Targeted delivery of the hydroxylase inhibitor DMOG provides enhanced efficacy with reduced systemic exposure in a murine model of colitis

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Targeting hypoxia-sensitive pathways has recently been proposed as a new therapeutic approach to the treatment of intestinal inflammation. HIF-hydroxylases are enzymes which confer hypoxic-sensitivity upon the hypoxia-inducible factor (HIF), a major regulator of the adaptive response to hypoxia. Previous studies have shown that systemic (intraperitoneal) administration of hydroxylase inhibitors such as dimethyloxalylglycine (DMOG) is profoundly protective in multiple models of colitis, however the therapeutic potential of this approach is limited due to potential side-effects associated with systemic drug exposure and the fact that orally delivered DMOG is ineffective (likely due to drug inactivation by gastric acid). In order to overcome these issues, we formulated DMOG in a liquid emulsion drug delivery system which, when coated with specific polymer coatings, permits oral delivery of a reduced dose which is released locally throughout the colon. This colon-targeted DMOG formulation demonstrated increased relative colonic bioactivity with reduced systemic exposure and provided a similar degree of protection to systemic (intraperitoneal) administration at a 40-fold lower dose in DSS-induced colitis. In summary, targeted delivery of DMOG to the colon provides local protection resulting in enhanced efficacy with reduced systemic exposure in the treatment of colitis. This novel approach to targeting hydroxylase inhibitors to specific diseased regions of the GIT may improve its potential as a new therapeutic in inflammatory bowel diseases such as ulcerative colitis.

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1. Introduction

Molecular oxygen (O₂) is the primary electron acceptor of the mitochondrial electron transport chain and as such, a sufficient oxygen supply is essential to maintain metabolic homeostasis and cell survival. Over the course of evolution, metazoans have developed a highly conserved and effective ability to respond to the metabolic threat of reduced oxygen (hypoxia) with the induction of an adaptive transcriptional response which promotes tissue oxygenation. The hypoxia-inducible factor (HIF) is a key regulator of this response which controls the expression of a array of genes that promote survival through adaptation to hypoxia. HIF-dependent genes encode proteins which promote hypoxia-adaptive processes such as angiogenesis (e.g. vascular endothelial growth factor), erythropoiesis (e.g. erythropoietin) and glycolytic metabolism (e.g. pyruvate dehydrogenase kinase).

2-oxoglutarate-dependent hydroxylases are a family of dioxygenases, a subset of which play an important role in cellular oxygen-sensing through the conferral of oxygen-dependence upon hypoxia-sensitive transcriptional regulators including the HIF and Nuclear factor κB (NF-κB) [1,2]. Pharmacologic hydroxylase inhibitors including the 2-oxoglutarate mimetic DMOG have been demonstrated to be protective in multiple models of intestinal inflammation including chemical-, toxin-, radiation- and ischemia/reperfusion-induced intestinal injury [3–10]. The mechanism(s) of anti-inflammatory action of hydroxylase inhibitors in the intestine are multifactorial and may be HIF-dependent or HIF-independent [11]. Firstly, it appears that hydroxylase inhibition, through the activation of a panel of HIF-dependent barrier protective genes including intestinal trefoil factor, Mucin-3, p-glycoprotein, ecto-5'-nucleotidase and the adenosine A2B receptor leads to enhanced epithelial barrier function [12,13]. Secondly, hydroxylase inhibition in intestinal epithelial cells leads to a reduction in epithelial cell death through apoptosis which also contributes to enhanced intestinal barrier function [9,14]. Thirdly, in a HIF-independent manner, hydroxylase inhibitors decrease interleukin-1β-induced pro-inflammatory signaling and subsequent gene expression [15]. Fourthly, hydroxylase inhibition can impact upon inflammatory processes through the HIF-independent regulation of neutrophil survival [16]. Therefore, hydroxylase inhibitors represent a promising new therapeutic approach to the treatment of inflammatory
diseases of the gastrointestinal tract through both HIF-dependent and HIF-independent mechanisms [17].

