

Original Research Article

Colon-targeted doxycycline–procaine azo mutual prodrug attenuates acetic-acid–induced ulcerative colitis in mice via modulation of CRP, IL-6, IL-10, and TNF- α

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Abstract

Purpose: To investigate the anti-inflammatory effects of doxycycline-procaine (Dox-Proc) compared to Mesalazine, doxycycline, and procaine on acetic acid-induced ulcerative colitis (UC) in mice.

Methods: A total of 70 mice were equally randomized into 7 groups ($n = 10$). Ulcerative colitis was induced with intracolonic 5 % acetic acid. Group 1 was the control group, group 2 received acetic acid induction without treatment, group 3 received low-dose Dox-Proc (100 mg/kg), group 4 received high-dose Dox-Proc (300 mg/kg), group 5 received Mesalazine (200 mg/kg), group 6 received Doxycycline (64.2 mg/kg) and group 7 received procaine (34.1 mg/kg) every day for seven days. Serum C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumour necrosis factor- α (TNF- α) were measured following standard procedures.

Results: Acetic-acid colitis significantly raised CRP, IL-6, and TNF- α , and decreased IL-10 compared to control ($p < 0.0001$), which were normalized following administration of Dox-Proc. Doxycycline and procaine alone significantly elevated levels of CRP, IL-6, and TNF- α ($p < 0.0001$).

Conclusion: The Dox-Proc azo prodrug modulates the levels of CRP, TNF- α , IL-6, and IL-10, demonstrating significant anti-inflammatory effects in acetic-acid induced UC.

Keywords: Anti-inflammatory, Azo prodrug, Doxycycline, Procaine, Ulcerative colitis

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INTRODUCTION

Ulcerative colitis is a chronic, idiopathic inflammatory bowel disease characterized by persistent mucosal inflammation extending proximally from the rectum to the colon [1]. The pathophysiology is multifaceted, including genetic predisposition, environmental stimuli, dysbiosis of gut microbiota, and disruption of epithelial barrier and regulatory immunological processes [2]. A fundamental feature is the

sustained activation of NF- κ B in intestinal epithelium and immune cells of the lamina propria, which stimulates synthesis of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and other chemokines. This enhances leukocyte recruitment and prolongs tissue damage [3]. Chronic inflammation not only results in severe symptoms such as diarrhoea, rectal bleeding, and abdominal discomfort but also elevates the risk of colitis-associated colorectal cancer following prolonged oxidative

stress, DNA damage, and epigenetic modifications [4]. Present pharmacotherapy depends on 5-ASA derivatives, systemic or topical corticosteroids, thiopurines, methotrexate, calcineurin inhibitors, and biologics that target TNF- α , integrins, or interleukin pathways [5]. Despite the transformative effect of these drugs on ulcerative colitis care, they are constrained by incomplete or diminished efficacy, need for parenteral administration, and significant side effects, including infections and malignancies [6]. As a result, there is an increasing interest in colon-targeted drug delivery methods and prodrugs that may localize pharmacological effects to the affected mucosa while reducing systemic exposure [7].

Doxycycline, a derivative of tetracycline, is widely used as a broad-spectrum antibiotic with non-antimicrobial properties, such as matrix metalloproteinase inhibition, attenuation of NF- κ B activation, and reduction of pro-inflammatory cytokine production [8]. Procaine, a local anaesthetic, has been shown to regulate autonomic and immunological responses and may affect inflammatory signals [9]. A mutual azo prodrug, Dox-Proc, which links doxycycline and procaine, was systematically engineered to be hydrophilic and is absorbed little in the upper gastrointestinal tract, allowing it to pass through the small intestine mostly unaltered. Bacterial azoreductases in the colon break the azo bond, liberating free doxycycline and procaine into the colonic lumen, where they influence mucosal immunological pathways and dysbiotic microbiota [10]. The prodrug has been produced, structurally validated using FT-IR and NMR spectroscopy, and shown in preclinical rat experiments to diminish colonic inflammation and enhance survival. However, its effects on systemic cytokine profiles in a murine UC model have not been thoroughly described [11]. This study therefore investigated the anti-inflammatory effects of Dox-Proc compared to Mesalazine, doxycycline, and procaine on acetic acid-induced ulcerative colitis in mice.

