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ORIGINAL ARTICLE



A novel synthetic small molecule DMFO targets Nrf2 in modulating proinflammatory/antioxidant mediators to ameliorate inflammation

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ABSTRACT

Inflammation is a protective immune response against invading pathogens, however, dysregulated inflammation is detrimental. As the complex inflammatory response involves multiple mediators, including the involvement of reactive oxygen species, concomitantly targeting proinflammatory and antioxidant check-points may be a more rational strategy. We report the synthesis and anti-inflammatory/antioxidant activity of a novel indanedione derivative DMFO. DMFO scavenged reactive oxygen species (ROS) in *in-vitro* radical scavenging assays and in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. In acute models of inflammation (carrageenan-induced inflammation in rat paw and air pouch), DMFO effectively reduced paw oedema and leucocyte infiltration with an activity comparable to diclofenac. DMFO stabilised mast cells (MCs) in *in-vitro* A23187 and compound 48/80-induced assays. Additionally, DMFO stabilised MCs in an antigen (ovalbumin)-induced MC degranulation model *in-vivo*, without affecting serum IgE levels. In a model of chronic immune-mediated inflammation, Freund's adjuvant-induced arthritis, DMFO reduced arthritic score and contralateral paw oedema, and increased the pain threshold with an efficacy comparable to diclofenac but without being ulcerogenic. Additionally, DMFO significantly reduced serum TNF α levels. Mechanistic studies revealed that DMFO reduced proinflammatory genes (IL1 β , TNF α , IL6) and protein levels (COX2, MCP1), with a concurrent increase in antioxidant genes (NQO1, haem oxygenase 1 (HO-1), Glo1, Nrf2) and protein (HO-1) in LPS-stimulated macrophages. Importantly, the anti-inflammatory/antioxidant effect on gene expression was absent in primary macrophages isolated from Nrf2 KO mice suggesting an Nrf2-targeted activity, which was subsequently confirmed using siRNA transfection studies in RAW macrophages. Therefore, DMFO is a novel, orally-active, safe (even at 2 g/kg p.o.), a small molecule which targets Nrf2 in ameliorating inflammation.

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Introduction

Inflammation is the body's natural protective immune response to invading pathogens and is comprised of multiple signalling pathways and mediators [1]. However, an uncontrolled inflammatory response is debilitating and can lead to cell, tissue and organ destruction. Inappropriately triggered chronic inflammation underlies several pathologies such as rheumatoid arthritis (RA), autoimmune diseases, diabetes, cardiovascular diseases, cancer, pulmonary diseases, and

neurological diseases [2–4]. The most commonly prescribed class of anti-inflammatory agents, namely, the nonsteroidal anti-inflammatory drugs (NSAIDs), nonspecifically target COX isozymes to reduce prostaglandins throughout the body. However, this drug class has been associated with gastrointestinal (GI) side effects such as ulcers [5,6], kidney damage [7] and heart attacks [8].

In the search for newer NSAIDs with improved safety profiles, attention has focused on understanding the molecular mechanisms and the cell types driving

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inflammation. It is now recognised that inflammatory and immunologic responses induce reactive oxygen species (ROS) whilst conversely, ROS drive up proinflammatory responses [9–11]. Indeed, oxidative stress can lead to chronic inflammation by activating a variety of transcription factors including NF- κ B, AP1, HIF1 α , PPAR γ , and Nrf2, leading to the gene expression of pro and anti-inflammatory mediators [12–14]. In pinpointing the major proinflammatory cell types, it is now clear that not only do macrophages produce large amounts of ROS for their phagocytic activity [15], but that mast cells (MCs) are another subset of immune cells involved in inflammatory reactions. These cells release multiple proinflammatory mediators such as histamine, tryptase, and arachidonic acid metabolites together with increased ROS production, which is involved in MC degranulation and histamine release [15,16]. Owing to the role of MCs in the pathophysiology of several conditions such as inflammation, cardiovascular diseases, tumour biology, and host defence, stabilising MCs may be an attractive therapeutic strategy for inflammatory conditions mediated by MC degranulation [17–19].

Nrf2 is a master regulator of endogenous antioxidant defences and there is significant cross-talk between Nrf2 and the proinflammatory transcription factor NF- κ B [20–22], suggesting that targeting Nrf2 may be a highly effective strategy to tackle inflammation [23]. Moreover, Nrf2 activation induces the expression of the cytoprotective enzyme heme oxygenase 1 (HO-1), which protects and regenerates mucosal tissues thereby affording protection against ulcerogenicity [24,25]. Additionally, recent evidence suggests that HO-1 plays a down-regulatory role in NF- κ B nuclear translocation leading to an inhibition of the NF- κ B signalling pathway [26]. Hence, Nrf2 activators may be superior to traditional NSAIDs in their dual action by lowering inflammation whilst maintaining a healthy gut mucosa.

In this study, we evaluated 4b,9b-Dihydroxy-6-methoxy-8-(3-oxo-but-1-enyl)-4b,9b-dihydro-5-oxa-indeno [2,1- α] inden-10-one (DMFO), a novel small molecule indanedione derivative which we now show to have Nrf2 activating ability, for its anti-inflammatory activity in four animal models of acute and chronic inflammation. We studied its mechanism of anti-inflammatory action against the backdrop of pivotal pathways that govern inflammatory conditions, and its effect on important immune cells involved in inflammation such as mast cells and macrophages. Primary macrophages derived from Nrf2 KO mice and siRNA transfection studies in RAW 264.7 cells were used to confirm an Nrf2-targeted activity for DMFO. Our data showed that Nrf2 activators like DMFO lessen mediators of inflammation and stabilise inflammatory cells in

both *in vitro* and *in vivo* models of chronic and acute inflammation without ulcerogenic consequences, suggesting that activating Nrf2 with small molecule activators may be an alternate, safer and potentially superior strategy to existing treatment modalities to lessen inflammation.

Materials and methods

General

Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, A23187 (calcium ionophore), λ -carrageenan and compound 48/80 (C48/80) were purchased from Sigma-Aldrich Co. LLC, St. Louis, MO. Difco (Detroit, MI)TM Freund's adjuvant (5 mg/mL, *Mycobacterium butyricum*, catalog no. 264010) was from Difco Laboratories, Detroit, MI). Diclofenac sodium and ketotifen fumarate were gifts from Hetero Drugs Ltd., Hyderabad, AP, India and Microlabs Ltd., Bangalore, India, KA, India respectively. Fetal bovine serum (FBS) was from Gibco (CA)[®], Life Technologies Corporation, NY, USA. The COX2(#4842S) and HO -1(NBP1-31341) antibodies were purchased from Cell Signalling Technology Inc, Danvers, MA, and Novis Biological respectively. The IRDye[®] 800CW Donkey anti-rabbit and IRDye[®] 680RD Goat anti-mouse secondary antibodies and cell stain 700 were purchased from LI-COR GmbH (Germany). All other consumables used for protein expression were from Bio-Rad Laboratories Inc, Hercules, CA.

Synthesis and characterisation

DMFO was synthesised and recrystallised using previously reported methods from our laboratory [27]. Briefly, equimolar (4 mmol) quantities of 1, 2, 3-Indantrione monohydrate was made to react with dehydrozingerone in 10 mL of 30% v/v acetic acid medium by stirring on a magnetic stirrer at 45 °C for 4 h and the reaction mixture was kept at room temperature for 30–60 min until solid phase separation. Product formation was monitored using TLC (solvent system: chloroform: methanol (9.5:0.5)). The product was recrystallised from methanol and characterised by IR, MS, ¹H NMR, ¹³C NMR, and purity determined by HPLC.

In-vitro antioxidant studies

DPPH[•] and ABTS^{•+} radical scavenging

For DPPH[•] scavenging activity, equal volumes of DMFO (at different concentrations) and DPPH (200 μ M), were added to a 96-well microtitre plate, which was then

incubated in the dark at room temperature for 20 min. The absorbance was recorded at 517 nm and IC_{50} was calculated [28]. For ABTS^{•+} radical scavenging activity, 0.3 mL of ABTS radical cation was added to 1.7 mL of phosphate buffer and 0.5 mL of DMFO at various concentrations. The absorbance was recorded at 734 nm and IC_{50} was calculated [28]. The experiment was performed in duplicate and results are expressed as mean $\pm$ SEM.

Cell culture and maintenance

Murine cell-lines (macrophage RAW 264.7 and MC p815) were purchased from the National Centre for Cell Science, Pune, MH, India. Primary bone marrow-derived macrophages (BMDMs) from male *Nrf2* wild-type (WT) and knockout (KO) mice (10 weeks of age, 20–25 g) were isolated. *Nrf2* knockout mice (B6.129X1-Nfe212^{tm1Ywk}/J) were obtained from the Jackson Laboratory, maintained as a heterozygous colony and mated to produce homozygous knockouts and WT litter mates. The cell lines and primary BMDMs were cultured in the recommended culture medium, and maintained at 37 °C in a CO₂ incubator. Trypan blue dye exclusion was used to assess the cell viability. The viability of cells used in the various assays was always >90%.

