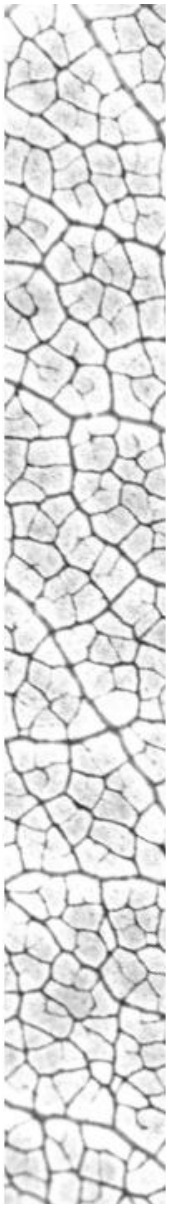

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The therapeutic effects of apremilast (phosphodiesterase 4 inhibitor) on histological outcome, inflammatory biomarkers and oxidative stress parameter in experimental Induced Colitis

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Abstract
Ulcerative colitis (UC) is idiopathic, chronic, relapsing inflammation of the intestines with no effective line of treatment. Therefore, novel and safer drugs with sufficient therapeutic efficacy are needed. The aim of the current study is to investigate the effect of apremilast on histological outcome, inflammatory biomarkers and oxidative stress parameter in experimentally induced colitis. **Material and method**: Experimental colitis was induced in rats by 4% acetic acid (vol/vol) enemas. Rats with colitis were received either apremilast 25mg/kg or sulfasalazine 100mg/kg orally for 7days. Macroscopical and microscopical assessment and the measurement of the colonic cytokines (IL-4 and TNF-α), oxidative stress marker myeloperoxidase (MPO), and adhesion molecule (E-Selectin). **Result**: Results showed that both apremilast and sulfasalazine significantly reduced the macroscopical and histological injury in colon that induced by acetic acid. In addition to the downregulation of colonic proinflammatory cytokines, MPO and E-Selectin. **Conclusion**: These results concluded that artemisinin had effective role in experimental colitis in rats through anti-inflammatory and antioxidant actions with downregulation the colonic adhesive molecule E-Selectin.

Keywords: Acetic acid, apremilast, IL-4, E-Selectin, Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is idiopathic, chronic, relapsing inflammation of the gastrointestinal tract. It is a mucosal disease with continuous ulceration always restricted to the mucosa of the rectum that can extend proximally into the colon (Zhang and Li, 2014; Wang et al, 2019). Although etiology of UC still unknown, but is thought to be due to the interactions of a patient's gene susceptibility, environmental and microbial factors, producing an abnormal immune host response which followed by initial tissue injury(Sartor,2006; Puneet et al, 2014). There is an evidence for inappropriate local immune responses associated with a large number of immunocytes infiltration resulting in mucosal disturbance and ulceration. Activation of these infiltrating immunocytes results in the release of various pro-inflammatory mediators that play a pivotal role in tissues destruction and propagation of the inflammatory responses (Zhang *et al*, 2018). Although, the wide spectrum of therapeutic aminosalicylates, glucocorticoids, immunosuppressants, and biological drugs, the increased proportion of deleterious effects at the same time with insufficient curative, therefore, novel and safer drugs with more therapeutic efficacy are needed (Braus and Elliott, 2009; Motavallian et al, 2019).

PDE4 inhibitor, has been reported to exhibit anti-inflammatory properties. Accumulating researches indicated that PDE4 inhibition could modulate both innate and adaptive responses, inhibition of PDE4 showed regulatory activities in macrophages, neutrophils, monocytes, and dendritic cells (Kim and Cheon,2017). Apremilast is a medication for the treatment of certain types of psoriasis and psoriatic arthritis,it also, may be useful for other immune system related inflammatory diseases (Li and Tang,2018), it is a selective inhibitor of the enzyme PDE4,thus increasing intracellular cAMP levels, which in turn down-regulates expression of a number of pro-inflammatory factors

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(Schafer *et al*,2014) Therefore, it was thought worthwhile to study the inductive remission of these drugs against colitis. Nevertheless, no adequate data are accessible with reference to the therapeutic outcome of apremilast in induced colitis. Hence, we aimed to assess the protective effect of apremilast versus experimental colitis.

