Effect of 5-lipoxygenase inhibition on events associated with inflammatory bowel disease in rats

Vijay Pal Singh, Chandrashekar S Patil & Shrinivas K Kulkarni*
Pharmacology Division, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160 014

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Leukotrienes play a part in inflammatory response. The unique role of the enzyme 5-lipoxygenase (5-LOX) in the production of leukotrienes makes it a likely therapeutic target for inflammatory conditions like asthma, rheumatoid arthritis, psoriasis, and inflammatory bowel disease (IBD). The aim of the present study was to evaluate the effect of zileuton, an orally active selective 5-LOX inhibitor against the events associated with dextran sodium sulphate-induced colitis in a rat model of IBD. The animals were administered simultaneously zileuton (100mg/kg) or sulphasalazine (100mg/kg) orally for 7 days. On day eight, rats were sacrificed, and distal colon isolated to determine myeloperoxidase activity, in vivo superoxide dismutase activity, prostaglandin E₂ levels and histological examination. Both zileuton and sulphasalazine significantly prevented the development of inflammatory events associated with colitis. The effect of zileuton was more pronounced towards reducing myeloperoxidase activity and increasing PGE₂ levels in distal colon. The results show that chemotactic leukotrienes are responsible for inflammatory surge in damaged colon and, zileuton, significantly improved healing by inhibition of neutrophil recruitment and indirectly through increase in prostaglandins at the site of inflammation. It is suggested that inhibitors of 5-LOX enzyme may have useful therapeutic role in the treatment of chronic intestinal inflammation.

Keywords: Inflammatory bowel disease, 5-Lipoxygenase: 5-LOX, Colitis, Zileuton

Inflammatory bowel disease (IBD) is a general term for a group of chronic inflammatory disorders of gastrointestinal tract [Ulcerative colitis (UC) and Crohn's disease (CD)] 

Leukotrienes, the lipoxygenase product of AA, are one of the vital mediators highlighted in inflammatory cascade allied to IBD. Leukotrienes (mainly leukotriene B₄: LTB₄) have been found in inflamed colon mucosa in concentration known to induce deleterious effects (chemokinesis, cell aggregation, increasing vascular permeability). These concentrations are similar to those seen in human IBD. In addition, drugs known to be effective in the treatment of IBD are capable of reducing intestinal leukotriene production in both experimental models of colitis and human IBD. Consequently, the 5-lipoxygenase (LOX) enzyme responsible for the synthesis of leukotrienes has become a target for the development of new potential anti-inflammatory drug in ulcerative colitis and other inflammatory conditions.

Zileuton, an orally effective 5-LOX inhibitor has been shown to selectively inhibit in vitro synthesis of LTB₄ in a variety of intact cell and cell free systems. In patients with active ulcerative colitis, zileuton 600 mg, qid has shown to reduce the local release of leukotrienes into rectal dialysates. However, lipoxygenase inhibitors such as benoxaprofen and MK-591 did not offer meaningful advantage in relieving the IBD symptoms. Hendel et al., demonstrated no significant relationship with IBD activity and alteration in mRNA for 5-LOX in colonic biopsies. This suggests a further scrutiny of potential of 5-LOX inhibitors in IBD.
The present study has been aimed to evaluate the effect of zileuton in comparison to sulphasalazine in an experimental model of dextran sodium sulfate (DSS)-induced colitis in rats.

Materials and Methods

Animals—Wistar rats weighing 150-200 g body weight (Central Animal House, Panacea Biotec Ltd., Lahiru) of either sex were used. They were housed in plastic cages in a room at 25°C±0.5°C and 12:12 hr L:D cycle. Animals were given food and water ad libitum. Experiments were carried out between 0900 and 1500 hrs. The experimental protocols were approved by the Institutional Animal Ethics Committee.

Drugs and regimen—Sulphasalazine (Panacea Biotec Ltd., New Delhi, India), zileuton (Archechem, Mumbai India), dextran sodium sulphate (DSS, mol wt 5000) and nitro blue tetrazolium (NBT, Himedia Laboratories Ltd, India) were used. Sulphasalazine (100 mg/kg) and zileuton (100 mg/kg) was suspended in tween 80 and administered orally once daily in the dose volume of 10 ml/kg, 24 hr before the induction of colitis until the day of sacrifice. DSS (5% w/v) was administered in drinking water.

Induction of inflammatory bowel disease/experimental colitis in rats and drug regimen—For the induction of experimental colitis, rats received standard meal with normal drinking water replaced with water containing 5% w/v DSS for 7 days. Sulphasalazine and zileuton treated group received standard meal, DSS containing water, and respective drugs orally, once a day. Control rats were given same food, plain water without DSS, and 0.5% CMC orally.

