_{app} = 1.2\text{--}8.9 \times 10^{-6}$ cm/s for concentrations of 0.0738–0.738 mmol/L, and undetermined for 7.38 mmol/L).

3.3.3 | DMF and MMF as potential P-gp substrates

DMF and MMF were assessed as substrates of P-gp by investigating their transport across the cell barrier, through measurement of DMF and MMF in the receiver compartment over an incubation period of 120 minutes.

At all timepoints, DMF displayed high P_{app} in both the A–B and B–A directions in WT and transfected MDCKII cells (P_{app} 23.0–67.0 $\times 10^{-6}$ cm/s). Negligible differences in DMF permeability were reported between WT and transfected cell lines, with net efflux ratios <2 in MDCKII-MDR1 cells. Net efflux ratios of DMF were unchanged in the presence of verapamil (net efflux ratios ≤ 1.22), supporting the finding that DMF is not a substrate of P-gp.

Similarly, the net efflux ratio reported for MMF (at 120 minutes) was $< 2 \times 10^{-6}$ cm/s at all concentrations investigated, indicating that MMF does not act as a substrate of P-gp.

In both DMF and MMF experiments, the probe substrate [³H]digoxin showed a net efflux ratio of >16.6, which was reduced in the presence of verapamil. These data confirmed that the test systems were able to assess P-gp substrate interactions.

3.3.4 | DMF and MMF as potential P-gp inhibitors

Caco-2 cell line

Experiments in the Caco-2 cell line in the absence of DMF and MMF demonstrated notable differences in P_{app} of [³H]digoxin in the B–A and A–B directions, with efflux ratios of ≥ 5.89 . As the concentration of DMF was increased, [³H]digoxin efflux ratios decreased, suggesting that DMF was acting as a weak inhibitor of P-gp (Table 2), with an estimated IC_{50} value (relative to the 0 mmol/L solvent control) of 1.5 mmol/L (Figure 6A).

For MMF (0.0738, 0.246, and 0.738 mmol/L) in Caco-2 cells, there were no differences in P_{app} values of [³H]digoxin in the presence of MMF when compared with [³H]digoxin alone (0 mmol/L

MMF). With 7.38 mmol/L MMF; however, the P_{app} for [³H]digoxin increased approximately 9-fold in the A–B direction. This may have been a consequence of cytotoxicity (cell layer damage was shown by high Lucifer yellow transport) or inhibition. At 0.0738, 0.246, and 0.738 mmol/L MMF, [³H]digoxin efflux ratios were unchanged. At 7.38 mmol/L MMF, the efflux ratio decreased by approximately 85% (Table 2).

In the presence of the reference inhibitor, verapamil, the [³H]digoxin efflux ratio was reduced by $\geq 80\%$ versus [³H]digoxin alone at 120 minutes. These data indicate that the experimental test system was able to assess P-gp inhibitory interactions.

MDCKII cell line

DMF and MMF inhibitory potential was also assessed in the MDCKII cell line. [³H]Digoxin net efflux ratios decreased with increasing concentrations of DMF, which suggested inhibition by DMF of P-gp (Table 2), with an estimated IC_{50} value of 0.9 mmol/L (Figure 6B).

At MMF concentrations of 0.0738, 0.246, 0.738, and 7.38 mmol/L, the net efflux ratio of [³H]digoxin was effectively unchanged (Table 2). These data demonstrate that MMF was not an inhibitor of P-gp at concentrations up to 7.38 mmol/L.

In the presence of verapamil, the efflux ratios of [³H]digoxin were reduced by a minimum of 61% in MDCKII cells, indicating that the experimental test system was able to assess inhibitory interactions.

3.3.5 | Caco-2 and MDCKII cell layer integrity

TEER results demonstrated that the electrical resistance in all wells used was $>1000 \Omega \cdot \text{cm}^2$ per well for Caco-2 cells and $>83 \Omega \cdot \text{cm}^2$ per well for MDCKII cells. This indicated the presence of intact monolayers and confirmed that the Caco-2 and MDCKII cell monolayers used were suitable for use in drug transport studies with DMF and MMF.