MATERIALS AND METHODS

Materials

Animals: Forty-eight Adult male albino rats (200-220g) were supplied from the animal house of college of science/Thi-Qar University. Animal were housed five per cage for 7 days before any experiments started to acclimatize to the animal room conditions of controlled temperature 28-30ºC, with a 12 h light /12 h dark cycle and had access to laboratory chow pellet and were allowed to drink tap water *ad libitum*.All ethical themes of the studies on animals were considered carefully and the experimental protocol was approved by the ethical committee in the college of medicine of Al-Nahrain University.

Chemicals and Drugs: Acetic acid and diethyl ether (BDH Chemical Ltd., England), sulfasalazine and apremilast (Sigma –Aldrich), immunohistochemistry kits (Abcam/UK), were purchased.

Experimental Design

This study was performed on 40 adult male albino –wister rats weighing 200-220g. Animals were divided into four group (n=10/group).Group I kept as control and received no treatment. Group II,III, IV were subjected to the induction of colitis by rectal administration of 4% acetic acid (AA) (v/v).One hour after the induction of colitis group II was given normal saline orally; group III and IV were treated orally with apremilast 25mg/kg and sulfasalazine 100mg/kg respectively for 7 days.

Induction of ulcerative colitis

Rats previously subjected to starvation for at least 24 hrs before the induction of colitis but were be allowed free access to tap water, during starvation, rats were kept in cages provided with a wide wire –mesh floor to avoid coprophagy (Robert and Dale,1971). On the day of the experimental colitis induction water was interrupted two hours before the procedure. according to the method described by(Manna et al,2017) with slight modification. Briefly, under light ether anesthesia rats were administered 5ml/kg of 4% acetic acid (AA) solution by transrectally using a flexible silicone plastic tube with an external diameter of 2mm was inserted into the colon to 8cm. then, rats were holed in head down position for 2 min to prevent AA solution leakage. Control animals submitted the same procedure using equal volume of normal saline instead of AA solution.

Preparation of drugs

The sulfasalazine and apremilast freshly prepared before administration on the day of the experiment. Investigated drug (apremilast) and the standard sulfasalazine were prepared as suspensions in distilled water.The doses of apremilast (25mg/kg) was selected based on other studies reporting TNFα suppressing effect and therapeutic potential for the treatment of other inflammatory conditions of this drug at this dose (Chen *et al*,2018). Sulfasalazine was used as standard therapy in a dose of 100mg/kg (Paula et al, 2012).

Assessment of colitis

After the ending of experiment, animals were sacrificed by high dose of diethyl ether inhalation and rapidly dissection of the abdomen, thereafter the colon was extracted.The colon specimens were opened longitudinally cleansed gently using normal saline, and observed normally for macroscopic assessment. At last, the colon samples was fixed in 10% formalin for histopathological and immunohistochemistry examination.

Macroscopic evaluation

Colon edema

The colon sample of each animal was incised along its mesenteric border and gently washed, the colon edema was determined by measuring the colon weight by a sensitive balance after plotting the tissue on a filter paper to discard excess water. It was used as indicator of tissue edema and the intensity of colitis (Atarbashe and Abu-Raghif, 2020).

Disease Activity Index (DAI)

The DAI defined by (Mao *et al.,*2019) was used to estimate the disease clinically which include bodyweight loss {(0) no reduction or weight gain; (1) 1-5 % reduction; (2) 6-10% reduction;(3)11-20% reduction; (4) more than 20% reduction},the grades of stool consistency {(0) Normal; (2) loose; (4) diarrhea}, and rectum bleeding{(0) normal; (2) mild;(4) severe bleeding}.The DAI was calculated as the sum of total scores.

Macroscopic colonic score

The colonic samples were examined visually. The macroscopic score based on the clinical features of the colon according to scoring system ranging from 0-6 as follows: 0= absence of inflammation; 1=redness or swelling; 2= swelling and redness; 3= one or two ulcers; 4= one large ulcer or more than two ulcers; 5= initial necrosis; 6, severe necrosis (Appleyard and Wallace,1995).