Histopathological evaluations—The distal colon segments from 0.5% CMC, DSS, sulphasalazine, and zileuton treated animals were isolated and immersed in 10% neutral buffered formalin till further processing. Sections were stained with hemotoxylin-eosin and investigated by light microscopy for the presence of inflammatory changes. At least three specimens per treatment were viewed to study the histopathological changes.

Myeloperoxidase (MPO) activity—Myeloperoxidase activity was determined following technique reported earlier by Singh et al. The isolated segments from different treatment groups were individually homogenized in 5 ml of phosphate buffer (0.01 M). Homogenized tissue was centrifuged at 10,000 rpm. Supernatant was mixed with o-phenylenediamine (600 μg/ml in phosphate buffer) and 300 mM of H₂O₂ was added to initiate the reaction. Absorbance was observed at 492 nm at an interval of 30 sec for 5 min. Change in the optical density per minute was calculated and the results were expressed as percentage increase of myeloperoxidase activity over the control.

In situ detection of superoxide (levels of oxidative stress)—Tissue superoxide estimation was performed as per Hagen et al. using Nitro Blue Tetrazolium (NBT) perfusion. In the presence of superoxide, NBT forms an insoluble blue formazan precipitate within the tissues. The method was adapted as follows. The animals from different treatment groups were anesthetized (thiopental sodium 25 mg/kg ip), a midline laprotomy was performed and a solution of 5ml 1% NBT in 0.9% sodium chloride (NaCl) at 37°C containing 0.2 mg/ml heparin was slowly (within 2 min) perfused in the dorsal aorta. This was followed by perfusion of 5 ml 0.9% NaCl at 37°C. The animals were killed immediately, the distal colon regions isolated and placed on a non-absorbent surface. They were then flushed with 0.9% NaCl (4°C) and fixed in neutral buffered formalin till further processing. Sections were stained with hemotoxylin-eosin for histological examination. The number of formazan precipitates in a total of ~270 mm² colonic mucosa was counted and expressed as number of cells containing formazan precipitates/100 mm² of the mucosa.

PGE₂ levels in distal colon—The PGE₂ were determined by modification in the method reported earlier by Tries et al. for determination of eicosanoids in the inflamed paw. Briefly, seven days after DSS or drug treatment the distal colon was isolated, and homogenized in ice cold buffer containing 33% (v/v) acetonitrile and 67% (v/v) of disodium hydrogen orthophosphate (Na₂HPO₄). The final solution was brought to pH 3 with 100 mM phosphoric acid. After centrifugation for 30 min (4000 g, 4°C), the supernatants were collected and kept at -13°C until extraction of eicosanoids. Extraction of eicosanoids was performed using C18 columns (Orochem Technologies, USA). The columns were initially saturated with the mobile phase, sample applied and then washed with water, ethanol 10% and hexane successively twice and finally eicosanoids were eluted with methylformate. The solvent was evaporated with N₂ under pressure.
and the residual thus obtained was resolved using reverse phase HPLC (Agilent Technologies, USA) and PGE₂ levels were quantified. The results are expressed as ng/gm of colon weight.

Statistical analysis—Data presented as mean ± S.E. were analyzed using one-way ANOVA followed by post hoc Dunnett’s test. *P<0.05* was accepted as the level of significant difference compared to time matched controls.

**Results**

*Morphological changes and histopathological examination*—All the rats treated with DSS developed noticeable inflammation in the distal colon; the middle and the proximal colon had fewer lesions. DSS-evoked colitis was characterized by thickening of muscles, shortening of crypts, edema, a massive immune cells infiltration, ulceration and significant areas of complete epithelial rupture (Fig. 1a,b).

Following sulphasalazine or zileuton treatment for 7 days, the hyperemic and ulcerative changes were almost normal. The crypts length was normalized with absence of inflammatory changes (Fig. 1c,d).

*MPO activity and oxidative stress*—Myeloperoxidase activity in animals with DSS was found to be 2.5 times higher than the control value. The increase in the myeloperoxidase activity was significantly (*P<0.05*) reduced in zileuton

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**Fig. 1**—Microphotographs of distal colonic segments from (a) control rats, (b) rats treated with DSS, (c) DSS and sulphasalazine (100 mg/kg), and (d) DSS and zileuton (100 mg/kg). [m: muscle, small arrow heads denote edema, large arrow heads indicate inflammatory infiltrate. ×125].
(100 mg/kg) or sulphasalazine (100 mg/kg) treatments. However, zileuton was more effective in reducing MPO activity when compared to sulphasalazine (Fig. 2).

NBT especially forms insoluble formazan precipitates in presence of superoxide. NBT perfusion led to the formation of formazan precipitates in the distal colon segment of DSS-treated rats. These precipitates were strictly localized inside the goblet cells of the colonic mucosal (Fig. 3). Formazan precipitates were increased twice the control value with DSS. Co-administration of sulphasalazine significantly prevented the increased formation of formazan precipitates by NBT, whereas zileuton had no significant effect as compared to DSS-treated group (Table 1).