Permeability marker compounds were assessed for permeability across the cell monolayers. For atenolol, all P_{app} values met the acceptance criterion of $< 2 \times 10^{-6}$ cm/s, which indicated that atenolol behaved as a low permeability compound. For propranolol, all P_{app} values were $> 10 \times 10^{-6}$ cm/s, thus meeting the acceptance criterion and indicating that propranolol behaved as a medium to high permeability compound.

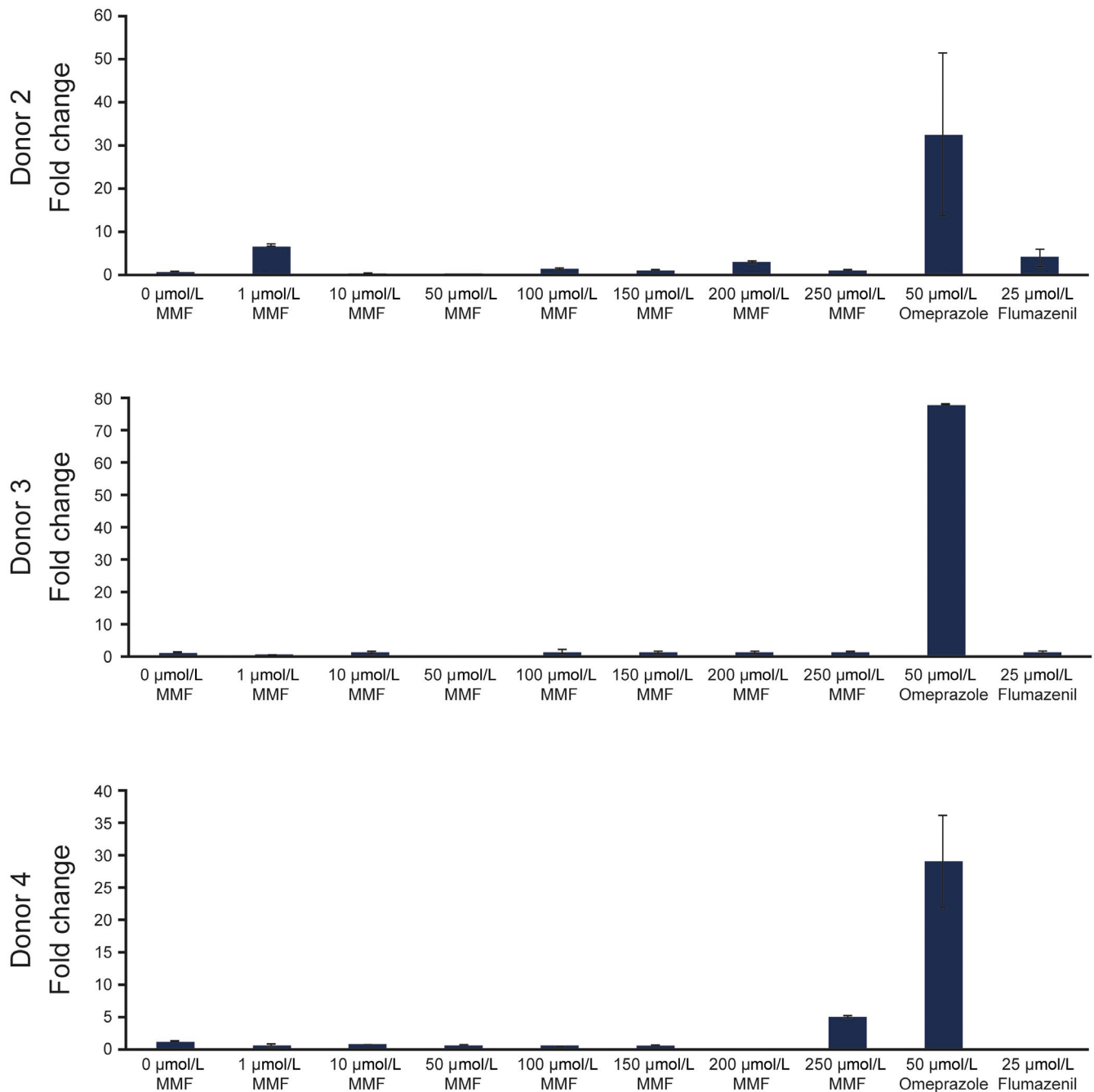


FIGURE 3 Assessment of the potential for MMF to induce human CYP1A2 mRNA levels in donors 2 (XKU), 3 (CDP), and 4 (EJW) (omeprazole and flumazenil also shown). Data presented as mean of 2 or 3 replicates with standard deviation. CYP, cytochrome P450; MMF, monomethylfumarate; mRNA, messenger ribonucleic acid