Histopathological examination

The colonic samples were fixed in 10% formalin at room temperature. Dehydration, paraffin embodiment, and deparaffinization were done on the specimen, prepare 4 µm thick section from each colonic sample and stained with Hematoxylin and eosin (H&E).Slides were examined and scored for histopathological

Table 1. Macroscopic and histopathological parameters in study and healthy control groups

Macroscopic and Histopathological Parameters	Groups (n=10/group)			
	Healthy control	colitis	Colitic rats treated with Sulfasalazine	Colitic rats treated with Apremilast
Colonic weight (gram)	1.27±0.15 A	3.12±0.2 B	1.61±0.07 C	1.5±0.09 C
Disease activity index	0.0+0.0 A	10.33+1.1 B	2.0+2.11 C	3.0+2.11 C
Macroscopic score	0.0±0.0 A	3.67±0.52 B	1.7±1.34 C	1.4±0.7 C
Histopathology	0.0±0.0 A	3.0±0.0 B	1.5±0.71 C	1.2±0.63 C

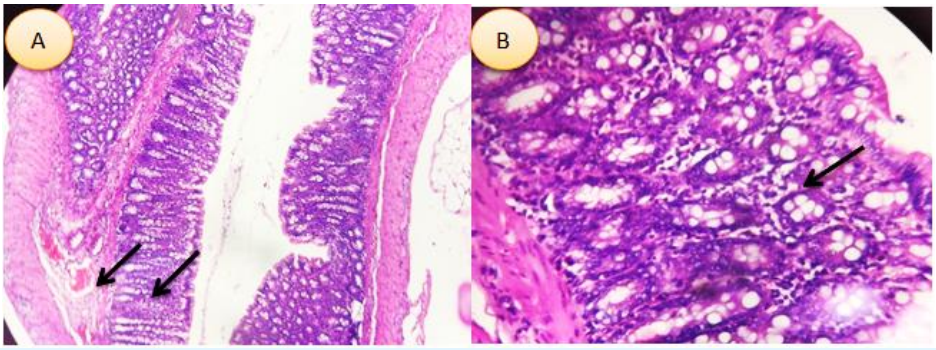

Fig. 1. Histological section through colonic wall showing normal mucosal and submucosal pattern with no evidence of inflammation and preservation of colonic gland with goblet cells (arrow); A: 20X; B: 40 X; H and E stain

evaluation in a blinded fashion by experienced histopathologist and results evaluated according to scoring system ranging from 0-3 (0:normal,1:focal, 2:zonal, 3: diffuse) which estimated the extension of: epithelial damage, and/ or glandular crypts dilation, loss of goblet cells, inflammatory cells infiltration, crypt abscesses, edema, mucosal hemorrhage and dysplasia(Cooper et al,1993).

Immunohistochemistry

Immunohistochemistry (IHC) techniques exhibit the benefit of directly demonstrating cells in the affected tissue (Bertevello et al,2005). The immunohistochemical reactions were produced by the presence of specific antibodies, concomitantly the estimation of the production of a number of biochemical markers in intestinal tissue specimens that were paraffin-embedded so as to measure the colonic cytokines, adhesion molecule, and oxidative stress markers. “Quantification of IHC was carried out in accordance to the following semi quantitative scores (Hernández-Rodríguez et al, 2004) : 0, no staining;1, ≤ 25%; 2, 26-50%; 3, 51- 75 %; and 4, 76-100%. that based on the percentage of positively stained cells.

Statistical analysis

All the data were presented as mean ± standard deviation, Unpaired t test was used for comparison of means of two groups, while ANOVA (analysis of variance) with post hoc Tukey test were used for comparison of means of parameters among four drug groups. Statistical package for social sciences (SPSS) version 23 were used to analyze the results. P value less than 0.05 were considered significant (Daniel,2009).

RESULTS

Effect of apremilast on macroscopic scores

The colonic mucosa of colitis untreated rats showed edematous inflammation, extensive ulceration and necrosis compared with normal colonic mucosa of healthy group, the rats that administered apremilast or sulfasalazine orally evolve significant lowering to the disease activity index and colonic weight. In addition, both drugs significantly ($p<0.01$) lower the macroscopical score as shown in **Table 1**.

Effect of apremilast on histopathological scores

The present study exhibits the histological changes in untreated colitis, primarily showed mucosal ulceration and necrotic tissue, heavy mononuclear inflammatory infiltrate, complete loss of goblet cells as displayed in **Fig. 2**. Furthermore, both sulfasalazine and apremilast treated groups evolve significant ($p<0.01$) attenuate in the histopathological score as evidenced by mucosal regeneration and glandular formation; mild inflammation and slightly depleted goblet cells as displayed in the **Fig. 3**. However, apremilast exhibited a higher significant reduction in the scoring of the microscopical parameter displayed in a **Table 1**.