Most of the experimental models which have demonstrated protective effects of hydroxylase inhibitors in intestinal inflammation to date have utilized systemic administration of the drugs [17]. However, systemic exposure to hydroxylase inhibitors results in unwanted side effects such as erythropoiesis and angiogenesis. Furthermore, oral administration of DMOG is not effective against colitis. This lack of efficacy is believed to be due mainly to susceptibility of the drug to acidic and enzymatic degradation in the stomach and small intestine. To overcome these issues, we hypothesized that targeted delivery of low dose DMOG to the colon would be of benefit in treating colitis as it would provide local colonic tissue protection without the risks associated with systemic exposure. The formulation technology used in this study was developed to permit colon-targeting and thereby enables DMOG release throughout the colon where it is then available for to provide a topical therapeutic effect. To test this hypothesis, we utilized this colon-targeted, minisphere-based technology which has been developed to target cargo drug release selectively throughout the colon with minimal release in the small intestine. This system is directed toward maximizing colonic delivery of the drug while minimizing systemic exposure. We compared the effectiveness of an orally administered, colon-targeted formulation of DMOG with systemic (intraperitoneal) administration with respect to markers of disease progression in the DSS model of murine colitis. We found that colon-targeted DMOG demonstrated enhanced efficacy in the treatment of colitis at a lower dose with significantly reduced systemic activity. In summary, we demonstrate that colon targeted, non-systemic delivery of a hydroxylase inhibitor provides a novel efficacious and safe potential alternative therapeutic approach to treating ulcerative colitis.

2. Materials & methods

2.1. Preparation of minispheres

Minispheres were prepared according to the protocol detailed in the US patent register (patent # WO 2008/122967) and outlined in Table 1. The source and chemical grades of the components used for formulation are outlined in Supplementary Table 3. Briefly, DMOG (Caymen Chemicals, >98% purity), Sodium Dodecyl Sulfate, D-Sorbitol and Type A porcine gelatin were added to water under constant agitation with a standard magnetic stirrer at 100–250 rpm. This is referred to as the aqueous phase. Transcutol HP, Cremophor EL and Miglyol 810 N were stirred at room temperature with a standard magnetic stirrer at 100–250 rpm until a clear solution was obtained. This is referred as the oil phase. The oil phase was mixed with the aqueous phase in a 1:10 ratio to form an emulsion that was stirred using a standard magnetic stirrer at 100–250 rpm to achieve complete homogeneity. The homogenous emulsion was then ejected through a single orifice (approximately 1 mm diameter) to form droplets which solidify in cooling oil medium (Miglyol 810 N) to form beads which were recovered by passing through a 500 μm sieve and then dried overnight at room temperature. These minispheres are referred to throughout the manuscript as the “uncoated DMOG minispheres”. Uncoated DMOG minispheres were weighed and coated with a combination of ethylcellulose:pectin (E:P) (98:2% w/w) using a Würster coater (Freund-Vector MFL01 Micro Fluid Bed Dryer; Supplementary Fig. 1). The degree of E:P coating thickness was calculated as a function of % dry weight gain achieved. The weight gain achieved was 6.5%. These minispheres are referred to throughout the manuscript as “coated DMOG minispheres”. The preparation efficiency using this approach is approximately 85% (input/output). Using this formulation approach, 90% of the minispheres harvested are within the 1.0–1.4 mm diameter range while 10% are between 1.4 and 2.0 mm in diameter. X-ray tomography and microscopic images of sample beads are shown in Supplementary Fig. 2.

After the minispheres are orally administered, the ethylcellulose–pectin coating layer starts to wet and hydrate which initiates the creation of channels in the polymer coating; the thickness of the coat correlates with the time when the cores starts to dissolve and release the soluble and intact DMOG into the gastrointestinal lumen. Due to the coating thickness of these beads, the release is delayed until the beads reach the colon. Once in the large intestine the pectin, which is included as a pore-former in the ethylcellulose is degraded by the bacteria that are present in the colon. This degradation creates pores in the coating layer through which the drug is released, further enhancing the colon-targeted release of DMOG. The inclusion of the oil phase exipients further modulate the rate of dissolution of the core and support the interaction between the drug and the colon tissue.