Cell viability assay

An MTT assay was performed in RAW 264.7 cells as previously reported [29]. Briefly, 5×10^4 cells/well were seeded in a sterile 96-well plate and treated with various concentrations of DMFO or vehicle for 24 h. MTT reagent of about 20 μ L (5 mg/mL in sterile PBS, pH 7.4) was added, and incubated for 4 h, after which the medium was removed. One hundred microlitres of 100% DMSO was then added to each well and kept on a vibrating shaker for 10 min and the absorbance was measured at 540 nm. The percentage cytotoxicity was calculated and the IC_{50} was determined from concentration-response curves. The experiment was performed in duplicate with $n = 3$ replicates at each concentration per experiment.

Intracellular ROS assay in LPS-stimulated macrophages

Intracellular ROS was measured using DCFH-DA (100 μ M) in LPS-stimulated RAW 264.7 cells as previously described by us [30], and the IC_{50} for ROS inhibition was determined. We also determined the effect of DMFO on ROS production in the absence of LPS. Experiments were performed in triplicate with $n = 3$ –6 replicates at each concentration per experiment.

Mast cell degranulation in compound 48/80 and A23187-stimulated mast cells

The assay was performed based on a previously reported method [31] with modifications. Briefly, 10^6 cells/500 μ L were transferred to 1.5 mL sterile microcentrifuge tubes and 500 μ L each of different concentrations of DMFO was added and incubated for 1 h at 37 °C. After incubation, 500 μ L of C48/80 (30 μ g/mL) or A23187 (30 μ M) in DMEM was added to each tube to cause MC degranulation (except the negative control, which received an equal volume of media), and incubated for another 30 min at 37 °C. The tubes were then immersed in an ice-cold water bath to terminate the action of stimulants. The tubes were centrifuged at 1000 rpm for 5 min at 4 °C and the histamine released into the supernatant was estimated according to the method of Shore [32], after slight modifications. Briefly, 100 μ L of supernatant was pipetted into a 96-well black plate and 50 μ L of 0.4 N NaOH was added, followed immediately by 10 μ L of 0.1% orthophthalaldehyde (OPT) and the plate was shaken for 5 min. 50 μ L of 0.1 N HCl was added and the fluorescence intensity was measured using a fluorescence microplate reader at λ_{ex} : 350 nm and λ_{em} : 450 nm. The percentage reduction in histamine release was calculated and the IC_{50} was determined from the dose-response curve. The experiment was performed in duplicate, with $n = 3$ replicates at each concentration per experiment and ketotifen used as a standard.

In-vivo studies

Animals, dose selection and treatment protocol

Male Sprague-Dawley rats (180–220 g) were used for all acute and chronic inflammatory studies, except the ovalbumin-induced mast cell degranulation assay which was performed in BALB/c (20–25 g) mice. Experimental animals were inbred and maintained at the Central Animal Research Facility, Manipal Academy of Higher Education, Manipal, Karnataka, India, in sterile polypropylene cages with autoclaved paddy husk as bedding under controlled conditions of light (12 h/12 h light-dark cycle), temperature (25 ± 2 °C), air exchange (10–12/h) and humidity ($50 \pm 5\%$), with free access to standard pellet diet and water, was provided *ad libitum*. The Institutional Animal Ethics Committee (IAEC), KMC, Manipal approved the experimental protocol (IAEC/KMC/50/2011-2012). The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government Offices of India, were followed in conducting the studies. Based

on previous reports of this novel class of compounds synthesised in our laboratory [28], safe dose testing was performed. DMFO was found to be safe even up to 2 g/kg p.o. The maximum dose selected for *in-vivo* studies was 200 mg/kg p.o. (1/10 of the safe dose (2 g/kg p.o)). All treatments were administered as a suspension in 0.25% carboxymethylcellulose (CMC) p.o. For the mechanistic studies, *ex vivo* studies using primary macrophages, B6.129X1-Nfe212^{tm1Ywk/J} Nrf2 KO male mice and WT littermates were bred and housed at the Alfred medical research and education precinct (AMREP) precinct animal centre (PAC) animal facility, Melbourne, Australia. All mouse experiments were approved by the AMREP animal ethics committee and investigations confirmed to National Health and Medical Research Council (NHMRC; Australia) guidelines.

Carrageenan-induced paw oedema in rats

According to the method of Winter et al. [33], animals were randomised into six treatment groups ($n=5$) and treated with 0.25% CMC (vehicle) or diclofenac sodium in 0.25% CMC (10 mg/kg p.o) or DMFO in 0.25% CMC (20 mg/kg, 100 mg/kg, or 200 mg/kg p.o.). One hour post-treatment, all rats were given a subplantar injection of 0.05 mL of 1% suspension of carrageenan into the hind paw, except for the saline control group which received saline. The paw volume was then measured immediately using a plethysmograph (7140-Digital plethysmograph, Ugo Basile Srl, Comerio, VA, Italy) and designated as the zero (0 h) hour volume. Paw volume was recorded at 1, 3 and 6 h after carrageenan challenge. The increase in paw volume at 3 h for each group was calculated and results are expressed as a mean increase in paw volume $\pm$ SEM. The most effective dose of DMFO was used for further *in-vivo* studies.

Ovalbumin-induced mast cell degranulation in mice

Active cutaneous anaphylaxis, involving the release of histamines from mast cells in response to ovalbumin (OVA), was evaluated in 6–8-week-old mice [34,35]. This was determined by estimating the leakage of Evan's Blue dye into surrounding ear tissue, known as extravasation, in response to the antigen OVA. Animals were randomised ($n=6$) and on day 0, animals were sensitised to antigen by administering 100 μ L containing 40 μ g of OVA in 1 mg of alum i.p., and the control group received an equal volume of sterile saline. Animals were treated once daily with 0.25% CMC or ketotifen fumarate (1 mg/kg p.o) or DMFO (200 mg/kg p.o) from 1 h postinjection on day 0 to day 14. On day 14, 1 h after CMC, ketotifen or DMFO treatment, the

animals were anaesthetised, blood was withdrawn from the retro-orbital plexus, serum separated by centrifugation (6000 rpm at 25 °C for 10 min) and stored at -80°C to measure IgE. The animals were then rechallenged by administering 20 μ L containing 10 μ g of OVA antigen, intradermally into the ear, followed by 0.3 mL of 1% Evan's Blue saline solution i.v. into the tail vein. After 30 min, the animals were culled, and the ears were collected for measurement of extravasated dye [36]. Briefly, the ear was kept overnight in 1 N KOH at 37 °C. A mixture of 0.6 N phosphoric acid and acetone (5:13) was added and shaken vigorously to dissolve the tissue. Following filtration, the absorbance was measured at 620 nm. The amount of dye was quantified from Evan's Blue standard curve and expressed as Evan's Blue μ M/mg of protein using BCA assay (PierceTM BCA Protein Assay Kit, Thermo Fischer Scientific). Dye extravasation was expressed as mean $\pm$ SEM. The IgE in serum (ng/mL) was determined using commercially available mouse IgE ELISA kits according to the manufacturer's protocol. Serum IgE levels were expressed as mean serum IgE (ng/mL) $\pm$ SEM.

Carrageenan-induced inflammation in a rat air pouch assay

A previously established method was employed [37]. On day 1, rats were anaesthetised and the dorsal side of the animal was shaved (1 cm²) under ether anaesthesia and an air pouch was produced at this site by injecting 20 mL of air s.c. using a 28 gauge needle. On day 3, an additional 20 mL of air was injected into the pouch. On day 5, rats were randomised ($n=5$) and treated with 0.25% CMC or diclofenac sodium in 0.25% CMC (10 mg/kg p.o) or DMFO in 0.25% CMC (200 mg/kg p.o.). On day 5, an acute inflammatory response was induced within the air pouch by injecting 1 mL of 1% w/v carrageenan in saline into the pouch (except for the saline control which received an equal volume of saline). Rats were culled 6 h post-carrageenan injection, and 5 mL of ice-cold saline was injected into the pouch and gently massaged. The pouch was cut open and the exudates were collected. The total leucocyte count in the exudates was obtained using a veterinary blood cell counter (PCE-210VET, Erma Inc, Tokyo, Japan) and the results are expressed as mean WBC count (1×10^3 cells/mg of protein) $\pm$ SEM. A nitrite assay was performed using freshly prepared Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid in a 1:1 ratio) [38]. Results are expressed as mean NO released per mg of protein (μ M/mg) $\pm$ SEM. A myeloperoxidase (MPO) assay to evaluate neutrophil activation was performed based on the

method of Graff et al. [39]. Briefly, 50 μ L sample or standard or 50-mM phosphate buffer (pH-6) (blank) was added to a 96-well microtitre plate, together with 1 μ L of 0.5% H_2O_2 per mL of o-dianisidine hydrochloride (ODA) solution (0.167 mg/mL). Two hundred and fifty microlitres ODA solution was then added to each well. The absorbance was measured at 490 nm after 15 min. Results are expressed as mean MPO per mg of protein (ng/mg) $\pm$ SEM.