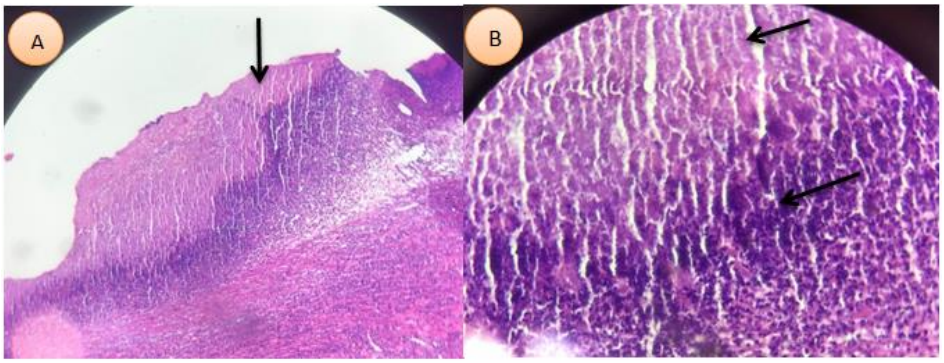

Fig. 2. Histological section through colonic wall showing mucosal ulceration and necrotic tissue ;heavy mononuclear inflammatory infiltrate in experimentally induced colitis in rat; A: 20X; B: 40 X; H and E stain

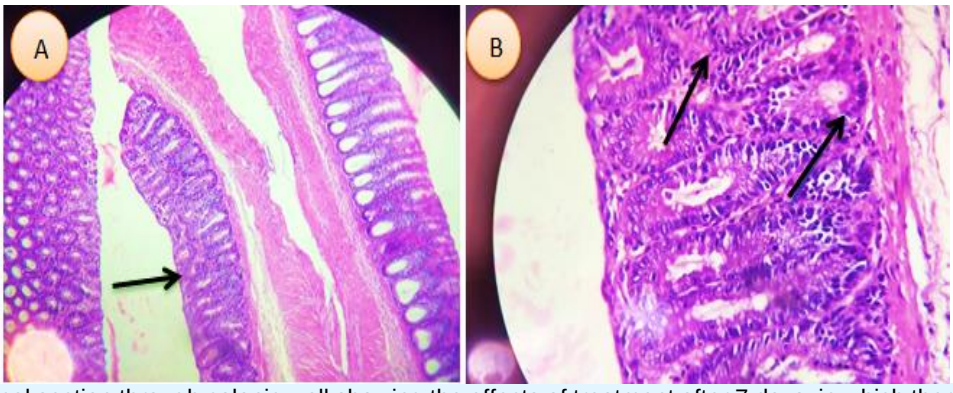

Fig. 3. Histological section through colonic wall showing the effects of treatment after 7 days in which there is evidence of mucosal regeneration and glandular formation ;mild inflammation and slightly depleted goblet cells ; A: 20X; B: 40 X; H and E stain

Table 2. Immunohistochemical score for cytokines, oxidative stress and adhesive molecule

cytokines, oxidative stress and adhesive molecule Parameters	Groups (n=10/group)			
	Healthy control	colitis	Colitic rats treated with Sulfasalazine	Colitic rats treated with Apremilast
Tumor necrosis factor-α	1.0±0.0 A	3.5±0.55 B	1.0±0.0 A	1.0±0.0 A
Interleukine-4	1.0±0.0 A	4.0±0.0 B	1.5±0.53 C	1.2±0.42 C
Myeloperoxidase	1.0±0.0 A	4.0±0.0 B	1.6±0.52 C	1.2±0.42 C
E-selectin	1.0±0.0 **A**	4.0±0.0 B	1.7±0.48 C	1.3±0.48 C

Effect of apremilast on the **cytokines TNF-α and IL-4.**

As shown in **Table 2**, colonic levels of TNF- α and IL-4 showed significant elevation after acetic acid introduction compared to those of control group. these values were significantly ($p<0.01$) diminished in rats treated with apremilast and sulfasalazine.

