Dimethyl Fumarate Reduces Inflammatory Responses in Experimental Colitis

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Abstract

Background and Aims: Fumaric acid esters have been proven to be effective for the systemic treatment of psoriasis and multiple sclerosis. We aimed to develop a new treatment for colitis.

Methods: We investigated the effect of dimethylfumarate [DMF, 10-30-100 mg/kg] on an experimental model of colitis induced by dinitrobenzene sulphuric acid [DNBS]. We also evaluated the therapeutic activity of 7 weeks’ treatment with DMF [30 mg/kg] on 9-week-old IL-10KO mice that spontaneously develop a T helper-1 [Th1]-dependent chronic enterocolitis after birth, that is fully established at 8–10 weeks of age. The mechanism of this pharmacological potential of DMF [10 μM] was investigated in colonic epithelial cell monolayers [Caco-2] exposed to H₂O₂. The barrier function was evaluated by the tight junction proteins.

Results: The treatment with DMF significantly reduced the degree of haemorrhagic diarrhoea and weight loss caused by administration of DNBS. DMF [30 and 100 mg/kg] also caused a substantial reduction in the degree of colon injury, in the rise in myeloperoxidase [MPO] activity, and in the increase in tumour necrosis factor [TNF]-α expression, as well as in the up-regulation of ICAM-1 caused by DNBS in the colon. Molecular studies demonstrated that DMF impaired NF-κB signalling via reduced p65 nuclear translocalisation. DMF induced a stronger antioxidant response as evidenced by a higher expression of Mn-superoxide dismutase. Moreover, DMF protected human intestinal epithelial cells against H₂O₂-induced barrier dysfunction, restoring ZO-1 occludin expression, via the HO-1 pathway.

Conclusions: DMF treatment reduces the degree of colitis caused by DNBS. We propose that DMF treatment may be useful in the treatment of inflammatory bowel disease.

Keywords: Inflammatory bowel diseases; DNBS; dimethyl fumarate

1. Introduction

Crohn’s disease [CD] and ulcerative colitis [UC] collectively referred to as inflammatory bowel disease [IBD], are relatively common diseases of gastrointestinal tract. IBDs are characterised by dysfunction of mucosal immune response, abnormal cytokines production with increase in tumour necrosis factor [TNF]-α, and interleukin [IL]-1β augmentation in adhesion molecules expression and cell infiltrate, that ultimately lead to epithelial cell apoptosis and mucosal damage.1 Although the aetiology of IBD remains unknown, there is circumstantial evidence supporting a central role for disregulation of mucosal CD4⁺ T helper-1 [Th1] effector cell responses to the normal enteric bacterial flora, as a common disease mechanism.2

Several animal models of IBD have been developed. Among these, the model of colonic inflammation induced by dinitrobenzene...
sulphuric acid [DNBS] delivered intrarectally to normal mice displays human CD-like features, notably predominant NF-κB p65-dependent Th1 activation.¹

Conventional therapy uses anti-inflammatory and immunosuppressive corticosteroids to treat acute-phase symptoms. However, the low remission rate and severe side effects of these therapies are not satisfactory.² Thus, there is urgent medical need for new therapeutic strategies. Recent articles have shown the many beneficial actions resulting from the use of fumaric acid esters [FAEs] in moderate to severe psoriasis.³ The efficacy of FAEs in psoriasis may therefore be mediated by immunomodulatory mechanisms, which might also play a role in the treatment of other inflammatory diseases.

Several anti-inflammatory effects of FAEs have been suggested, including suppression of adhesion molecules and inhibition of cytokine production.⁴ The most pharmacologically effective molecule among the FAEs is dimethyl fumarate [DMF]. DMF is a new orally available disease-modifying agent that was recently approved by the US Food and Drug Administration [FDA] and the European Medicines Agency [EMA] for the management of relapsing forms of multiple sclerosis.⁵ Data from human and animal studies suggest that this compound has both anti-inflammatory and anti-oxidant properties.⁶ In addition, it has been demonstrated the neuroprotection and immunomodulation mediate by [Nrf]-2 of DMF⁷.