4 | DISCUSSION

Although DMF has been in use for some decades as part of a mixture of FAE, the introduction of DMF as a monotherapy and its demonstration of noninferiority to the long-established FAE mixture³ have generated renewed interest in this mode of treatment.^{14,15} The work presented here was prompted by regulatory requirements. For transparency, these data should be made publicly available because the relevant data points on the potential effect of DMF and its major

metabolite on CYP enzymes (with accompanying implications for DDIs) and the kinetics of drug transport^{10-12,16,17} are not currently included in available summaries of product characteristics.^{2,13} Since the metabolism of DMF does not involve the CYP system, the likelihood that DMF has DDIs is low.¹⁸ In line with this, no safety issues involving a potential DDI have been identified in clinical trials or reported during postmarketing surveillance (up to June 2019).

DMF did not directly inhibit CYP3A4/5 activity in HLM experiments, as confirmed by 0% inhibition at the highest (supratherapeutic)

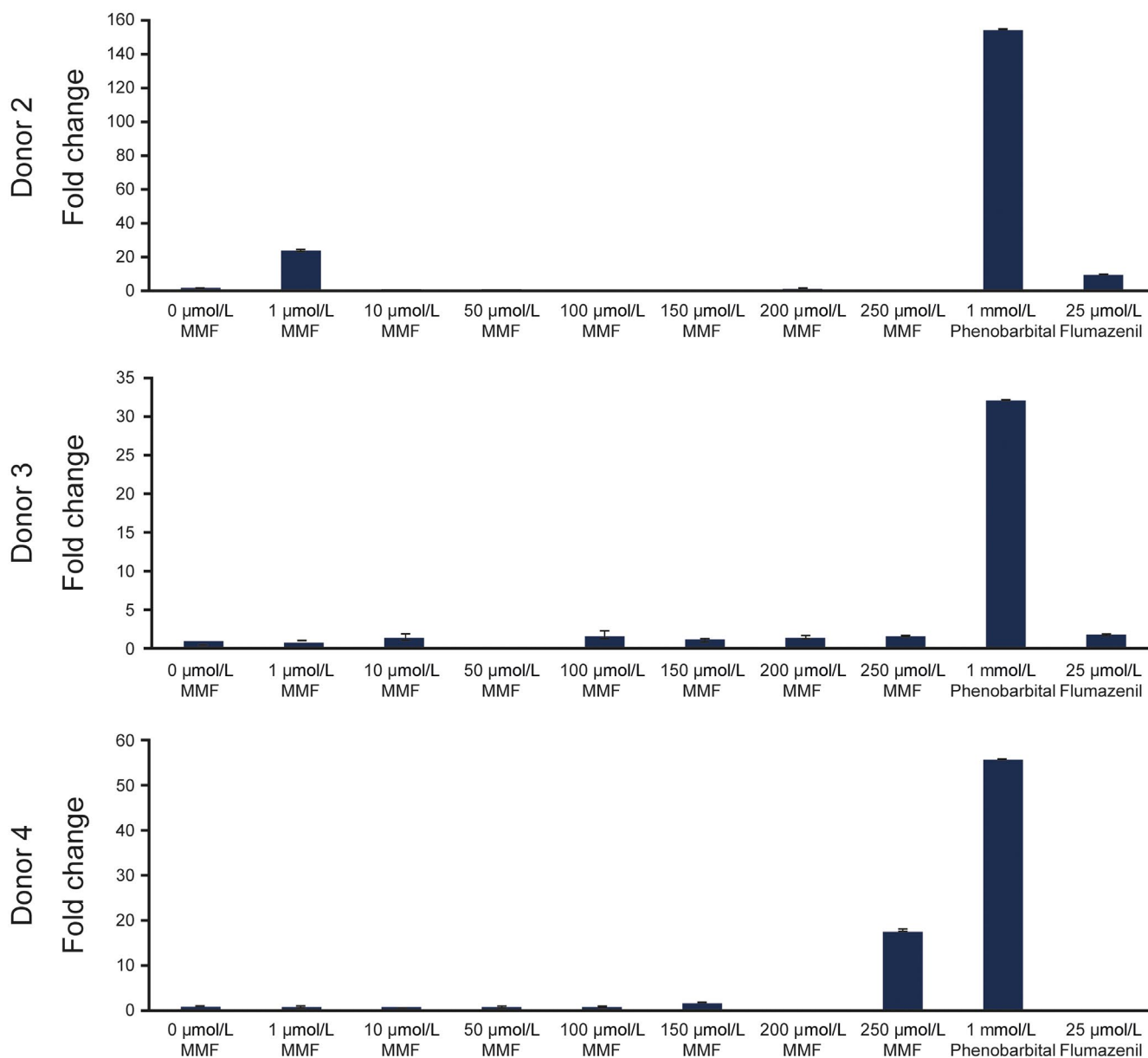


FIGURE 4 Assessment of the potential for MMF to induce human CYP2B6 mRNA levels in donors 2 (XKU), 3 (CDP), and 4 (EJW) (phenobarbital and flumazenil also shown). Data presented as mean of 2 or 3 replicates with standard deviation. CYP, cytochrome P450; MMF, monomethylfumurate; mRNA, messenger ribonucleic acid