EXPERIMENTAL

Animal

A total of 70 mice were equally and randomly assigned into 7 groups ($n = 10$). The animals were housed under conventional research laboratory conditions ($25 \pm 2^\circ\text{C}$, 12 h light / dark cycle) with unrestricted access to standard food and water to acclimatize for 9 days. Ethical approval was obtained from the Pharmacy College, Uruk University (approval number:

263A) and complied with institutional protocols for the care and use of laboratory animals [12].

Induction of ulcerative colitis

Following an overnight fast with unrestricted access to water, all groups (except the control group) were subjected to mild anesthesia and ulcerative colitis was induced by intracolonic administration of 0.1 – 0.2 mL of 5 % (v/v) acetic acid using a flexible plastic catheter inserted about 3 cm into the rectum. Animals were positioned with their heads facing downwards for 30 s to avert leaking, and returned to their cages. Control group was administered an equivalent amount of normal saline in place of acetic acid [13,14].

Treatment protocol

Two days after acetic acid induction (day 3), daily oral treatments commenced and persisted for seven consecutive days. Group 1 was the normal control group, group 2 received acetic acid induction without treatment (colitis control group), group 3 received low-dose Dox-Proc (100 mg/kg), group 4 received high-dose Dox-Proc (300 mg/kg), group 5 received Mesalazine (200 mg/kg), group 6 received Doxycycline (64.2 mg/kg) and group 7 received procaine (34.1 mg/kg) every day for seven days. The normal and colitis control groups were administered a corresponding amount of the vehicle. All treatments were delivered daily by oral gavage [15].

Sample collection

At the end of the treatment period, mice were fasted overnight, euthanized, and whole blood (5 mL) was collected from the jugular vein in a gel tube, centrifuged, and the serum was collected for further analysis.

Evaluation of parameters/indices

Serum levels of CRP, IL-6, IL-10, and TNF- α

Serum levels of CRP, IL-6, IL-10, and TNF- α were determined using ELISA kits (Elabscience Biotechnology, USA) following the manufacturer's instructions. Standards and samples were added to pre-coated microplates, incubated with biotinylated detection antibodies and streptavidin–HRP conjugate, developed with TMB substrate, and read at 450 nm.

Clinical disease activity scoring

Following induction of colitis, a modified DAI scoring method was used to assess the consistency and condition of the faeces from the mice. The DAI scores (0 – 4) were computed based on consistency and presence of blood in faeces [16].

Statistical analysis

Data were analyzed using GraphPad Prism version 8 (RRID: SCR_002798). Continuous data were presented as mean $\pm$ standard deviation (SD) and analyzed using analysis of variance (ANOVA). Tukey's multiple comparisons post hoc test was used for comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Serum CRP

Group 2 demonstrated significantly higher serum CRP levels compared to group 1 ($p < 0.0001$). There was no significant difference in serum CRP levels in groups 3 and 4 compared to control (group 1; $p > 0.05$). Furthermore, groups 6 and 7 exhibited significantly higher CRP levels compared to control ($p < 0.0001$; Table 1).

Table 1: Serum level of CRP (mg/L; mean $\pm$ SD)

Group	CRP
1	10.4 $\pm$ 2.00
2	22.85 $\pm$ 2.54*
3	12.56 $\pm$ 2.60
4	10.68 $\pm$ 1.35
5	11.9 $\pm$ 3.29
6	20.75 $\pm$ 4.33*
7	19.67 $\pm$ 3.46*

* $P < 0.0001$ vs control (group 1), group 2 (acetic acid-induced), group 3 (100 mg/kg Dox-Proc), group 4 (300 mg/kg Dox-Proc), group 5 (200 mg/kg mesalazine), group 6 (64.2 mg/kg doxycycline), group 7 (34.1 mg/kg procaine)

Serum TNF- α

Group 2 demonstrated significantly higher serum TNF- α compared to group 1 ($p < 0.0001$). However, treatment with Dox-Proc reduced TNF- α to baseline with no significant difference compared to the control and values comparable to mesalazine ($p > 0.05$). Also, groups 6 and 7 exhibited significantly higher TNF- α levels compared to control ($p < 0.0001$; Table 2).

Serum IL-6

Group 2 demonstrated significantly higher IL-6 levels compared to group 1 ($p < 0.05$). Groups 3 and 4 showed lower IL-6 levels closer to group 5. In contrast, groups 6 and 7 exhibited significantly higher IL-6 levels compared to control ($p < 0.0001$; Figure 1).