The principal current hypothesis for the pharmacodynamic effect of DMF is based on the concept that it influences pro-inflammatory signal transduction pathways by modulating the intracellular redox system.⁸ There is evidence that changes in this system contribute to a decreased translocation of NF-κB, leading to an inhibition of the expression of pro-inflammatory cytokines including TNF-α and interleukin [IL]-1β. In addition, because inhibition of NF-κB is expected to inhibit tumours which are dependent on NF-κB activity, such as multiple myeloma and acute myeloid leukemia [AML],⁹ it follows that DMF causes tumour cells death; indeed, it has been shown that DMF reduced metastasis in a mouse model of melanoma,¹⁰ in HL-60 AML cells and in other cell types.¹¹ DMF directly sustained endothelial tight junctions, inhibited inflammatory cytokine expression, and attenuated leukocyte transmigration. It is also known that DMF protects endothelial cells from TNFα-induced apoptosis.¹²

Here we investigate the effects of DMF on the inflammatory response and colon injury caused by intra-colonic administration of DNBS. To demonstrate that DMF exerts this beneficial therapeutic effect by interfering with neutrophilic infiltration and release of

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**Figure 1.** Effects of DMF treatment on clinical expression of DNBS-induced colitis, body weight, and macroscopic damage score changes. Colon tissues from sham-treated mice, colon tissues from DNBS-treated mice at 4 days post DNBS administration, colon tissues collected from DNBS-treated mice which have received DMF at 10, 30, and 100 mg/kg treatment [A]. The macroscopic damage score [C] was made by two independent observers. A significant reduction in body weight increase [B] was observed 4 days after DNBS administration. Treatment with DMF at 100 mg/kg significantly reduced the increase in body weight loss [A] and in macroscopic damage score [C]. Data are means ± SEM (standard error of the mean) of six mice for each group. *p < 0.01 vs sham; #p < 0.01 vs DNBS.

DMF, dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; MPO, myeloperoxidase; TNF, tumour necrosis factor; SD, standard deviation; ND: not detectable.
pro-inflammatory mediators [e.g., reactive oxygen species, TNF-α] we studied the effects of DMF on: [i] the degree of colonic injury after DNBS administration; [ii] the rise in myeloperoxidase [MPO] activity [mucosa]; [iii] the production and the expression of TNF-α; [iv] the rise of oxidative stress as well as the increased expression of ICAM-1 and decreased expression of MnSOD; [v] the expression of MMP-9 and -2 caused by DNBS in the colon; [vi] the NF-κB nuclear translocation and IκB-α degradation; [vii] Nrf-2 involvement; and [viii] barrier dysfunction in human intestinal epithelial cells.

2. Materials and Methods

2.1. Cell culture

The human intestinal colorectal adenocarcinoma cell line, Caco2, was cultured in Dulbecco’s modified Eagle’s medium. Culture media contained 10% fetal bovine serum [FBS], 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids. Cells were cultured at 37°C in a humidified 5% CO₂ and 95% filtered air, and the culture medium was replaced every 2 days. After washing in phosphate-buffered saline [PBS], cells were trypsinised with 0.25% trypsin-EDTA at 37°C for 5 min, centrifuged at 1 000 ×g for 3 min and then re-suspended in the appropriate medium. Cell viability was determined by trypan blue staining. The concentration of DMF used in this study was chosen on the basis of viability testing performed in our laboratory [data not shown] and on the basis of recent literature data.

2.2. Animals

IL-10KO mice and corresponding wild-type [WT] controls were purchased from Jackson Laboratories [Charles River, Italy]. Male adult CD1 mice [25–30 g, Harlan, Milan, Italy] were housed in a controlled environment and provided with standard rodent chow and water. Mice were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light, 12-h dark cycle. The animals were acclimatised to their environment for 2 weeks under standard conditions, with free access to tapwater and standard rodent diet. The study was approved by the University of Messina Review Board for the care of animals. All animal experiments were carried out in the accordance with the national law on animal protection [D.M. 116192], Europe [O.J. of E.C. L 358/1 12/18/1986] and USA [Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA].

2.3. Experimental groups

CD1 mice were randomly divided into the following groups [n = 6 for each group].