concentration of DMF (666 µmol/L). Additionally, insufficient inhibition following a 30-minute pre-incubation with cofactor meant that IC_{50} values could not be determined (>666 µmol/L) and suggested that DMF is also unlikely to be a time-dependent inhibitor of CYP3A4/5 within the therapeutic concentration range. Similarly, considering the high IC_{50} value for MMF reported in this study (>750 µmol/L), should time-dependent inhibition occur, it would only be relevant at supratherapeutic concentrations that would never be reached in clinical practice. These data are in contrast with ciclosporin, another systemic psoriasis agent that is a known CYP3A4 inhibitor.²

Hepatocyte viability data confirmed the validity of the conclusions drawn from CYP induction experiments with MMF. There was

no evidence of cytotoxic effects on human hepatocytes following incubation with MMF up to a concentration of 250 µmol/L. No concentration-dependent increases exceeding 2-fold in CYP1A2 and CYP2B6 mRNA levels were reported with MMF at concentrations of up to 200 µmol/L. At 250 µmol/L, a 4.78-fold increase of CYP1A2 and a 17.6-fold increase of CYP2B6 mRNA expression were seen in one of the donors. However, no concentration-dependent increases above 2-fold in CYP3A4 mRNA levels were reported with MMF at concentrations up to 250 µmol/L. As 250 µmol/L is >22 times higher than the clinically relevant maximum plasma concentration (C_{max}) of MMF (11.2 µmol/L for a 240 mg dose),¹⁹ the present results suggest that, should induction of CYP enzymes occur, it will only take place to any relevant extent at concentrations that are never reached in