Freund's adjuvant-induced arthritis in rats

Previously reported methods were employed [40,41]. Briefly, on day 0, paw volumes were measured using a digital plethysmometer (Catalog No. 37140- Digital plethysmograph, Ugo Basile Srl, Comerio, VA, Italy) and 0.1 mL complete Freund's adjuvant (100 mg of *Mycobacterium butyricum* triturated with 20 mL of light liquid paraffin & autoclaved for 20 min at 120 °C) was injected intraplantarly (0.1 mL/paw) into the right paw (ipsilateral paw). On day 14, paw oedema was calculated as the change in paw volume (mL) (day 0–14), and animals were randomised into treatment groups ($n=6$) such that the mean contralateral paw volume of each group was equal. Animals were grouped and treated with 0.25% CMC or diclofenac sodium in 0.25% CMC (10 mg/kg p.o) or DMFO in 0.25% CMC (200 mg/kg p.o.) once daily for 7 days from day 14 to day 21. On day 21, arthritis scoring was done based on clinical score, mobility score, and joint stiffness [40,41]. Briefly, clinical scoring for the severity of arthritis (0–4) was carried out as: 0 = no signs of arthritis, 1 = swelling and/or redness of the paw or 1 digit, 2 = 2 joints involved, 3 = >2 joints involved, 4 = severe arthritis of the entire paw and digits [41]. Mobility scoring was carried out as: 0 = no movement, 1 = crawls using fore paw, 2 = walks with protective behaviour towards both hind paws only partially using contralateral paw, 3 = walks with protective behaviour toward both hind paws, 4 = walks with protective behaviour towards ipsilateral paw only partially using the paw, 5 = walks with protective behaviour towards ipsilateral, 6 = normal walking according to Nagakura et al. [40]. Joint stiffness was scored as: 0 = freely moving joint, 1 = restriction of ankle movement in either bending or extension, 2 = restriction of ankle movement in both bending and extension according to Nagakura, et al. [40]. Paw oedema (change in paw volume from day 0–21) during the treatment period was calculated. Mechanical hyperalgesia (pain threshold) was measured based on the method of Randall-Selitto [42] using an Analgesy-metre (Catalog No. 37215, Ugo Basile, Srl, Comerio, VA, Italy). Briefly, the pain threshold was assessed in terms of paw

withdrawal response/attempts to increasing pressure (0–250 g). The results are expressed as the mean pain threshold (g) $\pm$ SEM. ($n=6$). Radiography of the paw for soft tissue swelling and joint involvement was performed. All scoring was performed by a blinded observer. All results are represented as mean $\pm$ SEM.

Mechanistic studies

Protein expression by western blotting and MCP-1 ELISA in RAW 264.7 macrophages

A quantity of 8×10^4 RAW 264.7 cells were stimulated with LPS (0.1 μ g/mL) and after 1 h, treated with DMFO (5 μ M) for 18 h. Whole cell lysates were prepared using RIPA buffer with protease inhibitors. Protein concentration was determined using a BCA assay and 15 μ g of protein were loaded and run in a precast SDS-PAGE gel (Bio-Rad, Hercules, CA). Proteins were transferred to an immuno-blot PVDF membrane (Bio-Rad), and the membrane was stained (REVERT Total Protein Stain, LI-COR GmbH) and imaged for total protein quantification and subsequent normalisation according to the manufacturer's instructions. The membrane was blocked for 1 h at room temperature with Odyssey Blocking buffer (TBS) (LI-COR GmbH) and cut into two strips around 40 kD. The upper strip (>40 kD) was incubated overnight at 4 °C with COX2 (1:2500, ref 12282, cell signaling) and alpha-tubulin (1:5000) antibodies while the lower strip (<40 kD) was incubated with HO-1 (1:2500, ref NBP1-31341, Novus Biological). The blots were subsequently incubated with a 1:10000 dilution of IRDye[®] 800CW Donkey anti-rabbit secondary antibody (Ref 925–32 213, LI-COR GmbH) alone for the lower strip or in combination with a 1:10000 dilution of IRDye[®] 680RD Goat anti-mouse secondary antibody (Ref 926–68 070, LI-COR GmbH) for the upper strip, for 1 h. Three independent experiments were performed and the blots were analysed using the Odyssey CLX imaging system (LI-COR GmbH) at 2 wavelengths (700 and 800 nm for red and green bands respectively). The cell culture supernatant was subjected to ELISA assay for MCP1. The assay was performed according to the manufacturer's instructions (Mouse CCL2/JE/MCP-1 Quantikine ELISA kit, R&D Systems, Minneapolis, MN). Three independent experiments were performed with $n=6$ replicates per experiment.

In cell westerns

A second independent method was performed to quantitate the protein response of RAW 264.7 cell macrophages to DMFO. Briefly, RAW 264.7 cells were

seeded at 80 000/well in 24-well plates 24 h prior to treatment with either DMSO (vehicle) or DMFO (50 μ M) for 1 h before the addition of LPS (0.1 μ g/mL) for 18–20 h. Cells were then fixed with neutral buffered formalin (4% final concentration) containing 0.1% Triton X-100 for 30 min, washed twice with PBS 0.1% Triton X-100 before the addition of Odyssey blocking buffer (PBS, LI-COR) diluted 1:2 with ddH₂O and incubation at RT for 1 h. Antibodies specific for COX2 (Cell Signalling, 1/1000), or HO-1 (Novus Biological, 1/2000), were added to the cells for 2 h before detection using IRDye® 800CW Donkey anti-rabbit secondary antibody (LI-COR GmbH) and cell stain 700 (LI-COR) for 1 h to allow for normalisation to cell density. The cells were imaged using the Odyssey CLX imaging system (LI-COR). Three independent experiments were performed with five replicates per experiment.

Studies in primary bone marrow-derived macrophages

Primary BMDMs were plated at 8000 cells per well and were stimulated with LPS (0.1 μ g/mL) and DMFO (5, 50 μ M) for 18–20 h before gene expression changes of proinflammatory and oxidative stress markers were assessed by real-time quantitative RT-PCR.

Quantitative real-time PCR

RNA was extracted from cultured cells and cDNA produced as described by Kantharidis et al. [43]. Gene expression was determined by qRT-PCR using SYBR or the TaqMan system based on real-time detection of accumulated fluorescence (ABI QS3/5, Perkin-Elmer Inc, PE Biosystems, Foster City, CA). Probes and primers are shown in [Supplementary Table 1](#). Gene expression was normalised to 18S mRNA and reported as a ratio compared to the level of expression in untreated control cells, which was given an arbitrary value of 1. Each experiment included $n = 6$ –8 replicates.

siRNA transfection studies in RAW 264.7 macrophages

RAW 264.7 cells were transfected with siRNA specific for Nrf2 (Silencer Select s9492 and s9491 NFE2L2, Ambion) or scrambled control using Lipofectamine RNAiMAX (Invitrogen, Paisley, UK) as per manufacturer's instructions. Post transfection cells were incubated for 48 h in 10% FBS/Optimem (Gibco) before treatment with DMFO (50 μ M) for 18 h and antioxidant responsiveness determined by gene expression. Each experiment included $n = 6$ –8 replicates.

Data analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA and *post hoc* Tukey's test. $p < .05$ was considered statistically significant (GraphPad Prism Software; version 7.03, Graph Pad Software, Inc, San Diego, CA).

Results

Chemical composition and purity

The schema of DMFO synthesis is shown in [Figure 1\(A\)](#). All other characteristics are shown in [Table 1](#). The purity of DMFO as evaluated by HPLC was $>99\%$.

Antioxidant and mast cell stabilising activity in-vitro

Free radical scavenging assays that have different mechanisms of action, such as DPPH• and ABTS•⁺, give an indication of the nature of the reaction of antioxidants with free radicals. DPPH• radical scavenging is mediated by the transfer of electrons or hydrogen radicals [44], whereas the scavenging of the ABTS•⁺ radical cation takes place through hydrogen transfer [45]. A greater potency in ABTS•⁺ scavenging activity (compared to DPPH) by DMFO suggests a dominant antioxidant mechanism of hydrogen donation ([Table 2](#)).

The effect of different concentrations of DMFO on cell viability was evaluated before screening for *in vitro* anti-inflammatory and MC stabilising activity. DMFO was found to be safe ($IC_{50} > 250 \mu$ M), indicating a relatively low cytotoxicity ([Table 2](#)). In LPS-stimulated RAW 264.7 macrophages, DMFO inhibited intracellular ROS production, measured using DCFH-DA, with an IC_{50} of 39.04 μ M ([Figure 1\(B\)](#)). DPI (5 μ M) used as standard showed $62.18 \pm 1.78\%$ ROS inhibition (data not shown). DMFO also reduced NO levels in LPS-stimulated macrophages with an IC_{50} of 48.27 μ M. In additional experiments, we showed that DMFO alone (at the highest *in vitro* dose used, 50 μ M) had no effect on ROS production in the absence of LPS, as measured by dichlorofluorescein (DCF) fluorescence (see [Supplementary Figure 1](#)).