Figure 2. Effects of DMF treatment on colon injury and histological score. No histological or macroscopic alterations were observed in the colon tissue from sham-treated mice [A, F]. Mucosal injury was produced after DNBS administration, characterised by absence of epithelium and a massive mucosal and submucosal infiltration of inflammatory cells [B, F]. The histopathological features of colon tissues from DNBS-injected mice at 4 days post DNBS administration showed severe signs of colon injury [B, F]. Treatment with DMF reduced the severity of colon inflammation with statistically significant effects at the dose of 100 mg/kg [E, F] compared with the lower doses of 10 and 30 mg/kg [C, D, and F]. Data are means ± SD of 6 mice for each group. *p < 0.01 vs SHAM; #p < 0.01 vs DNBS. DMF, dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; SD, standard deviation; ND: not detectable.
1. Sham + vehicle group: vehicle solution [saline] was administered by oral gavage for 4 days.
2. Sham + dimethylfumarate [10 mg/kg]: DMF [10 mg/kg] was administered by oral gavage for 4 days.
3. Sham + dimethylfumarate [30 mg/kg]: DMF [30 mg/kg] was administered by oral gavage for 4 days.
4. Sham + dimethylfumarate [100 mg/kg]: DMF [100 mg/kg] was administered by oral gavage for 4 days.
5. DNBS + vehicle: vehicle [saline] was administered by oral gavage for 4 days starting from 3 h after the administration of DNBS.
6. DNBS + DMF [10 mg/kg]: DMF was administered by oral gavage at 10 mg/kg every 24 h, starting from 3 h after the administration of DNBS.
7. DNBS + DMF [30 mg/kg]: DMF was administered by oral gavage at 30 mg/kg every 24 h, starting from 3 h after the administration of DNBS.
8. DNBS + DMF [100 mg/kg]: DMF was administered by oral gavage at 100 mg/kg every 24 h, starting from 3 h after the administration of DNBS.

The doses of DMF [10, 30, and 100 mg/kg] used here were based on previous dose-response and time-course studies by our laboratory.

Moreover, as therapeutic approach, we evaluated the therapeutic activity of DMF [30 mg/kg] for 7 weeks on 9-week-old IL-10KO mice. IL-10KO mice spontaneously develop a Th1-dependent chronic enterocolitis shortly after birth, that is fully established at 8–10 weeks of age.2

2.4. Induction of experimental colitis

Colitis was induced by intrarectal administration with a very low dose of DNBS [4 mg per mouse] by using a modification of the method first described in rats.16 In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anaesthetized by Enflurane. 2, 4, 6-dinitrobenzene sulphonic acid (DNBS: 4 mg in 100 μl of 50% ethanol) was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Vehicle alone [100 μl of 50% ethanol] was administered in control experiments [sham]. Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. After colitis and sham-colitis induction, the animals were observed for 4 days.

At the end of the experimental period, animals were weighed, killed, and the colon was removed, opened along the anti-mesenteric border, rinsed, weighed, and processed for histology, immunohistochemistry, cell separation, and western blot analysis. Colon damage [macroscopic damage score] was evaluated and scored by two independent pathologists as described previously,17 according to the following criteria: 0, no damage; 1, localised hyperaemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of ulcers with inflammation at one site; 5, two or more major sites of ulcers with inflammation at several sites; 6, confluent inflammation throughout the colon.

dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; MPO, myeloperoxidase; TNF, tumour necrosis factor; SD, standard deviation;

Figure 3. Effects of DMF treatment in IL-10KO 16-week old mice. Effect of DMF administration on colonic MPO activity [D], macroscopic damage score [E], and colonic TNF-α levels [F]. Treatment with DMF at 100 mg/kg significantly reduced macroscopic damage score in IL-10KO 16-week-old mice [C and E] compared with vehicle [B and E]. MPO activity was significantly increased in IL-10KO 16-week-old DNBS-treated mice in comparison with IL-10WT 16-week-old mice [D]; treatment with DMF at 100 mg/kg significantly reduced the colonic MPO activity in IL-10KO 16-week-old mice. Colonic TNF-α levels were increased after DNBS injection in IL-10KO 16-week-old mice compared with IL-10WT 16-week-old mice [F]; treatment with DMF at 100 mg/kg significantly reduced the colonic TNF-α levels in IL-10KO 16-week-old mice [F]. Data are means ± SD of six mice for each group. *p < 0.05 vs IL-10WT 16-weekold mice; #p < 0.01 vs IL-10KO 16-week-old mice + vehicle. DMF, dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; MPO, myeloperoxidase; TNF, tumour necrosis factor; SD, standard deviation;
inflammation and ulceration extending more than 1 cm along the length of the colon; and 5–8, one point is added for each centimetre of ulceration beyond an initial 2 cm.

2.5. Histological examination
After fixation at room temperature in buffered formaldehyde solution [10% in phosphate-buffered saline], samples were dehydrated in graded ethanol and embedded in Paraplast [Sherwood Medical, Mahwah, NJ]. Thereafter, 7-μm sections were deparaffinised with xylene, stained with haematoxylin-eosin, observed with a AxioStar Plus equipped with AxioCamMRc [Zeiss, Milan, Italy], and studied using an Imaging computer program [AxioVision, Zeiss, Milan, Italy]. The following morphological criteria were considered as reported by Cannarile et al.: score 0, no damage; score 1 [mild], focal epithelial oedema and necrosis; score 2 [moderate], diffuse swelling and necrosis of the villi; score 3 [severe], necrosis with presence of neutrophil infiltrate in the submucosa; and score 4 [very severe], widespread necrosis with massive neutrophil infiltrate and haemorrhage. Colon damage [sections n = 6 for each animal] was scored by two independent pathologists blinded to the experimental protocol.