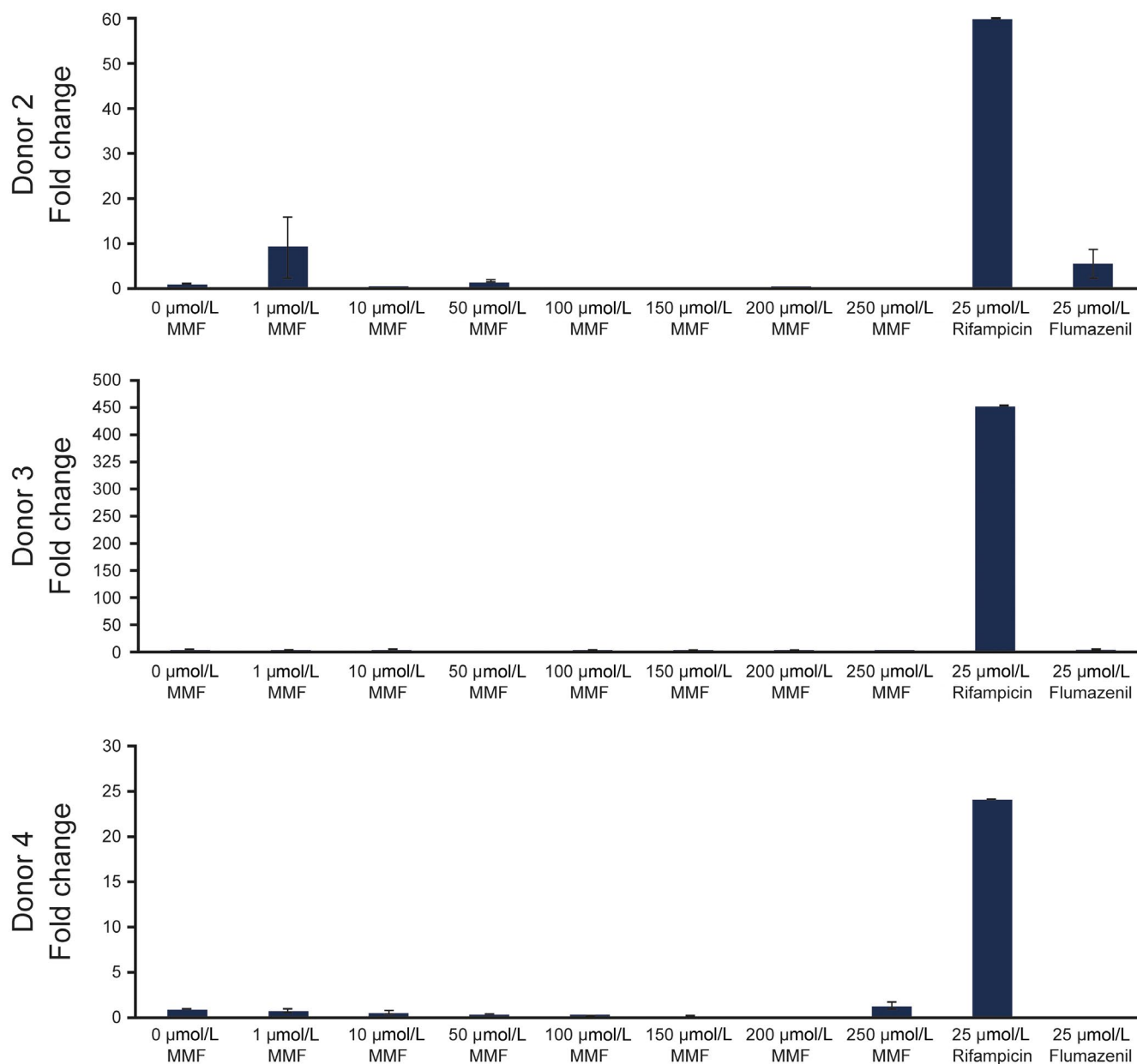


FIGURE 5 Assessment of the potential for MMF to induce human CYP3A4 mRNA levels in donors 2 (XKU), 3 (CDP), and 4 (EJW) (rifampicin and flumazenil also shown). Data presented as mean of 2 or 3 replicates with standard deviation. CYP, cytochrome P450; MMF, monomethylfumurate; mRNA, messenger ribonucleic acid

clinical practice. MMF is therefore not expected to alter the disposition of drugs that are metabolized by CYP1A2, CYP2B6, and CYP3A4 in the clinical setting.

The reductions in concentration of MMF in media noted over the final 24-hour dosing interval could have been due to hepatocyte uptake, possibly coupled to metabolism. The slight reductions in MMF concentrations noted in no-cell control plates may indicate nonspecific binding of MMF to the culture plates used in these experiments. Despite these reductions in concentration, levels were still quantifiable.

Although not a substrate for P-gp (see later), DMF is likely to be a weak inhibitor of P-gp, because efflux and net efflux ratios of [^3H]digoxin decreased with increasing DMF concentrations, with

estimated IC_{50} values of 1.5 mmol/L in Caco-2 cells and 0.9 mmol/L in transfected MDCKII-MDR1 cells. This effect should not have any clinical relevance because DMF is hydrolyzed very rapidly in vivo:¹⁹ the hydrolysis half-life of DMF in the intestine is less than 2 minutes due to the pH conditions and presence of gut esterases.²⁰ These findings are similar to those of apremilast and ciclosporin, other systemic psoriasis agents that have also been shown to be weak inhibitors of P-gp.^{2,21} In addition, in vitro experiments carried out to predict the P-gp component of DDIs suggest that CYP interactions are likely to have greater clinical relevance.²² The impact of DDIs mediated solely by P-gp inhibition would not be remarkably high if P-gp inhibition occurs in isolation and without accompanying inhibitory effects on CYP.²³