Effect of apremilast on the **myeloperoxidase**

After apremilast and sulfasalazine treatment, the high colonic MPO level in the induced group was found to be significantly ($p<0.01$) diminished **Table 3**. Even so, apremilast exhibited a higher reduction in the scoring of MPO.

Effect of apremilast on the E-selectin (CD62)

The elevated colonic CD62 in the colitis group was found to be significantly ($p<0.01$) decreased after apremilast and sulfasalazine treatment. However, apremilast exhibited a better reduction results in the scoring of the CD62 parameter displayed in a **Table 2**.

Comparison expressed by letters; dissimilar letters denotes significant difference. The expression of values as mean ±Standard deviation (SD).

Comparison expressed by letters; dissimilar letters denotes significant difference. The expression of values as mean ±Standard deviation (SD).

DISCUSSION

Phosphodiesterase 4 belongs to a group of enzymes that catalyze the breakdown of cAMP in various types of cells, involving inflammatory cells, and is play an important role in the inflammatory response. drugs targeting these enzymes represent a beneficial strategy for the treatment of inflammatory disturbances, including

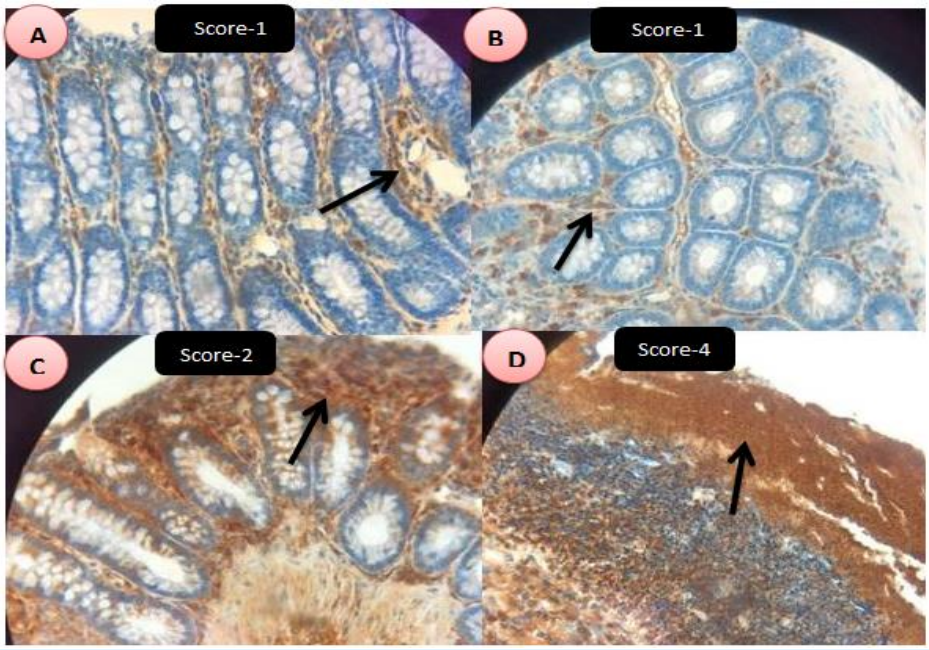

Fig. 4. Immunohistochemical expression of: (A) Tumor necrosis factor-α (TNF-α) reveals membranous and secretory pattern (brown color in the stromal cells) ;(B) Interleukine-4 reveals secretory pattern (brown color in the stromal cells); (C)Myeloperoxidase (MPO) reveals cytoplasmic pattern (brown color in the stromal cells); (D)CD 62 reveals membranous pattern (brown color in the stromal cells)

ulcerative colitis (Martinez and Gil,2014; Spadaccini et al, 2017). Additionally, PDE4 inhibition displayed superior effects on T cell receptor induced activation of T cells, showing in the depression releasing of cytokines and chemokines from T helper-1 (Th1), Th2, and Th17 cells (Sakkas et al, 2017). Li et al.in 2019 found that PDE4 inhibitor (apremilast) had significant role in reducing clinical signs of intestinal inflammation following induction of colitis through interfering with mucosal immunity, an observation that in accordance with the findings of the present study.