2.2. Dissolution of minispheres

Minispheres were added to 1 ml Dulbecco’s modified Eagle cell culture medium in 1.5 ml Eppendorf tubes. The tubes were placed on a rotating platform for 12 h to allow complete dissolution and DMOG release. The resulting mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was collected and filtered through a 0.22 μm filter for sterile filtration (Millex-GP filter unit; Millipore).

2.3. Luciferase assay

Hela cells were transfected with a luciferase reporter construct encoding the firefly luciferase gene under the control of a concatamer of hypoxia response elements (HRE). Cells were cultured for 24 h before treatment with filtered supernatant containing the dissolved spheres for up to 6 h after which cell lysates were harvested at room temperature following washing using 200 ul of 1 × luciferase lysis buffer (Promega, UK). Lysates were transferred to Eppendorf tubes and centrifuged at room temperature at 14,000 rpm for 6 min. 20 ul of lysate was added to 100 ul luciferase substrate solution (Promega, UK) and mixed vigorously using a pipette for 5 s. The 5 ml test-tube was immediately placed in a desktop luminometer (Berthold Technologies Junior LB 9509) and emitted light units were read over a 20 s period. The cumulative light units were displayed. The read-out is relative light units or RLU.

2.4. Oral administration of colon targeted spheres

The spheres were administered orally using a modified oral gavage needle (Supplementary Fig. S3). The sharp tip of a 16.5 gauge needle was cut and the edges were smoothened by a metal file. A 50 mm piece of clear, flexible PVC tube with the internal diameter 1.5 mm was fixed on the needle and secured using glue. The minispheres were loaded in the PVC tube. The needle was then fixed on to a 1 ml syringe containing 50 μl of PBS and 800 μl air. The needle and syringe assembly is positioned at the esophagoreal opening and the sphere was released.
by pressing the syringe plunger. The force created with air in the syringe should be sufficient to deliver the mini-capsule to the stomach of the mouse. The PBS acts as a delivery medium and facilitates delivery of the sphere.

2.5. In vivo imaging (IVIS)

Female mice expressing a transgene encoding firefly luciferase under the control of a concatamer of NF-κB response elements (Caliper LS) were dosed with vehicle (oral), uncoated DMOG minispheres (0.2 mg DMOG/mouse/oral), coated DMOG minispheres (0.2 mg DMOG/mouse/oral), DMOG (8 mg DMOG/mouse/intraperitoneal) or IP E. coli-derived LPS (Enzo Life Sciences; 2 mg/kg/intraperitoneal) 24 h prior to in vivo imaging. The mice were anesthetized and injected with luciferin (150 mg/kg IP). In vivo luciferase activity (indicative of NF-κB activation), measured as photons/s/cm²/sr, was quantified using Living Image Software (version 3.0.2; Caliper LS). Following whole animal in vivo imaging, colons were harvested for ex vivo imaging.

2.6. Dextran sodium sulfate (DSS) model of colitis

For DSS colitis experiments, C57BL/6 female mice in the age range of 10–12 weeks were used (Charles River U.K.). All procedures described were approved by the University College Dublin Animal Research Ethics Committee (AREC-09-06-Tambuwala-Taylor). Animal license (B100/3762) was obtained from the Irish Department of Health.

Colitis was induced by administering dextran sodium sulfate (DSS) (4000–5000 in drinking water. Acute disease was induced in C57BL/6 mice with 2.5% DSS dissolved in the drinking water for 6 days. Disease activity index was determined by the assessment of weight loss, stool consistency and presence of blood in feces. The scoring system based on these symptoms is shown in Supplementary Table 1. On termination of the experiment, mice were sacrificed by cervical dislocation.