TABLE 2 Assessment of DMF and MMF as potential inhibitors of P-gp following a 120-minute incubation

DMF concentration	A-B ^a	B-A ^a	Efflux ratio ^a	% Control relative to 0 mmol/L DMF
Caco-2 cells				
0 mmol/L	0.562	4.16	7.40	100
0.333 mmol/L	0.720	4.75	6.60	89.2
0.666 mmol/L	0.739	4.31	5.83	78.8
3.33 mmol/L	1.16	2.87	2.47	33.4
6.66 mmol/L	1.76	2.85	1.62	21.9
MMF concentration	A-B ^a	B-A ^a	Efflux ratio ^a	% Control relative to 0 mmol/L MMF
Caco-2 cells				
0 mmol/L	0.971	5.72	5.89	100
0.0738 mmol/L	1.20	5.43	4.53	76.9
0.246 mmol/L	1.12	5.06	4.52	76.7
0.738 mmol/L	0.998	5.87	5.88	99.8
7.38 mmol/L	9.08	7.75	0.854	14.5
DMF concentration	Efflux ratio: WT ^a	Efflux ratio: P-gp ^a	Net efflux ratio ^b	% Control relative to 0 mmol/L DMF
MDCKII cells				
0 mmol/L	2.30	16.2	7.04	100
0.333 mmol/L	1.89	9.42	4.98	70.7
0.666 mmol/L	1.67	7.67	4.59	65.2
3.33 mmol/L	1.32	1.11	0.841	11.9
6.66 mmol/L	0.764	0.623	0.815	11.6
MMF concentration	Efflux ratio: WT ^a	Efflux ratio: P-gp ^a	Net efflux ratio ^b	% Control relative to 0 mmol/L MMF
MDCKII cells				
0 mmol/L	2.22	22.5	10.1	100
0.0738 mmol/L	NC	19.3	NC	NC
0.246 mmol/L	3.28	>23.0	>7.01	>69.4
0.738 mmol/L	2.93	>26.1	>8.91	>88.2
7.38 mmol/L	1.20	>16.7	>13.9	>138

> values reported as *greater than* because concentration in receiver compartment was below the limit of detection and therefore below the limit of detection used to calculate P_{app} . A, apical; B, basolateral; NC, data not calculable.

^a[³H]Digoxin was used as the probe substrate. A-B and B-A data expressed as permeability coefficient P_{app} ($\times 10^{-6}$ cm/s) for [³H]digoxin.

Efflux ratio = $(P_{app}(B-A)/P_{app}(A-B))$.

^bNet efflux ratio = efflux ratio in transfected cells/efflux ratio in wild-type cells.

In contrast, MMF is neither a substrate nor an inhibitor of P-gp (at concentrations of up to 7.38 mmol/L). Considering that the maximum concentration tested (7.38 mmol/L) was equivalent to the concentration in the intestinal lumen following administration of the maximum clinically relevant DMF dose (240 mg per 250 mL), and was much greater than the clinically relevant MMF C_{max} of 11.2 μ mol/L,¹⁹ the absorption and distribution of co-administered P-gp substrate drugs is not expected to be affected by MMF.

The permeability findings indicate that DMF is expected to undergo moderate-to-high absorption across the gastrointestinal tract in humans, as shown by the P_{app} A-B transport values noted in Caco-2

cells. P_{app} results also indicated that MMF is likely to undergo very low absorption in humans. After 120 minutes, P_{app} values for A-B transport were in accordance with low permeability in vivo (ie $P_{app} < 2 \times 10^{-6}$ cm/s).

Neither DMF nor MMF is a substrate of P-gp, as shown by negligible differences in DMF permeability between WT and transfected MDCKII-MDR1 cell lines. The net efflux ratio for [³H]digoxin was $> 2 \times 10^{-6}$ cm/s. This decreased in the presence of verapamil. It is noted also that atenolol and propranolol were found to be suitable and predictable controls for the study of drug permeability and active transport in the Caco-2 and MDCKII cell monolayers.