2.6. Myeloperoxidase activity
At 4 days after intracolonic injection of DNBS, the colon was removed and weighed. The colon was analysed for myeloperoxidase [MPO] activity, an indicator of polymorphonuclear leukocyte accumulation, using a spectrophotometric assay with tetramethylbenzidine as substrate, according to a method previously described. Each piece of tissue was homogenised in a solution containing 0.5% hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer pH 7, and centrifuged for 30 min at 20,000 xg at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H$_2$O$_2$. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37°C and was expressed in U/g wet tissue.

2.7. Thiobarbituric acid-reactant substances measurement
As a marker of lipid peroxidation, thiobarbituric acid-reactant substances measurement was determined, as previously described, in the colon tissue at 4 days after DNBS administration. Thiobarbituric acid-reactant substances were calculated by comparison with OD532 of

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**Figure 4.** Effects of DMF treatment on lipid peroxidation. A significant increase in thiobarbituric acid-reactant substances [TBARs] [A] were observed in the colon tissues collected at 4 days after DNBS administration when compared with sham-treated mice. TBARs were significantly attenuated by DMF treatment [A]. In addition, Mn-SOD and Nrf-2 expression was measured by western blot analysis. Mn-SOD and Nrf-2 expression was reduced in colon tissue from DNBS-injected mice compared with sham groups [B and C]. Treatment with DMF [100 mg/kg] demonstrated increased Mn-SOD and Nrf-2 levels [B and C]. The relative expression of the protein bands was standardised for densitometry analysis to β-actin levels. Data are means ± SD of six mice for each group. *p < 0.01 vs SHAM; #p < 0.01 vs DNBS. DMF, dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; SD, standard deviation.
standard solutions of 1, 1, 3, 3-tetramethoxypropan 99% malondialdehyde bis [dymelacetal] 99% [MDA] [Sigma, Milan]. The absorbance of the supernatant was measured by spectrophotometer at 532 nm.

2.8. Western blot analysis

Western blot analysis for manganese superoxide dismutase [Mn-SOD], IxB-α, NF-xB, Nrf-2, IL-1β, TNF-α, MMP-9, MMP-2, ZO-1, occludin, and HO-1. Tissue or cellular samples from the terminal colon were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1 000 x g for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in Buffer B containing 1% Triton X-100, 150 mMNaCl, 10 mMTris–HCl pH 7.4, 1 mM EDTA, 0.2 mM PMSF, 20 μM leupeptin, 0.2 mM sodium orthovanadate.

After centrifugation for 30 min at 15,000 g at 4 °C, the supernatants containing the nuclear protein were stored at −80 °C for further analysis. The expression of Mn-SOD, ZO-1, HO-1, occludin, IxB-α,IL-1β, TNF-α, MMP-9, and MMP-2, was quantified in cytosolic fraction from colon tissues collected 4 days after DNBS-induced colitis. IxBβ and Nrf-2 expression were quantified in nuclear fraction from colon tissues collected 4 days after DNBS-induced colitis. The filters were blocked with 1 x PBS, 5% [w/v] non-fat dried milk [PM] for 40 min at room temperature and subsequently probed with specific Abs anti-MnSOD [1:500; Millipore] or anti-HO-1 [1:1 000; StressGen Biotech], or anti-ZO-1 [1: 1 000; Cell Signaling], or anti-occludin [1:500; ThermoFisher], or anti-IxB-α [1: 1 000; Santa Cruz Biotechnology], or anti-IL-1β [1:500; Santa Cruz Biotechnology], or anti-NF-MMP-9 [1:500; Millipore], or anti-MMP-2 [1:500; Millipore], or anti-NF-xB [1:1 000; Santa Cruz Biotechnology], or Nrf-2 [1:500; Santa Cruz Biotechnology], or anti-TNF-α [1:250; Abcam], in 1 x PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 [PMT] at 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G [IgG] secondary antibody or peroxidase-conjugated goat anti-rabbit IgG [1:2 000, Jackson ImmunoResearch, West Grove, PA] for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteins, they were also incubated in the presence of the antibody against β-actin protein [cytosolic fraction 1:1 000 Sigma–Aldrich] or lamin A/C [nuclear fraction 1:500 Sigma–Aldrich]. Signals were detected with enhanced chemiluminescence [ECL] detection system reagent according to the manufacturer’s instructions [SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA]. The relative expression of the protein bands of Mn-SOD [~ 24 kDa], HO-1 [~ 32 kDa], ZO-1 [~ 220 kDa], occludin [~ 65 kDa], IxB-α [~ 37 kDa], NF-xB p65 [~ 65 kDa], IL-1β [~ 31 kDa], TNF-α [~ 17kDa], MMP-9 [~ 92 kDa], MMP-2 [~ 72 kDa], and Nrf-2 [~ 61kDa] was quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer [GS-700, Bio-Rad Analyst, IBM] and a computer program [Molecular Analyst, IBM], and standardised for densitometric analysis to β-actin or lamin A/C levels. A preparation of commercially available molecular weight markers [Precision Plus Protein Standard, Bio-Rad, Hercules, CA, USA], consisting of proteins of molecular weight 10–250kDa, was used to define molecular weight positions and as reference concentrations for each molecular weight.