C48/80 and A23187 are nonantigenic stimuli that elicit MC degranulation by promoting calcium internalisation, through different pathways, leading to histamine release. DMFO showed a better activity against A23187-induced MC degranulation than C48/80 stimulated MCs (IC_{50} 163.7 μ M vs. IC_{50} 356.9 μ M) ([Figure 1\(C\)](#)), suggesting that DMFO may be interfering in signalling events at the level of, or downstream of, calcium entry. Ketotifen, used as a standard, inhibited A23187-induced

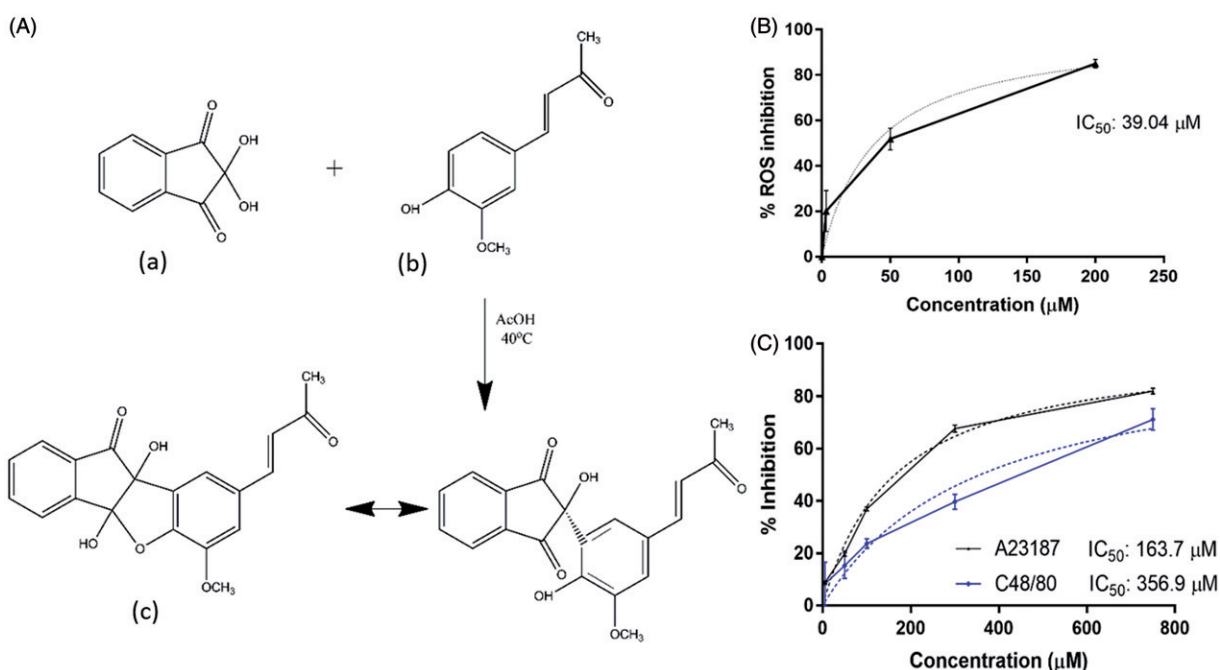


Figure 1. (A) Schema of the synthesis of DMFO. Equimolar quantities of 1, 2, 3-Indantrione monohydrate (a) was made to react with dehydrozingerone (b) in acetic acid medium and final product (DMFO) formation (c) was monitored using TLC. (B) Effect of DMFO on the percentage ROS inhibition in LPS-induced RAW 264.7 cells. DCFH-DA was used to estimate intracellular ROS. Fluorescence intensity was measured at λ_{ex} : 485 nm and λ_{em} : 530 nm. IC_{50} represents the concentration resulting in a 50% inhibition of ROS. (C) Effect of DMFO on MC degranulation in C48/80 and A23187-induced p815 mast cells. Positive dose-response curves showing the IC_{50} of DMFO in preventing histamine release into the medium after treatment with the two mast cell degranulation agents C48/80 and A23187 are shown. The histamine released into the media was determined by fluorescence intensity measured at λ_{ex} : 350 nm and λ_{em} : 450 nm. Best fit curves were applied using GraphPad Prism software to calculate the IC_{50} s. IC_{50} represents 50% inhibition of histamine release.

Table 1. Properties of DMFO.

Sl. No	Property	Description
1	Physical appearance	Yellowish crystalline solid
2	Yield (recrystallized)	55%
3	DSC melting peak	136.5 °C
4	UV λ_{max} (methanol)	233 nm
5	Molecular ion peak at m/e	351
6	Molecular formula	$\text{C}_{20}\text{H}_{16}\text{O}_6$
7	Molecular weight	352.34 g/mol
8	IR $\nu_{\text{max}}/\text{cm}^{-1}$ (KBr)	3448.84 cm^{-1} (–OH str), 1710.92 cm^{-1} (s, C=O str), 1636.65, 1202.66 cm^{-1} (s, C–O str), 740.69, 577.70 cm^{-1}
9	^1H NMR ppm (400 MHz in DMSO- d_6)	2.074 (3H,s) (–CH ₃), 3.795 (3H,s) (–OCH ₃), 6.641 & 6.6 (1H,d), 6.675 (1H,s), 6.781 & 6.802 (1H,d) (aryl-H), 7.105 & 7.125 (1H,d), 7.282 (1H,s), 7.538 & 7.497 (1H,d), 8 (3H,s), 8.298 (1H,s), 9.692 (1H,s)
10	^{13}C NMR ppm (100 MHz in DMSO- d_6)	199.82 & 197.49 (–C=O*2), 150.40, 148.42, 145.70 (*2), 141.30 (*2), 136.66 (*2), 125.99, 124.42, 123.93, 122.21 (*2), 116.09, 111.94, 72.90, 56.15 (–OCH ₃), 45.90 (–CH ₃)
11	Purity (HPLC)	99.9%

MC degranulation with an IC_{50} $415.98 \pm 49.00 \mu\text{M}$, suggesting that DMFO was far superior in its action in preventing histamine release into the medium.

Effect of DMFO on OVA-induced mast cell degranulation in sensitised mice

In this study, mice were treated with 200 mg/kg DMFO for 14 days post sensitisation to OVA. DMFO at

200 mg/kg (which is 1/10th the safe dose) administered orally was well tolerated with no adverse effects noted. Antigen-induced MC degranulation, mediated by OVA-IgE binding, leads to the release of histamine and 5-hydroxytryptamine [46]. Histamine, in turn, increases the permeability of the capillaries to leukocytes, to allow them to engage perceived pathogens. Evan's Blue dye extravasation is used as a marker for the extent of histamine-induced vascular leakage. In our

model of OVA-induced MC degranulation, DMFO significantly reduced Evan's Blue dye extravasation on day 14 after OVA rechallenge, with an efficacy comparable to a standard drug ketotifen (1 mg/kg) (Figure 2(A), and Supplementary Figure 2). Importantly, DMFO did not affect IgE levels, while ketotifen, reduced IgE levels (Figure 2(B)). This confirms that the mast cell stabilising activity of DMFO is mediated entirely by targeting MCs and not induced by a reduction in IgE levels. This is significant as this implies that DMFO does not have any adverse effect on IgE-mediated immune defences.

Effect of DMFO on carrageenan-induced rat paw oedema

Carrageenan induces an acute inflammatory reaction in the injected paw, leading to fluid extravasation and

leucocyte infiltration, manifesting in the classic symptoms of inflammation, namely oedema, pain, and redness. DMFO dose-dependently reduced paw oedema with 100 and 200 mg/kg showing significant reductions 3 h post-carrageenan challenge (Figure 2(C)). DMFO (200 mg/kg) was comparable in efficacy to the standard NSAID diclofenac (10 mg/kg). However, there was no prolonged anti-inflammatory activity in either the DMFO or diclofenac-treated group as observed by an absence of any effect on paw volume measured at 6 h postinjection (data not shown). The reduction in paw volume, 3 h after carrageenan injection, indicates that DMFO is effective in the second phase of the inflammatory response, when there is COX-driven PG production, infiltration and activation of polymorphonuclear leucocytes. NSAIDs are most effective against this phase where PG are synthesised [47].

Table 2. DPPH• and ABTS^{•+} Radical scavenging activity and cell viability of DMFO.

Assay	IC ₅₀ (μM)
DPPH• radical scavenging assay	136.54 ± 7.13
ABTS ^{•+} radical scavenging assay	6.70 ± 0.07
MTT cell viability assay	338.94 ± 30.88

Effect of DMFO on carrageenan-induced inflammation in rat air pouch

Carrageenan, on entering the pouch lined with macrophages and fibroblasts, sets off an inflammatory