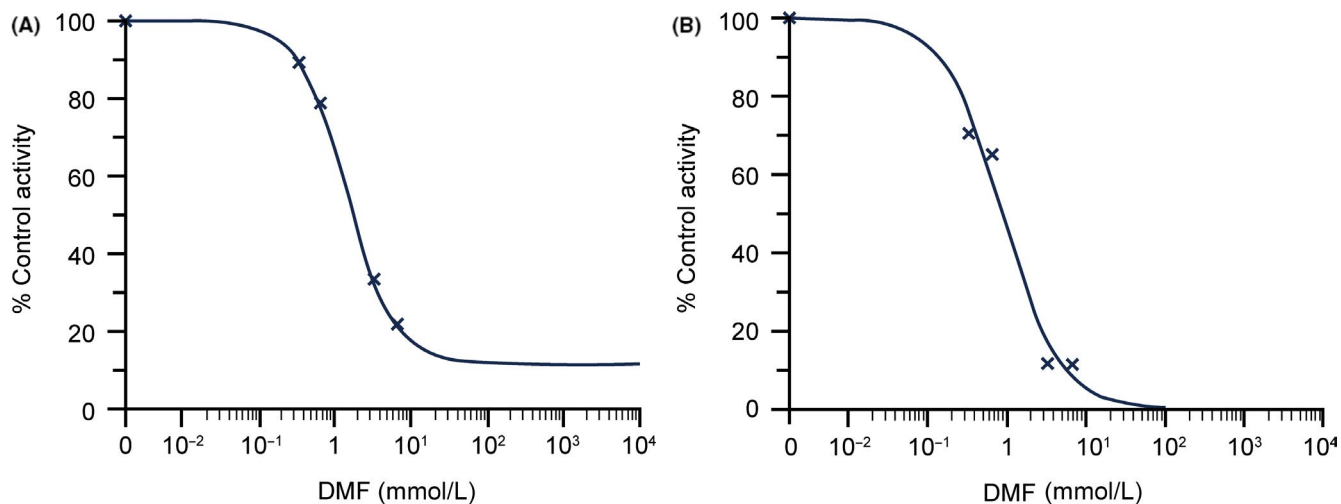


FIGURE 6 Inhibitory effects of DMF on P-gp using (A) the CacoReady™ Caco-2 cell monolayer and (B) the PreedyPort™ P-gp transfected MDCKII monolayer. DMF, dimethylfumarate; P-gp, P-glycoprotein; MDCKII, Madin-Darby Canine Kidney

In conclusion, this study demonstrates that neither DMF nor MMF is a direct inhibitor of any of the CYP enzymes investigated, nor are they likely to induce CYP at clinically relevant concentrations. They are therefore not predicted to alter the disposition of other compounds metabolized by CYP enzymes. DMF is not a substrate for P-gp within the therapeutic concentration range but may be a weak inhibitor at supratherapeutic concentrations unlikely to have any clinical relevance given the high IC₅₀ for DMF (in the millimolar range). MMF was neither a substrate nor an inhibitor of P-gp. DMF is expected to demonstrate moderate-to-high absorption across the gastrointestinal tract, while MMF is expected to demonstrate low permeability. Results show no evidence for MMF interaction with CYP enzymes and the clinically important P-gp transporter. Therefore, no interactions are expected with medicinal products metabolized or transported by these systems.

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DISCLOSURE

Jordi Aubets, Josep-Maria Jansat, and Miquel Salva are employees of Almirall S.A. Barcelona, Spain. Vicky M. Birks, Richard J. Cole, Michael Hall, Jenny Lewis, and Annabell Pitcher declared no conflicts of interest.

AUTHOR CONTRIBUTIONS

Participated in research design: Jordi Aubets, Josep-Maria Jansat, Miquel Salva, Michael Hall. *Conducted experiments:* Vicky M. Birks, Annabell Pitcher, Jenny Lewis. *Performed data analysis:* Jordi Aubets, Josep-Maria Jansat, Miquel Salva, Vicky M. Birks, Richard J. Cole, Jenny Lewis, Annabell Pitcher. *Wrote or contributed to the writing of the manuscript:* Jordi Aubets, Josep-Maria Jansat, Miquel Salva,

Vicky M. Birks, Richard J. Cole, Jenny Lewis, Annabell Pitcher, Michael Hall.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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