2.9. Immunohistochemicallocalizationof ICAM-1, P-Selectin and TNF-α

At 4 days after DNBS administration, colon tissues were fixed in 10% [w/v] PBS-buffered formaldehyde and 7-μm sections were stained with enhanced chemiluminescence [ECL] detection system reagent according to the manufacturer’s instructions [SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA]. The relative expression of the protein bands of Mn-SOD [~ 24 kDa], HO-1 [~ 32 kDa], ZO-1 [~ 220 kDa], occludin [~ 65 kDa], IxB-α [~ 37 kDa], NF-xB p65 [~ 65 kDa], IL-1β [~ 31 kDa], TNF-α [~ 17kDa], MMP-9 [~ 92 kDa], MMP-2 [~ 72 kDa], and Nrf-2 [~ 61kDa] was quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer [GS-700, Bio-Rad Laboratories, Milan, Italy] and a computer program [Molecular Analyst, IBM], and standardised for densitometric analysis to β-actin or lamin A/C levels. A preparation of commercially available molecular weight markers [Precision Plus Protein Standard, Bio-Rad, Hercules, CA, USA], consisting of proteins of molecular weight 10–250kDa, was used to define molecular weight positions and as reference concentrations for each molecular weight.
prepared from paraffin-embedded tissues. After deparaffinisation, endogenous peroxidase was quenched with 0.3% [v/v] hydrogen peroxide in 60% [v/v] methanol for 30 min. The sections were permeabilised with 0.1% [w/v] Triton X-100 in PBS for 20 min. Non-specific adsorption was minimised by incubating the section in 2% [v/v] normal goat serum in PBS for 20 min. Endogenous biotin- or avidin-binding sites were blocked by sequential incubation for 15 min with biotin and avidin [Vector Laboratories, Burlingame, CA], respectively. Sections were incubated overnight with: [i] purified goat polyclonal antibody directed towards P-selectin [Santa Cruz Biotechnology, C-20:sc-6941, 1:200 in PBS, v/v]; or [ii] purified hamster anti-mouse ICAM-1 [CD54] [AbDSerotec, MCA1371Z, 1:100 in PBS, w/v]; or [iii] purified goat polyclonal antibody directed towards TNF-α [Santa Cruz Biotechnology, C-20:sc-6941, 1:200 in PBS, v/v]. Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex [Vector Laboratories, Burlingame, CA].

2.10. Materials

All compounds were obtained from Sigma–Aldrich [Milan, Italy]. All chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline [0.9% NaCl; Baxter, Italy, UK].

2.11. Statistical evaluation

The authors performed three distinct experiments with six animals per groups for each experiment. All values in the figures and text are expressed as mean ± standard deviation [SD] of N observations. For the in vivo studies, N represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed. The results were analysed by one-way analysis of variance [ANOVA] followed by a Bonferroni post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of DMF treatment on the degree of colitis

No histological alteration was observed in the colon tissue from sham CD1 mice [Figure 1A, see macroscopic score 1G; 2A, see histological score 2F]. Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of caecum, colon, and rectum showed presence of mucosal congestion, erosion, and haemorrhagic ulcerations [Figure 1B, see macroscopic score 1G]. The histopathological features included a transmural necrosis and oedema and a diffuse leukocyte cellular infiltrate in the submucosa of colon section from DNBS-injected mice [Figure 2B, see histological score 2F]. DMF reduced the extent and