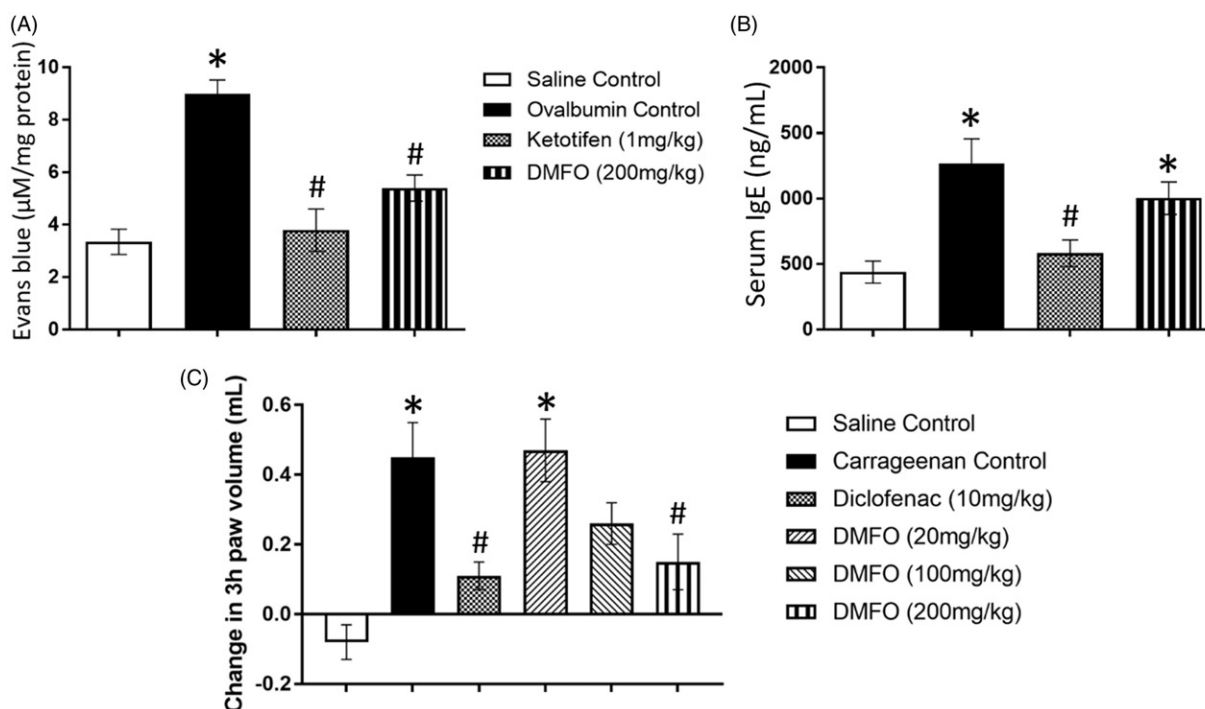


Figure 2. Effect of DMFO on OVA-induced mast cell degranulation in mice (A, B), and on Carrageenan-induced rat paw oedema (C). (A) On day 0, animals were pre-sensitised to antigen by administering OVA in alum i.p. Animals were dosed once daily. On day 14, animals were rechallenged with OVA intradermally in the ear followed by Evan's blue i.v. through the tail vein and the extent of dye extravasated into the ear (shown in Supplementary Figure 2) was quantified. Comparisons were made to a known inhibitory drug Ketotifen. (B) DMFO had no significant effect on serum IgE levels. (C) Carrageenan was injected into rats paws pretreated with different doses of DMFO or diclofenac (standard) and the change in paw volume was calculated after 3 h, using plethysmometry. Results are expressed as mean ± SEM; * $p < .05$ compared to saline control; # $p < .05$ compared to (A, B) OVA and (C) Carrageenan controls respectively.

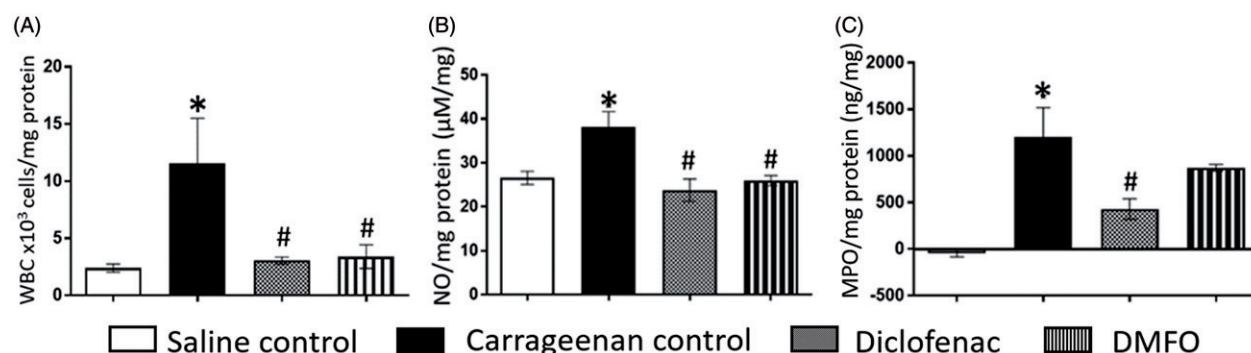


Figure 3. Effect of DMFO on Carrageenan-induced inflammation in a rat air pouch model. An air cavity was produced in rats by s.c. injection of air on day1 and day3. On day 5 post treatment with 200 mg/kg DMFO, carrageenan was injected into the pouch. After 6 h, pouch exudates were collected. Total leucocyte count (A), NO (B) and MPO levels (C) were determined and compared with diclofenac. Results are expressed as mean $\pm$ SEM; * p < .05 compared to saline control; # p < .05 compared to Carrageenan control.

granulomatous reaction involving fluid extravasation, the release of inflammatory mediators and influx of leukocytes [48]. Treatment with DMFO reduced the inflammatory reaction as effectively as the positive NSAID control, diclofenac. This was seen by (1) a significant reduction in the leucocyte count (Figure 3(A)) to the same extent as diclofenac; (2) a significant reduction in NO levels in the air pouch exudate, as effectively as diclofenac (Figure 3(B)), and (3) the suppression of classic symptoms of acute inflammation (redness and swelling) in the air pouch lining (Supplementary Figure 3). However, the reduction in MPO by DMFO was not as effective as diclofenac and failed to reach significance (Figure 3(C)).

Effect of DMFO on Freund's adjuvant-induced arthritis in rat paw

In the Freund's adjuvant-induced arthritis model (a model of immune-mediated polyarthritis [49] with significant paw oedema), Freund's adjuvant induced a significant increase in paw volume, in both the ipsilateral (Figure 4(A,C)) and contralateral (Figure 4(B,D)) paw of injected rats. DMFO significantly reduced oedema in the contralateral paw, (clearly observed in the photographs of the contralateral paw in Figure 4(B), and quantitated in Figure 4(D)), with a trend seen in the ipsilateral paw (Figure 4(A,C)) although this did not reach significance. Radiographic evaluation of the paws showed significant arthritic bone formation (joint space narrowing, bone destruction, synovial hyperplasia and pannus formation) in both the ipsilateral and contralateral paws after Freund's adjuvant treatment, which was significantly improved after DMFO treatment in both the ipsilateral and contralateral paws (Figure 5(A,B)). DMFO improved the pain threshold in the ipsilateral and contralateral paw (Figure 5(C,D) respectively), and additionally, significantly lessened the arthritis score

(Figure 5(E)) which was comparable to that seen with the standard drug diclofenac. Importantly, DMFO reduced serum TNF α levels to undetectable levels, more efficaciously than diclofenac (Figure 5(F)). Following 7 days of chronic dosing of DMFO at 200 mg/kg, rat stomachs showed no significant changes related to ulcerogenicity, unlike diclofenac, which produced haemorrhage and ulcerogenic lesions (Supplementary Figure 4). These data are therefore strongly suggestive of a nonulcerogenic role for DMFO in comparison to diclofenac (10 mg/kg).

Effect of DMFO on proinflammatory and antioxidant markers in LPS-stimulated macrophages

Macrophages play a pivotal role in chronic inflammation associated with RA [50]. NF κ B is a major regulator of the inflammatory response in macrophages through downstream mediators such as COX2 [51]. In order to investigate the effect of DMFO on this major inflammatory pathway, we investigated the effect of DMFO in mouse RAW 264.7 macrophages for its ability to inhibit COX2 protein expression by western blot. LPS treatment induced a robust increase in COX2 expression which was significantly inhibited in the presence of DMFO. (Figure 6(A,B) (normalised to total protein) and Supplementary Figure 5- normalised to the housekeeping gene α -tubulin.) We confirmed these results by performing a second independent in cell western experiment, and again showed a significant attenuation of the LPS-mediated increase in COX2 by DMFO (Figure 6(E)). DMFO also significantly increased gastroprotective HO-1 protein expression to the same extent as LPS, and led to an augmentation of HO-1 gene expression and protein levels in the presence of LPS (Figure 6(C,D)). Treatment with DMFO additionally reduced

(A) Ipsilateral



(B) Contralateral

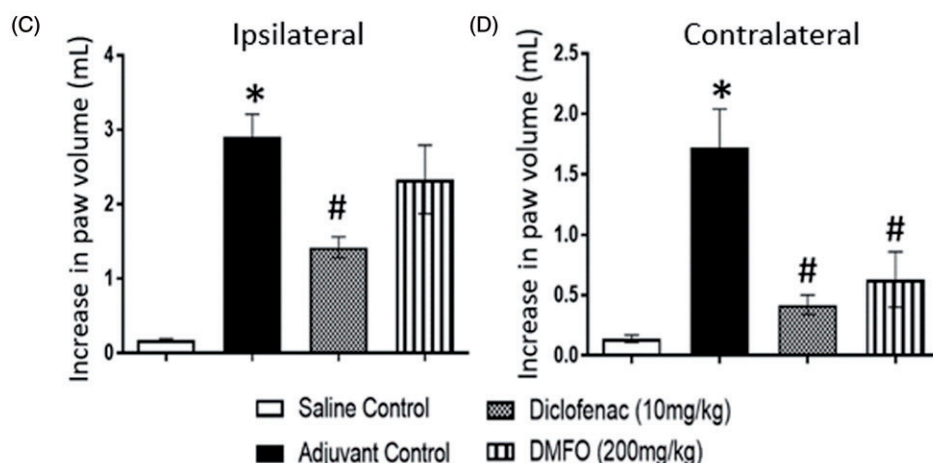
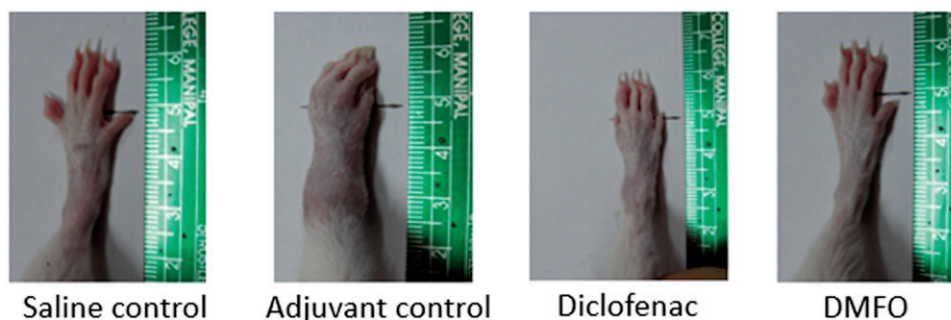