The present study showed that apremilast significantly reduced DAI, this finding is comparable with observation of El-Ashmawy et al.in 2018 who show the selective PDE4 inhibitor attenuated on the DIA in experimentally induced colitis in in roflumilast -treated rat than in non -treated rat. On the other hand the present study conducted that apremilast significantly reduces colonic weight in experimentally induced colitis in rats. Furthermore, In this work apremilast reduced macroscopic score and histopathological changes of colon in experimentally induced colitis and this finding in accordance with the finding of (Loher et al,2003; Videla et al, 2006; Li et al, 2019).

The proposed protective mechanism of of apremilast may be attributed to the more potent anti-inflammatory and anti-oxidant activity of apremilast, which have been estimated in the current study, it has been evidenced that PDE4 is predominant expressed in immune cells (Eskandari et al,2015).Increased intracellular level of cAMP by PDE4 inhibition exerts anti-inflammatory effects on neutrophils, lymphocytes and monocytes by activation of its receptor protein kinase A (PKA), manifesting in the lowering of release of cytokines and chemokines, so, when the level of cAMP is increased following administration of the PDE4 inhibitor (apremilast), inflammation cascade will be reduced significantly (Raker et al, 2016; El-Ashmawy et al, 2018).

From another point of view it has been suggested that mechanism for the protective role of apremilast against colitis may be due to the decrease in leukocyte adhesion and emigration induced by apremilast, which can be partly explained by its ability to significant down regulate E-selectin expression on the endothelial cell and this finding supported by observation of Sanz et al.in 2002. Selectins participate in the primary phases of leukocytes rolling to the epithelium of blood vessel, and endothelial selectin plays a major role in the emigration of leukocytes to the vascular wall and their adhesion to the endothelial cells (Anthoni,2006).

The protective effect of apremilast could be explained not only by a significant reduction of E-selectin expression but for a marked reduction of cytokines (TNF-α, IL-4) levels on colons. Tumor necrosis factor – α is a proinflammatory cytokines that has been demonstrated to mediate intestinal tract inflammation as well as, expression of TNF-α increases in colitis (Mark and Peter, 2016). which is evident by the fact that TNF blockers have proven to be a very efficacious in the treatment of colitis (Lopetuso et al,2017). After an initial damage to mucosal epithelial barrier, TNF-α is secreted by T cells, macrophages, and intestinal mucosal cells

causing release of chemokines and cytokines (Xiao et al, 2016). Interleukin-4 is a key immunoregulatory cytokine, its dysregulation may participate to many inflammatory disease, including ulcerative colitis. IL-4 is a critical T helper type2 cytokine which direct immune reactions (Kasaian et al, 2017). The current study evolved marked reduction in the immunohistochemical expression of TNF-α which is comparable with series of experiments conducted by PDE4 blockers (Rieder et al, 2013; El-Ashmawy et al, 2018). In addition the reduction of IL-4 on colons, this observation has been demonstrated by Schafer et al.in 2019, who proved that the apremilast decrease of cytokine protein IL-4 level in the dinitrochlorobenzene mouse model of atopic dermatitis.

Myeloperoxidase (MPO) is an enzyme essentially create in neutrophils and has been used as an effective index of granulocyte cells infiltration in both experimental and human colitis (Khan et al,2018)], however, MPO is active in inflamed mucosa in UC patients and participate to the progress of malignancies (Tian et al, 2017).The present study demonstrated that apremilast significantly reduced MPO immunohistochemical expression in rat colonic mucosa in comparison with colitis group. previous studies imply that PDE4 inhibitors raise cAMP can reduce immunological release of reactive oxygen species (ROS) from neutrophils and eosinophils and by this way minimizing the role of oxidative stress in mucosal damage (Loher et al, 2003; Videla et al, 2006; El-Ashmawy et al, 2018).Ansari et al also suggest that PDE4 inhibitors significantly reduced the oxidative stress markers in experimentally induced cardiotoxicity in rats (Ansari et al, 2019).

CONCLUSION

In conclusion, apremilast has a therapeutic effect through the reduction of the inflammatory mediators TNF-α, IL4 and MPO, down regulation of E-selectin which is comparable to that of sulfasalazine in experimentally induced colitis. These beneficial effects of apremilast may be useful in patients suffering from UC. Further studies are warranted to investigate the potential therapeutic efficacy of apremilast in UC and to identify other molecular mechanisms.

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