![Figure 6. Effects of DMF treatment on colon levels of cytokine production. Immunohistochemical analysis for TNF-α showed positive staining localised in the inflammatory cells in the injured area from DNBS-injected animals (B, B1, and D) compared with the sham-treated mice (A, A1, and D). Treatment with DMF reduced the increase of TNF-α in the colon after DNBS administration with statistically significant effects at the dose of 100 mg/kg [C, C1, and D]. IL-1β and TNF-α levels in the colon were increased after DNBS injection compared with the sham-treated mice [E and F]. DMF treatment significantly reduced the levels of IL-1β and TNF-α at the dose of 100 mg/kg [E and F]. Data are means ± SD of six mice for each group. *p < 0.01 vs SHAM; #p < 0.01 vs DNBS; ND: not detectable. DMF, dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; TNF, tumour necrosis factor; SD, standard deviation;](https://academic.oup.com/ecco-jcc/article-abstract/10/4/472/2571207)
severity of the macroscopic [Figure 1C, D, and E, see macroscopic score 1G] and histological signs of colon injury [Figure 2C, D, and E, see histological score 2F] with significant beneficial effects at the dose of 100 mg/kg [p < 0.01] [Figures 1E, 2E]. Four days after colitis induced by DNBS treatment, all mice had diarrhoea and a reduction in body weight [compared with the sham groups of mice] [Figure 1F]. DMF treatment reduced the loss of body weight with significant effects at the dose of 100 mg/kg [p < 0.01] [Figures 1F, 2E].

IL-10KO mice spontaneously develop a Th1-dependent chronic enterocolitis shortly after birth, that is fully established at 8–10 weeks of age. Therefore, we evaluated the therapeutic activity of DMF [30 mg/kg] on 9-week-old IL-10KO mice with fully established colitis. As Figure 3 shows, 7 weeks of treatment with DMF [30 mg/kg] significantly reduced the activity of the colitis, as assessed by colonic MPO activity [Figure 3, panel D], histological damage scores [Figure 3, panel E], and colonic TNF-α levels [Figure 3, panel F] at Week 16.

3.2. DMF reduced lipid peroxidation after DNBS administration and regulated Mn-SOD expression in colon tissues

The colitis caused by DNBS was also characterised by an increase in lipid peroxidation. Four days after DNBS administration, thiobarbituric acid-reactant substances [TBARs] levels were measured in the colon tissues as an indicator of lipid peroxidation. A significant increase in TBARs levels [Figure 4A] was observed in the colon tissues collected from DNBS-treated mice 4 days after the induction of experimental colitis when compared with sham-treated mice. TBARs were significantly attenuated by DMF [Figure 4A]. Reactive oxygen species, either directly or via the formation of lipid peroxidation products, may play a role in enhancing inflammation. To test whether DMF modulates the oxidative process, we analysed the colon expression of anti-oxidant enzyme Mn-SOD. A basal expression of Mn-SOD was observed in the colon tissues from sham-treated mice and DMF significantly reduced colon Mn-SOD [Figure 4B]. DMF treatment [100 mg/kg] significantly increased Mn-SOD expression [p < 0.01] [Figure 4B].

3.3. Effects of DMF on IκB-α degradation and NF-κB p65 translocation

Most inflammatory mediators are controlled by NF-κB transcription factor, which is kept inactive by IκB-α. By western blot analysis, we evaluated IκB-α in colon cytosolic extract and NF-κB p65 expression in colon nuclear extract. A basal level of IκB-α was detected in the colon tissues from sham-treated mice whereas, in DNBS-injured
mice, IκB-α expression was substantially reduced [Figure 5A]. IκB-α degradation was prevented in the colon tissues collected from DMF-100 mg/kg treated mice [Figure 5A]. Moreover, NF-κB p65 translocation was also significantly increased after DNBS instillation compared with the sham-treated mice [Figure 5B]. A significant reduction in NF-κB p65 nuclear expression was observed in the tissues from DMF-treated mice [Figure 5B], significantly only at higher doses.

3.4. Effect of DMF treatments on TNF-α and IL-1β expression

To test whether DMF modulates the inflammatory process, we analysed the colon expressions and levels of pro-inflammatory cytokines TNF-α and IL-1β. A substantial increase in TNF-α [Figure 6B, B1] and IL-1β expression [Figure 6E] was found in the colon tissues collected 4 days after DNBS administration. DMF treatment reduced in a dose-dependent manner colon expression of IL-1β [Figure 6E] in DNBS-injected mice and lowered the expression of TNF-α [Figure 6C, C1] with an important effect at the dose of 100 mg/kg [p < 0.01].