Figure 4. Effect of DMFO on (A) Ipsilateral and (B) Contralateral paw oedema in a model of Freund's adjuvant-induced arthritis. On day 0, complete Freund's adjuvant was injected intraplantarly into the right paw (ipsilateral paw). On day 14, Freund's adjuvant-induced arthritic animals were randomised and treated once daily from day 14 to day 21. On day 21, photography of the paws was undertaken. Representative photographs of Freund's adjuvant-induced arthritic rat paws are shown in A (ipsilateral) and B (contralateral). The oedema was quantitated and shown for the ipsilateral (C) and contralateral paw (D). Results are expressed as mean $\pm$ SEM; * $p < .05$ compared to saline control; # $p < .05$ compared to adjuvant control.

proinflammatory chemokine MCP1 levels in a dose-dependent manner as measured by ELISA (Figure 6(F)) in the cell culture media.

qRT-PCR studies revealed that DMFO dose-dependently reduced the expression of proinflammatory genes IL1 β , IL6, and TNF α in LPS-stimulated primary BMDMs (Figure 7(A–C)). Interestingly, DMFO dose-dependently enhanced the gene expression of the known downstream Nrf2 activated genes, NQO1, and Glo1 (Figure 7(D,E)). Glo1

activation has recently been reported to alleviate RA, suggesting that this may be one of the possible mechanisms of DMFO's antiarthritic activity [52].

Effect of DMFO on gene expression in Nrf2 KO and silenced macrophages

BMDMs isolated from WT and Nrf2 KO mice were stimulated with LPS followed by treatment with DMFO.

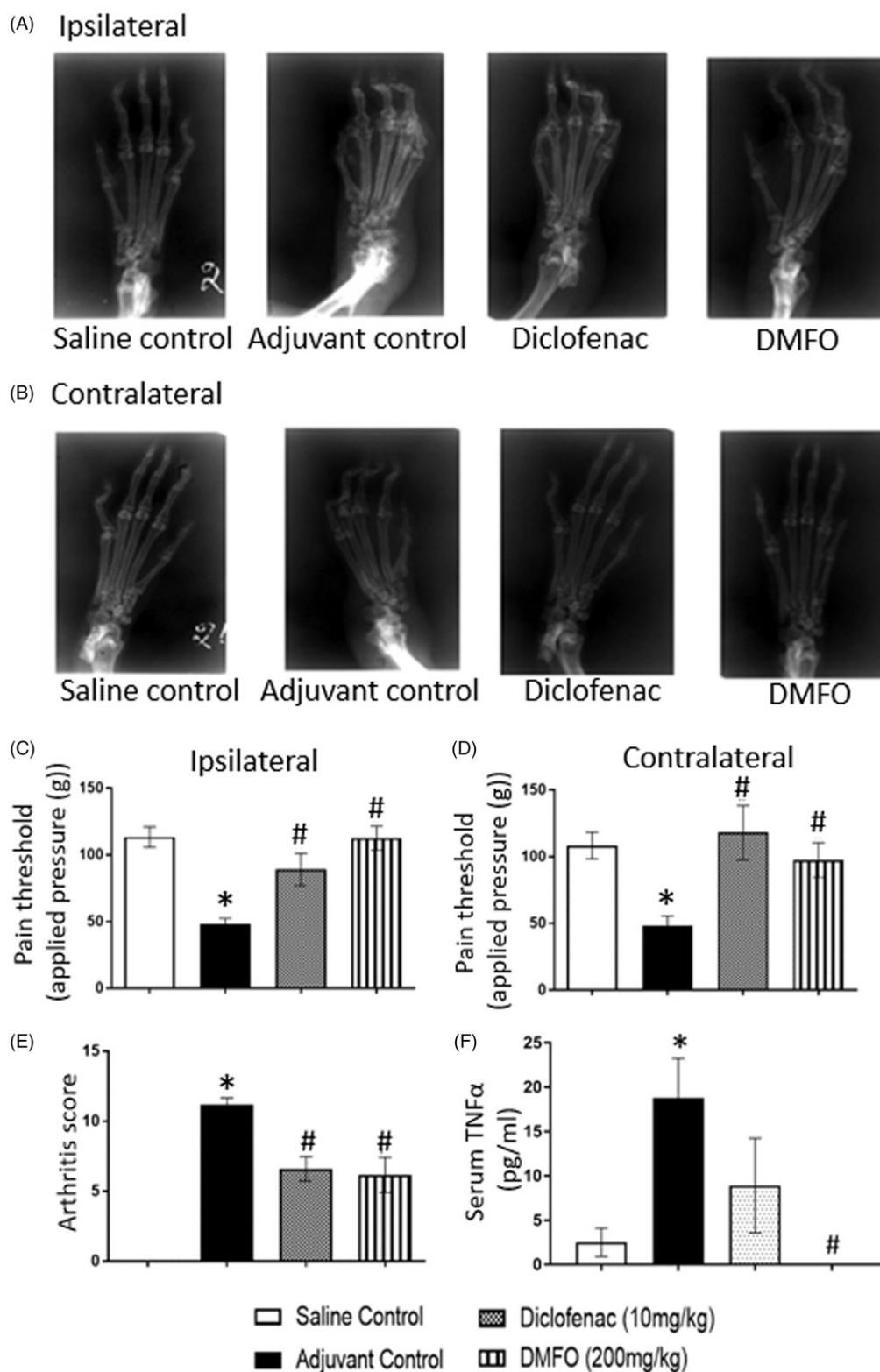


Figure 5. Effect of DMFO on arthritis score, pain threshold and serum TNF α levels in the Freund's adjuvant-induced arthritis model. Radiology of the paws was undertaken 21 days post treatment. Adjuvant control images show significant arthritic joint involvement in both the ipsilateral (A) and contralateral (B) paws. Treatment with both the positive control, Diclofenac and DMFO, prevented severe arthritic joint involvement. Paw pain thresholds after treatment with Diclofenac or DMFO are shown for both the ipsilateral (C) and contralateral (D) paw. Arthritis score, based on clinical score, mobility score and joint stiffness is shown in (E). Serum TNF α levels at the end of study period (day 21) are shown in (F). Results are expressed as mean $\pm$ SEM; * p < .05 compared to saline control; # p < .05 compared to adjuvant control.