Table 2: Serum level of TNF- α (pg/mL; mean $\pm$ SD)

Group	TNF- α
1	151.6 $\pm$ 12.28
2	354.3 $\pm$ 76.2*
3	156.2 $\pm$ 25.93
4	151.9 $\pm$ 15.88
5	146.5 $\pm$ 16.14
6	257.3 $\pm$ 63.55*
7	243.7 $\pm$ 31.24*

* $P < 0.0001$ vs control (group 1), group 2 (acetic acid-induced), group 3 (100 mg/kg Dox-Proc), group 4 (300 mg/kg Dox-Proc), group 5 (200 mg/kg mesalazine), group 6 (64.2 mg/kg doxycycline), group 7 (34.1 mg/kg procaine)

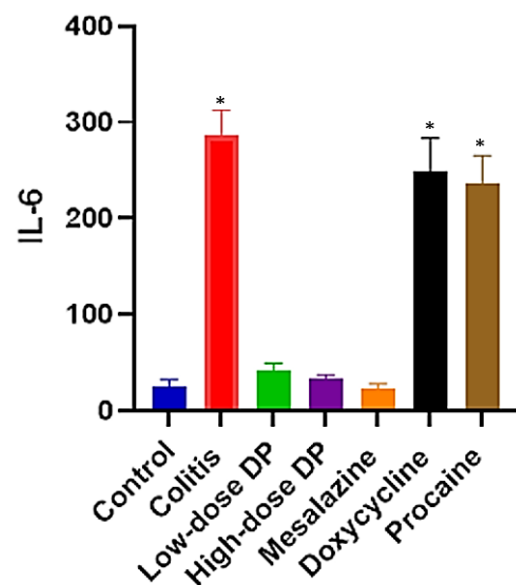


Figure 1: Serum level of IL-6 (pg/mL) for all groups. Mean $\pm$ SD. * $P < 0.0001$ vs control (group 1), group 2 (acetic acid-induced), group 3 (100 mg/kg Dox-Proc), group 4 (300 mg/kg Dox-Proc), group 5 (200 mg/kg mesalazine), group 6 (64.2 mg/kg doxycycline), group 7 (34.1 mg/kg procaine)

Serum IL-10

Serum IL-10 was significantly lower in group 2 ($p < 0.0001$) compared to control. Groups 3 and 4 showed slightly reduced levels of IL-10, which were not significantly different from control and group 5. Also, groups 6 and 7 showed significantly lower IL-10 levels compared to group 1 ($p < 0.0001$; Figure 2).

Clinical disease activity index (DAI)

The disease activity index for group 1 was 0, and 1 for groups 3, 4, and 5. Also, the DAI for group 2 was 4, and groups 6 and 7 were 3.

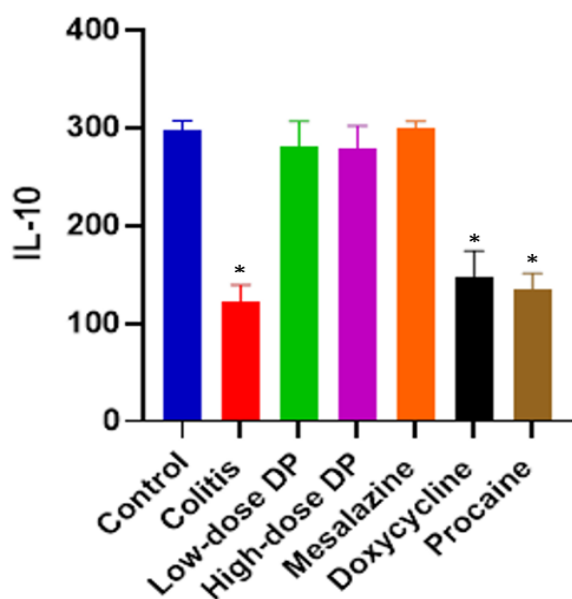


Figure 2: Serum level of IL-10 (pg/mL) for all groups. * $P < 0.0001$ vs control (group 1), group 2 (acetic acid-induced), group 3 (100 mg/kg Dox-Proc), group 4 (300 mg/kg Dox-Proc), group 5 (200 mg/kg mesalazine), group 6 (64.2 mg/kg doxycycline), group 7 (34.1 mg/kg procaine)