Eicosanoid levels—PGE₂ levels were estimated in the distal colon segments isolated after 7 days. Co-administration of zileuton significantly increased PGE₂ levels to 132.25±8.80 ng/g tissue (vs. DSS treated rats; 99.22±3.32 ng/g tissue). The change in the PGE₂ levels with co-administration of sulphasalazine was not significant (106.28±6.03 ng/g tissue vs. DSS-treated rats 99.22±3.32 ng/g tissue) (Fig. 4).

Discussion

The etiology of inflammatory bowel disease remains undefined, but it is recognized that during IBD, inflammatory mediators (prostaglandins, thromboxanes and leukotrienes) are generated within the colonic mucosa. Recent experimental studies have demonstrated the role of 5-LOX enzyme metabolites in IBD because of their potent stimulatory activity on inflammatory cells. Indeed, elevated concentration is detected in dialysates and biopsies of patients with active ulcerative colitis. In particular, LTB₄ has been reported to account for majority of chemotactic activities detected in rectal dialysates. Therefore, in the present study zileuton, a specific 5-LOX inhibitor was evaluated in well-characterized model of DSS-induced colitis in rats. This model shares many of the histopathological features of human ulcerative colitis and Crohn’s disease. Oral administration of DSS to rodents results in overt inflammation in the mid distal colon that is reminiscent of human IBD.

![Fig. 3](attachment:image_url)

Table 1—Number of formazan precipitates per 100mm² of colonic mucosa [values are mean ± S.E.]

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<tr>
<th>Treatment</th>
<th>No. formazan precipitates per 100mm² of colonic mucosa</th>
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<tbody>
<tr>
<td>Control</td>
<td>438.2±67.33</td>
</tr>
<tr>
<td>DSS</td>
<td>840.6±82.44*</td>
</tr>
<tr>
<td>Zileuton (100 mg/kg, po)</td>
<td>616.0±55.89</td>
</tr>
<tr>
<td>Sulphasalazine (100mg/kg, po)</td>
<td>457.0±49.87*</td>
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*P<0.05 as compared to control, *<0.05 as compared to DSS treated rats.
Administration of minute quantities of prostaglandins has been shown to provide protection against several experimental colitis models, which is suggestive of protective role of PGs in mucosal defense\textsuperscript{26,27}. This shift of AA metabolism with 5-LOX inhibitor has been identified in some of the studies\textsuperscript{28,29} but not significantly in others\textsuperscript{30,31}. This discrepancy in reports may relate to the time period at which eicosanoid levels were measured. Furthermore, whether this represents a true shift in AA metabolism or stimulation of other enzymes by zileuton further along AA biosynthesis cascade remains to be determined.

To further confirm the hypothesis that a relation exists between leukotrienes and leukocyte recruitment in IBD, rats were subjected to DSS colitis and sulphasalazine, which is well established in the management of human IBD. It acts as an inhibitor of prostaglandin synthase, 5-LOX and as a free radical scavenger\textsuperscript{32,33}. In the present study sulphasalazine significantly reduced MPO and SOD activity and increased PGE\textsubscript{2} contents. However, the effect of sulphasalazine was less marked for MPO activity and PGE\textsubscript{2} contents than that of zileuton. These findings further support the notion that the main effect of zileuton is on the synthesis of LTs and indirectly on the neutrophil accumulation.

Randomized, double blind clinical trials with zileuton (600 mg qid and 800 mg bid) have shown that the drug is well tolerated and is associated with a trend toward clinical benefit\textsuperscript{34}. Zingarelli et al. showed zileuton to be effective in attenuating the colitic lesions in trinitrobenzene sulphonic acid (TNBS)-induced IBD in rats\textsuperscript{35}. Other studies with zileuton have shown only marginal benefits, which could be due to low doses that were used\textsuperscript{36}. Preliminary study with other 5-LOX inhibitor, BWA4C has shown in vitro reduction in LT\textsubscript{B}\textsubscript{4} in colorectal biopsy specimens\textsuperscript{37}.

In conclusion, the results of the present study clearly show that chemotactic leukotrienes are responsible for inflammatory cascade in damaged colon and, zileuton a 5-LOX inhibitor, significantly improved healing in DSS-induced colitis in rats by inhibition of neutrophil recruitment and indirectly through increase in prostaglandins (which, may be protective) at the site of inflammation.

Therefore, inhibitors of 5-LOX enzyme may be useful therapeutic tools in the treatment of chronic intestinal inflammation.
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References

27. Fedorak R N, Empey L R & MacArthur, Misprostol provides a colonic mucosal protective effect during acute acetic acid-induced colitis in rats, Gastroenterology, 98(1990) 615.