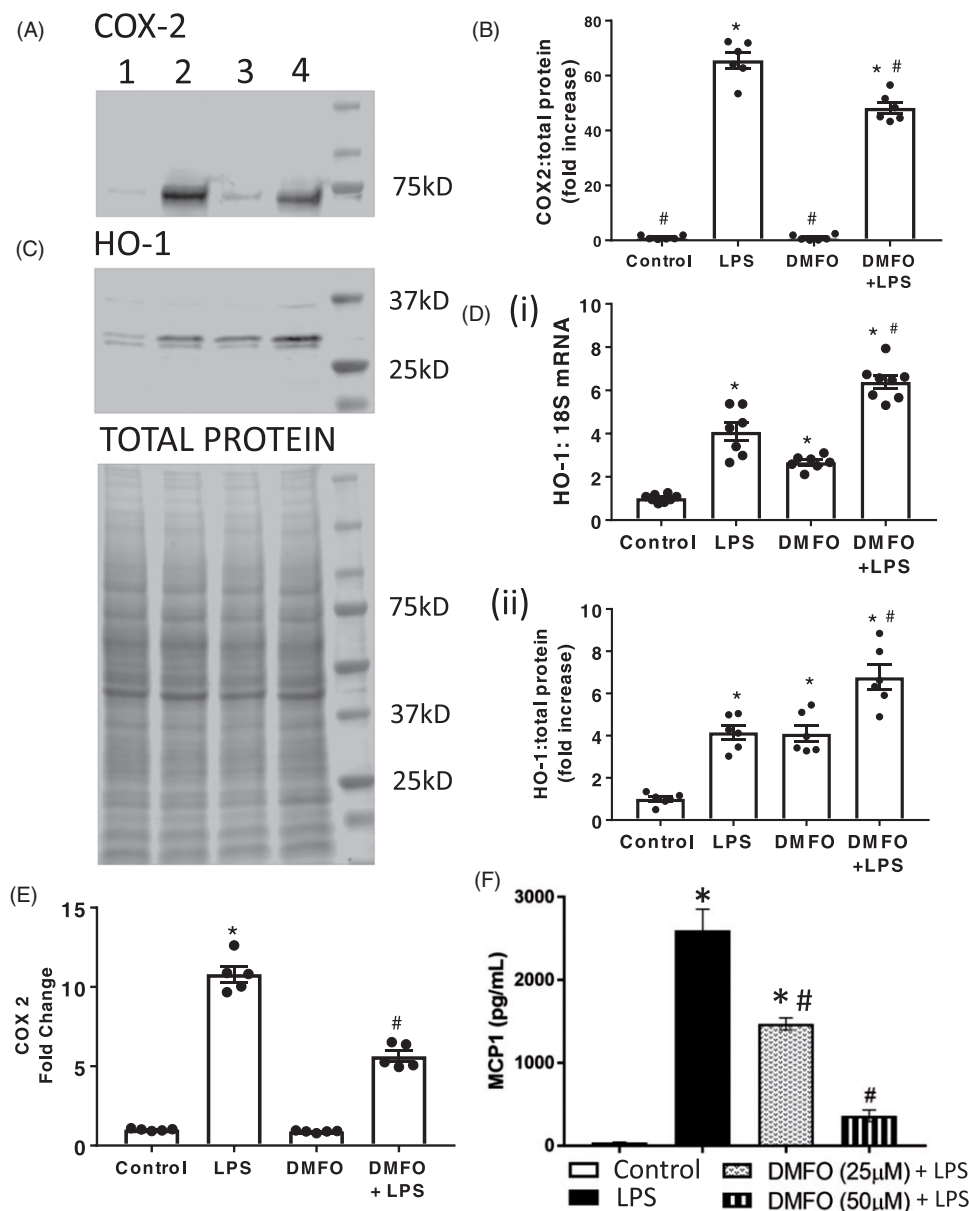


Figure 6. Effect of DMFO on COX2, HO-1 and MCP-1 expression in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were stimulated with LPS and treated with 50 μ M DMFO unless indicated otherwise. Western blots of Cox2 (p75) and HO-1 (p32) are shown in (A) and (C) respectively. Bands were quantitated and expressed relative to total protein levels in B (COX2) and D (ii) (HO-1). Gene expression for HO-1 is shown in D (i). (E) Determination of COX2 protein levels by In Cell Westerns in LPS-stimulated RAW 264.7 macrophages. (F) MCP1 levels in the supernatant were quantified using ELISA. Results are mean $\pm$ SEM; * $p < .05$ compared to media control; # $p < .05$ compared to LPS control.

DMFO caused a significant increase in Nrf2 gene expression in WT BMDMs (Figure 8(A)). As anticipated, BMDMs isolated from Nrf2 KO mice did not express the Nrf2 gene (Figure 8(A)). DMFO caused a significant ~ 2.5 -fold increase in the Nrf2 downstream gene NQO1 in WT BMDMs, which was absent in the Nrf2 KO BMDMs (Figure 8(B)). DMFO also significantly reduced IL1 β gene expression in LPS-stimulated Nrf2 WT BMDMs, but had no effect on LPS-stimulated Nrf2 KO

BMDMs (Figure 8(C)). In confirmatory experiments, RAW 264.7 macrophages were transfected with siRNA to silence Nrf2 (Figure 8(D)) –silencing of Nrf2 resulted in a $>95\%$ reduction in Nrf2 gene expression). DMFO failed to enhance Nrf2 (Figure 8(D)) or HO-1 (Figure 8(E)) gene expression in the presence of siRNA for Nrf2. Collectively, these experiments prove an Nrf2-targeted activity for DMFO in upregulating the known downstream genes of Nrf2 such as NQO1 and HO-1.

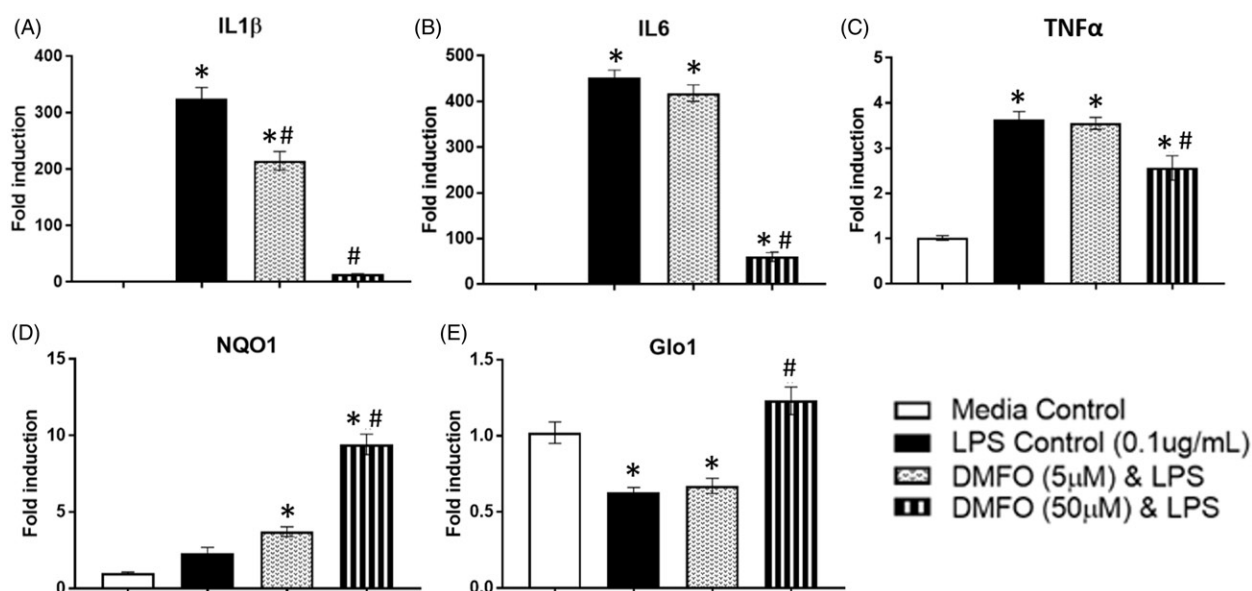


Figure 7. Effect of DMFO on proinflammatory gene expression in primary BMDMs stimulated with LPS. Proinflammatory gene expression of IL1 β (A), IL6 (B), and TNF α (C) was assessed in LPS-stimulated primary BMDMs after treatment with 5 and 50 μ M DMFO. The expression of downstream Nrf2 activated genes, NQO1 and Glo1, is shown in (D) and (E) respectively. Results are mean $\pm$ SEM; * p < .05 compared to media control; # p < .05 compared to LPS control.

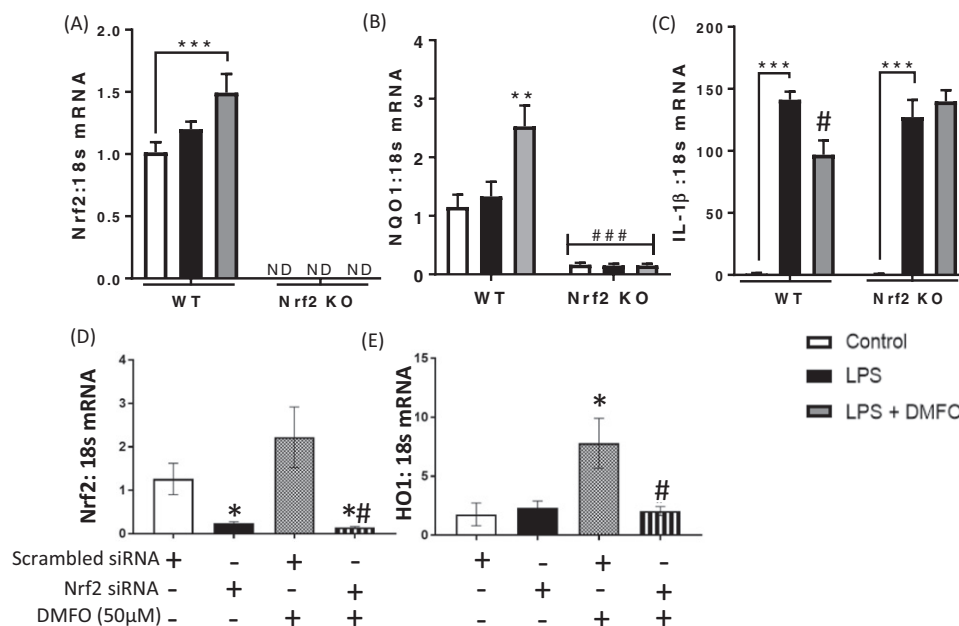


Figure 8. (A–C) Effect of DMFO on antioxidant and proinflammatory gene expression in WT and Nrf2 KO BMDMs stimulated with LPS. Cells were treated with 50 μ M (A) or 25 μ M (B) DMFO and/or LPS. Results are mean $\pm$ SEM; *** p < .001 and ** p < .01 compared to controls; ### p < .001 compared to WT counterparts; # p < .05 compared to LPS control. ND: not detected. (D, E) Effect of 50 μ M DMFO on Nrf2-siRNA transfected RAW 264.7 macrophages. Results are mean $\pm$ SEM; * p < .05 compared to Control siRNA; # p < .05 compared to Control siRNA + DMFO (50 μ M).