DISCUSSION

This study showed that the colon-targeted Dox-Proc azo mutual prodrug has a wide range of anti-inflammatory activity in acetic acid-induced ulcerative colitis in mice [17]. Acetic acid induced the anticipated pattern of increased systemic inflammation, characterized by higher levels of CRP, IL-6, and TNF- α , with reduced IL-10, indicating the activation of NF- κ B-dependent pathways and the inhibition of regulatory cytokine networks [18]. C-reactive protein (CRP) is an acute-phase protein produced by hepatocytes in response to IL-6 and TNF- α , and the reduction of CRP to baseline following treatment with Dox-Proc and mesalazine indicated the suppression of both hepatic acute-phase response and upstream cytokine activation, aligning with diminished colonic NF- κ B activity [19].

Tumour necrosis factor- α (TNF- α) is a primary mediator of intestinal inflammation, facilitating leukocyte recruitment and epithelial death. Reduction of TNF- α levels to baseline following treatment with Dox-Proc indicated that colonic administration of doxycycline effectively suppressed NF- κ B-mediated TNF- α

transcription, as doxycycline has been demonstrated to inhibit NF- κ B nuclear translocation at post-translational level [20]. Mesalazine decreases TNF- α by inhibiting cyclooxygenase and lipoxygenase pathways to reduce production of prostaglandins and leukotrienes, respectively. Mesalazine also reverses the antiproliferative effects of TNF- α , thus disrupting the effect of cytokines by reducing intestinal cell transcription of inflammatory mediators [21]. Furthermore, IL-6 is a multifunctional cytokine that enhances the acute inflammatory response and promotes CRP synthesis. The significant decrease in IL-6 following treatment with Dox-Proc indicated that the IL-6/STAT3 signalling pathway, a significant mediator of colitis development, was inhibited [22]. Mesalazine may also decrease IL-6 by suppressing NF- κ B pathway, which produces IL-6 [23]. Interleukin-10 (IL-10), produced by regulatory T cells and macrophages, is the primary anti-inflammatory cytokine in the colonic mucosa. The significant decrease in IL-10 observed in group 2 demonstrated the inhibition of the regulatory immune circuit due to acetic acid-induced mucosal injury. Restoration of IL-10 levels following treatment with Dox-Proc and mesalazine to levels close to control signified the re-establishment of mucosal immunoregulation, which is a defining characteristic of therapeutic remission in ulcerative colitis. This discovery is significantly important since neither doxycycline nor procaine alone could restore IL-10 levels, hence reinforcing the need for prodrug design in therapeutic effectiveness [24]. In previous studies, doxycycline and procaine, when taken as free agents, did not rectify the inflammatory profile, as CRP, IL-6, and TNF- α remained significantly higher [11]. The results indicated that the prodrug design, rather than the pharmacological properties of its constituents, is essential to the observed therapeutic effect [25].

Dox-Proc remains predominantly unabsorbed in the upper gastrointestinal tract and undergoes azo-reductive cleavage selectively in the colon, likely producing elevated local concentrations of doxycycline and procaine at the inflamed mucosa and within the dysbiotic microbial community, while minimizing systemic exposure [26]. Local doxycycline impedes NF- κ B activation, matrix metalloproteinases, and formation of reactive oxygen species, while procaine stabilizes membranes of neuronal and immune cells and modifies autonomic inputs that affect colonic blood flow and immunological tone. Collectively, these activities may alter the cytokine environment from a pro-inflammatory state to a more controlled profile, as observed in the restoration of IL-10 to baseline [27].

Limitations of the study

This study has some limitations. Detailed macroscopic and histopathological scoring of colonic tissue (assessment of oxidative stress markers or evaluation of mucosal barrier integrity) was absent. Furthermore, the study model represents an acute chemical colitis rather than a chronic immune-mediated disease, and the long-term safety of repeated Dox-Proc administration was not evaluated.

CONCLUSION

The Dox-Proc azo prodrug reduces the levels of CRP, TNF- α , and IL-6, while modulating IL-10, and thereby demonstrating significant anti-inflammatory effects in acetic acid-induced UC in mice.

DECLARATIONS

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None.

Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript writing (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions. All intellectual contributions, study analyses, and interpretations reported in the manuscript are the original work of the authors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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