3.5. DMF modulated ICAM-1 and P-selectin expression and reduced MPO activity

In this study we also evaluated the intestinal expression of ICAM-1 and P-selectin that contribute to cell recruitment during colon inflammation. Positive staining for ICAM-1 [Figure 7B, B1] and for P-selectin [Figure 7E, E1] was substantially [p < 0.01] in the vessels of the lamina propria and submucosa as well as in epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS-injected mice. Treatment with DMF at 100 mg/kg reduced the staining for ICAM-1 [Figure 7C, C1] and for P-selectin [Figure 7F, F1] in the colon tissues collected from DNBS-injected mice [p < 0.01]. The colitis caused by DNBS was also characterised by an increase in MPO activity [p < 0.01], an indicator of the neutrophil accumulation in the colon [Figure 7H]. This was consistent with observations made with light microscopy, that evidenced that the colon of DNBS-mice contained a large number of neutrophils. On the contrary, DMF significantly reduced the degree of PMN infiltration [determined as increase in MPO activity] in inflamed colon [Figure 7H].

3.6. Effect of DMF treatment on MMPs expression in colon tissues

To assess whether colon injury is associated with alterations in expression of secreted MMP-9 and MMP-2, mice were sacrificed at 4 days after DNBS administration and colons were subjected to western blot analysis. In this study we derived a basal level of total MMP-9 and MMP-2 from sham mice [Figure 8A, 8B]. A significant up-regulation of MMP-9 and MMP-2 expression was observed in the colon tissues from DNBS-treated mice, and DMF treatment at 10-30-100 mg/kg significantly prevented the increase of DNBS-induced MMP-9 and MMP-2 expression [p < 0.01] [Figure 8A, B].

3.7. Effect of DMF on H2O2-induced barrier dysfunction of human intestinal epithelial cells

After administration of H2O2 [500 μM], occludin and ZO-1 proteins were significantly restored by 10 μM DMF in Caco-2 [Figure 9A A1, BB1]. Moreover, the protection of human intestinal epithelial cells seem to be dependent on the HO-1 pathway [Figure 9C, C1].

4. Discussion

Inflammatory bowel diseases [IBDs] are complex pathological conditions that affect millions of people worldwide. The diseases are associated with inflamed intestinal and colonic mucosa in response to the dysregulated immune system. Indeed, because of their relapsing bouts and chronic course, both Crohn’s disease [CD] and ulcerative colitis [UC] can progress to fibrosis, resulting in pharmacologically unmanageable alterations that can be resolved by disabling surgical resections.19,20

![Figure 8](https://academic.oup.com/ecco-jcc/article-abstract/10/4/472/2571207/2571207)

**Figure 8.** Effects of DMF treatment on MMP-9 and MMP-2 activity. In contrast to sham-treated mice, colon tissues extracts from DNBS-treated mice showed an induction of MMP-9 and MMP-2 [A and B]. DMF at the dose of 100 mg/kg treatment reduced the degree of activation of MMP-9 and mmp-2 [a and b]. data are means ± sd of six mice for each group. *p < 0.01 vs sham; #p < 0.01 vs DNBS. DMF, dimethyl fumarate; DNBS, dinitrobenzene sulphuric acid; SD, standard deviation.
Dimethyl Fumarate and Colitis

The first novel compound for IBDs to be introduced was infliximab [Remicade®Centocor®]. The FDA officially approved infliximab also for the induction and long-term maintenance of remission in UC. Novel therapeutic approaches, including preliminary experience with biological therapies directed at TNF-α and other cytokines, adhesion molecules, growth factors, and probiotics have been reviewed. In particular, TNF-α-targeted therapies are a class of medication that has revolutionised the treatment of these diseases and the quality of life for patients, but it also poses risk of developing various side effects including infections, exacerbation of some neurological manifestations, and cutaneous lesions, or induces antibody production. Also, we observed that DMF significantly attenuated body weight loss.

Oxidative stress also plays a significant role in the pathogenesis of IBD. The intestinal mucosa is vulnerable to oxidative stress from constant exposure to reactive oxygen species [ROS] generated by the lumen contents. Oxidative stress can cause cell damage either directly or through altering signalling pathways. Recently, it has been demonstrated that DMF reduced ROS production and also oxidative stress. In particular, De-Hyung et al. showed that application of DMF leads to stabilisation of NF-E2 related factor 2 [Nrf2], activation of Nrf2-dependent transcriptional activity, and abundant synthesis of detoxifying proteins. Furthermore, endogenous antioxidants such as superoxide dismutase [SOD] are normally able to counteract oxidative stress in the intestinal mucosa. The present study confirmed the increase in oxidised membrane phospholipids in the colon tissues from DNBS-injected mice; also, we have shown that treatment with DMF reduced lipid peroxidation or MDA levels and increased the expression of anti-oxidant enzyme Mn-SOD.