The isolated colon was excised, washed in PBS and laid flat on moist tissue and colon length was measured and recorded. Sections (approximately 1 cm) of excised colonic tissue were fixed in 10% paraformaldehyde (pH 7.4; phosphate-buffered saline [PBS] buffered) and embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin. Histologic assessment and scoring of colon tissue sections was carried out in a blinded fashion as described in Supplementary Table 2. All tissue slides were imaged using the Aperio ScanScope XT Slide Scanner with ImageScope software v1.3.IV.

2.7. Erythropoietin (Epo) ELISA assay

Following experimental treatment, whole blood was collected by cardiac puncture. Serum was separated from the whole blood. The serum Epo levels were quantified using a Quantikine Mouse Epo ELISA kit according to manufacturer’s instructions (R&D systems).

2.8. Intestinal permeability measurements

Following experimental treatment, mice received oral administration of fluorescein isothiocyanate (FITC)-labeled dextran (4 kDa) by standard oral gavage. Mice were sacrificed 4 h later, and blood was removed by cardiac puncture. Serum was separated, and FITC levels in the serum were determined by fluorometry.

2.9. Statistical analysis

In vivo experiments were carried out on N = 5–6 individual mice. Statistical comparisons were made using one-way analysis of variance (ANOVA) or Student’s t-test using the GraphPad Prism 5 software package.

3. Results

We and others have demonstrated, using pre-clinical models, that pharmacologic hydroxylase inhibition represents a promising new therapeutic approach to colitis (11). However, because of potential side effects associated with systemic delivery of hydroxylase inhibitors (including angiogenic, metabolic and erythropoietic effects), it is desirable to develop new methods to target delivery of lower, yet still efficacious concentrations of hydroxylase inhibitors to specific regions of the intestine. The aim of this is to provide local therapeutic effects while limiting systemic exposure and associated side effects. To do this, we have utilized an emulsion-based drug delivery system. Initially, we demonstrated that the process of formulating DMOG into a novel emulsion-based colon-specific minisphere delivery system did not affect its pharmacologic activity. Coated DMOG minispheres were dissolved in tissue culture medium and the biological activity of the resultant dilute was compared with equivalent concentrations of non-formulated DMOG. Importantly, formulation of DMOG into coated minispheres did not significantly affect its ability to induce HIF-activity as assessed by HRE-luciferase reporter assay in Hela cells (Fig. 1). Therefore, the physical process of formulation of DMOG into the colon-specific drug delivery sphere system does not impact its biological activity.

We next investigated whether coated DMOG minispheres achieve enhanced colonic delivery when administered orally to mice in comparison to uncoated DMOG minispheres. To investigate this, we used transgenic mice which ubiquitously express the firefly luciferase gene under the control of a concatamer of NF-κB response elements (NRE) to assess NF-κB activity in vivo. NF-κB is a transcriptional regulator which is known to be protective in intestinal epithelial cells and has been previously demonstrated to be regulated in a hydroxylase–dependent manner [18,19]. Using this bioassay for hydroxylase inhibitory activity, we demonstrate that mice treated with the pro-inflammatory stimulus LPS demonstrate a dramatic increase in whole body and colonic NF-κB activity when compared to animals receiving the blank/empty minosphere (Fig. 2A–D). Intraperitoneal DMOG administration resulted in a mild increase in whole animal NF-κB activity without significantly affecting colon levels. In contrast, orally-delivered coated DMOG minispheres elicited a significant increase in colonic NF-κB activity without altering whole animal levels (Fig. 2A–D). Notably and as described previously, the degree of NF-κB activity elicited by DMOG was significantly less than that elicited by LPS. These data demonstrate that colon-targeted DMOG selectively increased basal NF-κB activity in the colon.