Discussion

Inflammation is a complex phenomenon involving a plethora of mediators and cross-regulatory, inter-dependent signalling pathways. Here, we identify that a novel synthetic small molecule, DMFO, exhibits

anti-inflammatory and antioxidant properties by targeting ROS and proinflammatory/antioxidant mediators in macrophages and by stabilising MCs against degranulation. Furthermore, our data identify that DMFO acts through an Nrf2 stimulated pathway to mediate its antioxidant and anti-inflammatory response. In *in vivo*

models, we show the efficacy of DMFO in lessening biological end-points of inflammation in models of RA and adjuvant-induced inflammation, with reduced side effects of ulcerogenicity compared with standard NSAIDs. Therefore, this novel Nrf2 targeting small molecule offers a rational therapeutic strategy to lessen inflammation by simultaneously modulating key proinflammatory and antioxidant mediators.

In initial *in-vitro* radical scavenging assays, we showed that DMFO is an effective antioxidant. Therefore, additionally, DMFO was tested for ROS inhibitory effects in LPS-stimulated macrophages. It is known that LPS, the gram-negative bacterial cell wall component [53], interacts with the cell surface pattern recognition receptor, toll-like receptor (TLR)4, on macrophages [54], leading to ROS generation [55]. Indeed, DMFO effectively reduced intracellular ROS and secreted NO levels in LPS-stimulated macrophages suggesting an inhibitory antioxidant effect of DMFO on LPS-mediated ROS generation.

Carrageenan-induced paw oedema is a widely accepted reproducible model of acute inflammation employed for screening NSAIDs [47]. The inflammatory response involves several phases, with the initial phase (0–1 h) being mediated by histamine, serotonin, and kinins. The second phase (1–3 h) involves COX-derived prostaglandin synthesis, and infiltration of leucocytes (especially polymorphonuclear leucocytes (PMNLs) such as neutrophils) resulting in visible signs such as swelling or oedema [47,56]. In our experiment, the reduction in paw volume or oedema, 3 h after carrageenan injection, indicates that DMFO is effective in the second phase of the inflammatory response, when there are infiltration and activation of PMNLs. This is in keeping with the known action of NSAIDs which are more effective against this second phase [47]. In another model of carrageenan-induced inflammation namely, the rat air pouch, carrageenan produces an acutely inflammatory air pouch lined with MCs, which together with the release of inflammatory mediators, fluid extravasation and influx of leukocytes [48], resembles the arthritic synovium [37,57]. In this model, we found that DMFO reduced the classic symptoms of acute inflammation, namely redness and swelling in the air pouch lining, in addition to reducing the leucocyte count and NO levels in the air pouch exudate, to the same extent as that seen by diclofenac. The lack of effect of DMFO on MPO distinguishes it from diclofenac which inhibits MPO in our carrageenan-induced inflammatory air pouch model. As we did not measure MPO in other models, it remains unknown whether this lack of effect by DMFO is specific to the air pouch model or is more widely applicable.

MC activation by different stimuli utilises different intracellular signalling pathways. Antigenic stimuli are responsible for IgE Fc receptors (FcεRI) crosslinking-mediated signalling events [58]. Nonantigenic stimuli such as C48/80 act via a receptor-independent peptidergic pathway [59], resulting in mobilisation of intracellular calcium. Another nonantigenic stimulus namely A23187 (calcium ionophore) directly delivers calcium ions to the cell interior via uptake through the plasma membrane [60]. Although DMFO inhibited both A23187 and C48/80-induced MC degranulation, DMFO more potently inhibited the former. This is suggestive of an action downstream of calcium mobilisation whilst affording a comprehensive MC stabilisation. We additionally studied the *in-vivo* MC stabilising activity of DMFO in a model of OVA-induced active cutaneous anaphylaxis in mice [61]. Cutaneous anaphylaxis is a localised hypersensitivity reaction to an antigen injected into the skin. Anaphylactic reaction to OVA is mediated by its binding to the IgE antibody bound to MCs, resulting in MC degranulation and release of histamine and 5-hydroxytryptamine [46]. The released histamine increases vascular permeability at the OVA-sensitised cutaneous site and induces extravasation of Evan's Blue dye [46]. Antigen-induced MC degranulation is a receptor-dependent process involving crosslinking of an allergen to IgE bound to FcεRI on MCs, leading to activation of signalling pathways that eventually culminate in degranulation [62]. In our experiments, DMFO significantly reduced dye extravasation in OVA-induced active cutaneous anaphylaxis, with an efficacy comparable to a standard drug ketotifen, and without affecting serum IgE levels. This is in agreement with our previously published observations of another indanedione-derivative DHFO which was effective in sheep-serum-induced active and passive anaphylaxis in rats [30]. Thus, DMFO stabilised MCs in both the antigen (OVA-induced active cutaneous anaphylaxis model) and nonantigen-induced (A23187 (calcium ionophore)) models, suggesting a multimodal activity which was independent of IgE. As ROS also play a significant role in MC degranulation, whether antigen-mediated or otherwise [63], the antioxidant activity of DMFO could also partly explain the MC stabilising activity. Indeed, the MC stabiliser ketotifen is a known antioxidant [64], suggesting that the MC stabilising activity observed by DMFO in this study may be related to upstream ROS inhibition.

Adjuvant-induced arthritis is a model of polyarthritis [49] resulting in inflammation of the synovium, destruction of cartilage and bone, and narrowing of joint spaces. Mechanical allodynia (responsiveness to pain)

and joint hyperalgesia (heightened sensitivity to pain), are two important features of RA, that develop as arthritis progresses [40]. In our Freund's adjuvant-induced arthritis model, DMFO significantly improved the arthritis score and pain threshold. However, DMFO appeared to reduce the pain and disability score more effectively than reducing oedema. Furthermore, DMFO offered greater protection to the contralateral paw in comparison to the ipsilateral paw, indicating a moderate systemic anti-inflammatory activity. It should, however, be noted that a therapeutic dosing strategy (treatment after induction of inflammation) being more clinically relevant, was employed in this aggressive model of chronic inflammation [65,66].

The stomach was observed for ulcerogenicity following a regimen of 7 days of DMFO dosing in our adjuvant-induced arthritis model. DMFO appeared to be nonulcerogenic, while diclofenac produced severe ulcers in addition to one case of mortality. NSAIDs are well described as nonspecifically blocking COX isozymes, and mediating ulcerogenicity by inhibiting constitutive COX1 which functions to produce gastroprotective prostaglandins [67]. As DMFO appears to be nonulcerogenic, a mechanism different from the nonspecific targeting of the COX isozymes by diclofenac, and more specifically to the targeting of COX2 could be involved and warrants further investigation. We additionally found that DMFO enhanced the gene expression of Nrf2 as well as antioxidant and cytoprotective enzymes downstream of Nrf2 activation such as NQO1, Glo1 and HO-1. Interestingly, HO-1 is gastroprotective [68,69] as it degrades free haem into cytoprotective CO and biliverdin/bilirubin [70]. The enhanced HO-1 expression may additionally contribute to the nonulcerogenic anti-inflammatory property of DMFO. Indeed, upregulation of HO-1 is reported to be an additional antiulcerogenic mechanism of proton pump inhibitors such as pantoprazole [69]. Overcoming toxicity is still considered a significant challenge in the treatment of chronic inflammatory conditions such as RA; therefore the non-ulcerogenicity of DMFO is seen as a particularly important advantage over-prescribed drugs.

In LPS-stimulated macrophages, DMFO inhibited COX2 expression and reduced chemokine MCP1 levels, implying an inhibitory role on the LPS-TLR inflammatory cascade. DMFO also reduced the gene expression of key proinflammatory cytokines such as IL6, TNF α , and IL1 β which could underlie its observed anti-inflammatory activity. It should be noted that DMFO also reduced serum TNF α levels to undetectable levels in the arthritis model. As LPS-TLR signalling induces the nuclear translocation of NF κ B which triggers an

inflammatory response and involves the upregulation of several proinflammatory mediators [55], DMFO may have interfered with NF κ B signalling in mediating its anti-inflammatory activity. Indeed, enhanced Nrf2 activity is known to negatively affect NF- κ B signalling [20] lending further support to this notion. Importantly, DMFO did not reduce proinflammatory IL1 β gene expression in macrophages derived from Nrf2 KO mice, and failed to increase the Nrf2-downstream gene NQO1 in Nrf2 KO macrophages, suggesting that the anti-inflammatory and antioxidant activity of DMFO is Nrf2-mediated. An Nrf2-targeted activity was further confirmed by highly-specific gene silencing experiments where DMFO did not affect the expression of HO-1 in Nrf2 siRNA transfected macrophages. DMFO may thus be a valuable anti-inflammatory agent because of an Nrf2-targeted anti-inflammatory and nonulcerogenic mechanism, in line with recent studies showing an anti-inflammatory effect of Nrf2 activators [71].

Conclusion

The novel synthetic anti-inflammatory small molecule DMFO was found to inhibit proinflammatory ROS production, one of the key mediators underlying the inflammatory response. Additionally, the compound also stabilised MCs, a major immune cell orchestrating the inflammatory immune response. Further studies revealed an action targeting the proinflammatory (COX2, MCP1, IL1 β , TNF α , IL6) and antioxidant (HO-1, NQO1, Glo1) mediators. Importantly, the anti-inflammatory activity demonstrated by DMFO was absent in response to a genetic deletion or silencing of Nrf2 confirming that DMFO targets Nrf2 in mediating the observed anti-inflammatory activity. Therefore, DMFO may be a promising novel, safe (even up to 2 g/kg), orally active, Nrf2-targeting anti-inflammatory drug, with the added advantage of being nonulcerogenic in its mode of action.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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