Several experimental evidences have clearly suggested that NF-xB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in the inflammation process. Therefore, we show that application of DMF to mice are more resistant to DNBS-induced colitis, with an important resolution of the macroscopic and histological signs of the inflammatory process. Also, we observed that DMF significantly attenuated renal complications are uncommon and poorly recognised.

For this, the aim of this study was to provide the most significant data regarding the therapeutic role of DMF in a mouse model of inflammatory bowel disease and to give an overview of biological and experimental drugs that will become available in the near future. DMF is the most pharmacologically effective molecule among the FAEs. It is a new orally available disease-modifying agent for the management of relapsing forms of multiple sclerosis. The anti-inflammatory action of DMF has been demonstrated. We show that DMF attenuated DNBS-induced colitis in mice. At the histological level, we observed that epithelial disruption was statistically significantly less in mice treated with DMF. Furthermore, DMF-treated mice are more resistant to DNBS-induced colitis, with an important resolution of the macroscopic and histological signs of the inflammatory process. Also, we observed that DMF significantly attenuated body weight loss.

Figure 9. Effects of DMF treatment on occludin, ZO-1, and HO-1 expression in Caco-2 cells. By western blot analysis, occludin and ZO-1 levels were lowered after H2O2 stimulation compared with the control group [A and B]. DMF treatment [100 mg/kg] increased the levels of occludin and ZO-1 in Caco-2 cells [A and B]. HO-1 levels were increased in Caco-2 cells after H2O2 stimulation and DMF treatment [100 mg/kg] relative to to basal expression in control group [C]. DMF, dimethyl fumarate.
associated with experimental colitis. However, recent findings suggest that NF-κB has not only pro-inflammatory but also tissue-protective functions. Many previous studies have demonstrated that DMF inhibits NF-κB by decreasing its phosphorylation and its entry into the nucleus. Thus, we report here that DNBS administration caused an increase in NF-κB nuclear translocation and IkB-α degradation in the colon inflamed tissues at 4 days, whereas DMF reduced NF-κB translocation and inhibited the IkB-α degradation.

Importantly, it was observed that DMF inhibits the TNFα-induced nuclear entry of NF-κB31 and degradation of IkB-α. It has been well demonstrated that TNF-α and IL-1β are the most important cytokines present in colon tissues and involved in the pathogenesis of colitis. The present study confirmed the expression of TNF-α and IL-1β in the colon tissues from DNBS-injected mice, and the treatment with DMF reduced these inflammatory cytokine levels. There is consistent evidence that, during acute and chronic colitis, the sustained production of pro-inflammatory cytokines plays a key role in the adherence and infiltration of leukocytes to endothelial cells, thus maintaining the chronic inflammation into the caecal and colonic interstitium. In the present study, we confirmed that DNBS administration induced the appearance of P-selectin on the endothelial vascular wall and up-regulated the surface expression of ICAM-1 on endothelial cells. Treatment with DMF abolished the expression of P-selectin and the up-regulation of ICAM-1 without affecting constitutive levels of ICAM-1 on endothelial cells. The absence of an increased expression of the adhesion molecule in the colon tissue from DMF-treated mice correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme MPO, and with the attenuation of the colon tissue damage as evaluated by histological examination.

It has been demonstrated that NF-κB regulates the expression of MMPs that are considered to be an important component in the progression of inflammation. There is also growing evidence that MMP-9 and -2 can be implicated in the pathophysiology of several intestinal inflammatory disorders. In fact, MMPs can be released from almost all connective tissue cells present in the bowel in response to inflammatory stimuli including colitis. The involvement of MMPs in inflammatory processes was demonstrated by studies in animal models that induced CD or UC and in cell cultures. Recently, different studies have demonstrated that DMF decreases matrix metalloproteinase expression and activity. In the present study, we confirmed the expression of MMP-9 and -2 in DNBS-injected mice, and the treatment with DMF reduced the degree of expression of MMP-9 and -2.

Nrf-2 and HO-1 protein expressions were up-regulated in those mice treated with DMF, and the activation of the NF-κB pathway was drastically ameliorated. Moreover, data showed the positive correlation of the antioxidant activity with the mechanism involved in the physiological maintenance of the integrity and function of the intestinal epithelium.

Taken together, the data presented in this study demonstrated that treatment with DMF exerts therapeutic activity on an animal model of colitis, suggesting that DMF could represent a target for therapeutic intervention in autoimmune/inflammatory disorders such as IBD.

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EE and SC planned experiments. MC, DI, and GB performed experiments. IP analysed the results and GC performed the biochemical analysis and prepared the manuscript. All authors read and approved the final manuscript.

References

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Conflict of Interest
The authors declare that they have no competing interests.
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