We next investigated whether the therapeutic efficacy of colon-specific DMOG delivery in a murine model of DSS-induced colitis is retained. Disease development was monitored in mice exposed to DSS in their drinking water with either IP DMOG (8 mg), uncoated DMOG minispheres (2 × 0.2 mg) or coated DMOG minispheres (2 × 0.2 mg). We first measured Disease Activity Index (DAI) and demonstrated that while uncoated DMOG minispheres were ineffective in ameliorating disease activity, coated DMOG minispheres demonstrated a comparable efficacy to systemic (IP) DMOG (Fig. 3A). Using colon length as a measure of disease, treatment of both IP DMOG and coated DMOG minispheres (but not uncoated DMOG minispheres) resulted in a significant reduction in DSS-induced colon shortening (Fig. 3B). Furthermore, quantified histological analysis of colonic tissues further supported a comparable protective effect of coated (but not uncoated) DMOG minispheres to that provided by systemic (IP) DMOG administration (Fig. 3C & D). Of note, a 40-fold lower dose of DMOG was given in the minispheres than was provided in the IP injection. Taken together, these data demonstrate that colon-targeted DMOG delivery offers equivalent protection at a significantly reduced dose in DSS-induced colitis.

Previous work has demonstrated that a significant proportion of the protective effects of DMOG observed in models of intestinal inflammation
Fig. 1. Formulation in coated minispheres does not affect DMOG’s pharmacologic activity. The pharmacologic activity of DMOG as reflected by HIF activation was assessed in Hela cells transfected with a HRE-luciferase reporter. Cells treated with dissolved coated DMOG minispheres (blue bars) or an equivalent dose of unformulated DMOG (purple bars) induced a similar level of HIF activity. Dissolved empty control spheres were used as a negative control (yellow bars). N = 3. NSD indicates not statistically significantly different as assessed by Student’s t-test.

Fig. 2. DMOG-induced NF-κB activity in reporter mice. (A) In vivo imaging of NF-κB activity in whole mice treated with 8 mg IP DMOG (top left), coated DMOG minispheres spheres containing 0.2 mg DMOG (top right), coated empty spheres (bottom left) or an intraperitoneal LPS injection (bottom right). (B) Total photon counts were measured to quantify signals from whole mouse reporter experiments. (C) Ex vivo imaging of NF-κB activity in colons from mice treated with 8 mg IP DMOG (top left), coated DMOG minispheres containing 0.2 mg DMOG (top right), coated empty spheres (bottom left) or IP LPS injection (bottom right). (D) Total photon counts were measured to quantify signals from ex vivo colonic tissue reporter experiments. Each control and experimental groups contain a minimum of 5 individual mice; *P < 0.05; **P < 0.01; and ***P < 0.001.
Fig. 3. Enhanced protection against DSS-induced colitis with colon-targeted DMOG delivery. (A) Disease activity index was assessed in mice treated with DSS and PBS vehicle (red line), DSS and uncoated DMOG minispheres (0.2 mg; black line), DSS and coated DMOG minispheres (0.2 mg; blue line) or DSS and IP DMOG (8 mg; green line). (B) Colon length in mice treated with DSS and vehicle, DSS and IP DMOG (8 mg), DSS and uncoated DMOG minispheres (0.2 mg) or DSS and coated DMOG minispheres (0.2 mg; blue line) over 6 days. (C) Histologic images of intestinal tissue from healthy mice or mice treated with DSS and vehicle, IP DMOG (8 mg), uncoated DMOG minispheres, or coated DMOG minispheres over 6 days. (Scale bar = 100 μm) (D) Histological scoring in mice treated with DSS and vehicle, DSS and IP DMOG (8 mg), DSS and uncoated DMOG minispheres (0.2 mg) or DSS and coated DMOG minispheres (0.2 mg; blue line) over 6 days. Each control and experimental group contained a minimum of 5 individual mice; *P<0.05; **P<0.01; and ***P<0.001.

Fig. 4. Reduced epithelial permeability in mice treated with coated DMOG minispheres. (A) Circulating EPO levels in mice treated with DSS and vehicle, DSS and IP DMOG (8 mg), DSS and uncoated DMOG minispheres (0.2 mg) or DSS and coated DMOG minispheres (0.2 mg; blue line) over 6 days. (B) Mice were treated with DSS with or without IP DMOG (8 mg), uncoated DMOG minispheres (0.2 mg) or coated DMOG minispheres. 4 kDa-FITC-labeled dextran was administered orally and serum levels of FITC were assessed. Each control and experimental groups contained a minimum of 5 mice; *P<0.05; **P<0.01; and ***P<0.001.
is a result of enhanced intestinal epithelial barrier function which diminishes the exposure of the mucosal immune system to luminal antigenic material. We investigated the impact of colon-targeted DMOG delivery on intestinal barrier function in mice exposed to DSS with and without DMOG along with an oral gavage of 4 kDa FITC labeled dextran. The amount of this compound entering the blood stream is reflective of intestinal permeability. The increase observed in intestinal permeability to FITC dextran in DSS treated mice was significantly diminished in animals treated with IP DMOG. Furthermore, coated DMOG minispheres provided an equivalent degree of protection to intestinal epithelial barrier function to IP DMOG while the uncoated DMOG minispheres did not significantly decrease DSS-induced intestinal barrier dysfunction (Fig. 4A).

To investigate systemic exposure to DMOG, circulating EPO levels, as a proxy for DMOG-induced systemic activity, were measured. EPO levels were reduced in animals treated with DSS when compared to healthy controls (Fig. 4B). There was a significant increase in circulating EPO with IP DMOG administration, uncoated DMOG minispheres and coated DMOG minispheres. However, the coated DMOG minispheres elicited a lower EPO response than the IP group, suggesting reduced systemic exposure to the drug. Of note, the total dose of colon-targeted DMOG was approximately 2.5% of the IP dose thus reflecting a significant advantage in terms not only of the benefits of limiting systemic exposure, but also of the lower dose of drug required for therapeutic effect when colon-targeted.

In summary, these data demonstrate that formulation of a reduced dose of DMOG in a colon-targeted manner allows the retention of therapeutic efficacy with the important added benefit of significantly reducing the systemic exposure. Therefore, we propose that targeted delivery of DMOG or other hydroxylase inhibitors may represent a new therapeutic approach to inflammatory bowel diseases such as ulcerative colitis.

4. Discussion

Hydroxylase inhibitors are becoming recognized as a potentially important new class of therapeutic agents [11]. Indeed, a number of clinical trials are currently in process to evaluate their efficacy in the treatment of anemia. A number of recent pre-clinical studies have indicated that the therapeutic utility of these agents may well extend beyond anemia to inflammatory diseases of the intestine such as inflammatory bowel disease (IBD) [9,10,20–23] However, for the treatment of IBD, several issues exist in relation to the effects of systemic exposure to hydroxylase inhibitors such as inappropriate erythropoiesis [2]. While desirable in the treatment of anemia this erythropoiesis would represent an unwanted side-effect in the treatment of IBD. Furthermore, there is a concern that unwanted pro-angiogenic side effects may also reduce the usefulness of systemic hydroxylase inhibitors for the treatment of IBD [11]. Responding to a need to regulate the dose and tissue-specific delivery of hydroxylase inhibitors such that the therapeutic benefits may be maximized while limiting unwanted systemic effects, this study reports data generated from a colon-targeted, emulsion-based formulation of DMOG in an in vivo model of colitis. Of note, while a murine model is used here (where transit-time, water content and intestinal luminal features such as pH may differ), the formulation process can be tailored for colonic release in human subjects.

Importantly, the formulation of DMOG into coated minispheres was without effect on its pharmacologic potency. Furthermore, coated DMOG minispheres elicited a localized activity of NF-κB in the colon without eliciting a systemic effect. IP-injected DMOG resulted in a heightened systemic expression of NF-κB, with limited colonic expression. Thus, colon-targeted coated DMOG minispheres targets DMOG to release throughout the colon where it acts locally while limiting systemic exposure. Importantly, the efficacy of DMOG released from coated DMOG minispheres was comparable to IP injected DMOG. However, the coated DMOG minisphere dose required for therapeutic efficacy was 40-fold less than that required when administered by IP injection.
Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.09.022.

References