Original Article

EFFECT OF AMINOSTEROID U74389G IN A MODEL OF INFLAMMATORY BOWEL DISEASE IN RATS

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Summary

Lazaroid U-74389G is a synthetic 21-aminosteroid with free radical-scavenging and anti-inflammatory effects. This study was designed to evaluate the anti-inflammatory activity of U-74389G on experimental 2,4-dinitrobenzene sulfonic acid hydrate (DNBS)-induced colitis in Wistar rats. Five experimental groups were formed: a sham control group, a control group, treated with 0.25 ml of 50% ethanol intrarectally (n=8), a group treated with DNBS (30 mg in 0.25 ml of 50% ethanol administered intrarectally, (n=8), a group treated with DNBS and U-74389G at a daily dose of 15 mg/kg i.p. (n=8), and a group treated with DNBS and sulfasalazine, orally, at a dose of 300 mg/kg. During the experiment, the bodyweight of the rats, food intake, stool consistency, and presence of blood in the stool were recorded as markers of clinical condition. On day 6, colonic tissues were excised and scored for macroscopic and histological damage. Blood samples were taken to measure levels of cytokines by ELISA methods. DNBS decreased significantly body weight (from 237.00±2.52 g to 212.50±6.25 g, p=0.04). The rats treated with U-74389G showed greater food intake and weight gain. U-74389G reduced ulceration index: the U-74389G score was 1.25±0.25, and the DNBS score – 3.87±0.61; $p<0.05$. All other macroscopic parameters assessed were significantly improved in rats treated with U-74389G. The levels of inflammatory cytokines IL-1, IL-6, and TNF-α, were significantly lower than those of the DNBS group, while U-74389G significantly elevated the level of anti-inflammatory IL-10. These findings indicate that U-74389G significantly inhibits colonic inflammatory damages in a rat model of inflammatory bowel disease.

Keywords: cytokines, experimental colitis, inflammatory bowel disease, lazaroid U-74389G

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Introduction

Inflammatory bowel diseases (IBDs) are characterized by inflammation with a chronic course and relapses caused by an inadequate immune response to the intestinal microflora in genetically predisposed individuals exposed to risk factors [1-3]. There are two forms of IBDs: ulcerative colitis (UC) and Crohn's disease (CD). Crohn's disease influences the entire layers of the whole intestinal tract, including the mouth and anus. Ulcerative colitis attacks the colonic mucosa [4]. The pathogenesis of the disease is not fully

understood. Literature suggests that activation and imbalance between Th1, Th2, and Th17 cells are responsible for the genesis of IBDs [5]. Activation of macrophages and increased production of cytokines such as TNF-alpha, IL-1, and IL-6 also contribute to the inflammatory response. Studies have shown that genetic factors play a role in the development of IBD by disrupting the integrity of the epithelial barrier [6], deficiency in autophagocytosis [7], congenital defects in receptor populations, and problems with lymphocyte differentiation, especially in Crohn's disease [8].

Despite the availability of various approaches, the treatment of IBDs is still a therapeutic problem. According to Tanida et al. [9], the conventional treatments for mild and moderate IBDs include nonsteroidal anti-inflammatory drugs containing 5-aminosalicylic acid derivatives, glucocorticosteroids, and purine *antimetabolites* (azathioprine and 6-mercaptopurine). Calcineurin inhibitors (cyclosporine and tacrolimus), TNF-α inhibitors (infliximab or adalimumab), or a neutralizing antibody (vedolizumab) against integrin α4β7 are second-line drugs, useful in patients not responding to the therapy mentioned above. Inhibiting annexin A2 and influencing the release of TNF-α [9] and annexin A5 with high affinity for Phosphatidylserine externalized on the colonic capillaries [10] may be a new therapeutic strategy to prevent inflammation in IBD. These drugs are beneficial for patients with refractory IBD but do not apply to all patients.

Glucocorticosteroid (GCS) therapy is effective in inducing remissions. Better results are achieved with UC than with CD in this respect [11]. However, GCSs cannot be recommended as first-line therapy to prevent recurrence of disease activity due to the lack of evidence of any benefit and the potential to cause significant short- and longer-term adverse effects [12].

Realizing that anti-inflammatory effect of GCS is not related to their hormonal activity stimulated the development of a group of non-glucocorticoid steroid analogues (21-aminosteroids) [13]. They have an anti-inflammatory effect and specifically inhibit oxidative dysbalance [14] without glucocorticoid or mineralcorticoid activity, thereby avoiding the complications of corticosteroid therapy [15]. They act as antioxidants and attenuate free radical-induced lipid peroxidation, to which cell membranes are highly sensitive [16,17].

Considering the findings mentioned above, we aimed to evaluate the effect of aminosteroid U74389G in the rat model of IBD.

Material and Methods

The experiments were approved by the Bulgarian Food Safety Agency and were conducted under the Animal Welfare Regulations.

Chemicals and reagents

2,4-Dinitrobenzenesulfonic acid hydrate (DNBS, CAS No 698999-22-3), U-74389G (CAS No 153190-29-5) sulfasalazine (CAS No 599-79-1), and all chemicals were obtained from Sigma-Aldrich Com Ltd. ELISA kits (Rat IL-1 alfa ELISA kit, Rat lL-6 ELISA kit, Rat lL-10 ELISA kit, and TNF alpha rat ELISA kit) were purchased from R&D systems.

Experimental animals

The study was carried out on 36 male Wistar rats (200-230 g, age four months). The animals were purchased from the Research and Laboratory Animal Breeding Center and were kept at the University vivarium for one month. They were raised under optimal laboratory conditions (temperature of 22±2° C and humidity of 50±10%) by providing a standard pellet diet and water ad libitum.

The experimental model of colitis and treatment

The experimental animals were divided into five groups randomly and anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg. The dose of 30 mg DNBS dissolved in 0.25 ml of 50% ethanol was inserted into the colon with a rubber catheter according to the procedure described by Morris et al. [18] and Wallace et al. [19]. Control rats were injected using the same procedure with either normal saline (sham control, n=6) or 50% ethanol (n=8). Test drugs were administered for 6 days, starting one day before inducing colitis. The rats with experimental colitis in the sulfasalazine (SS) group (n=8) were treated with sulfasalazine orally, at a dose of 300 mg/kg, once daily. In the U-74389G group (n=8), the lazaroid was injected intraperitoneally on the same days at a dose of 5 mg/kg. U-74389G

was dissolved in CS-4 solution (water solution of 20 mM citric acid monohydrate, 3.2 mM sodium citrate dehydrate, 77 mM NaCl, pH=3) in a 2 mg/mL concentration. Sulfasalazine was dissolved in phosphate-buffered saline, pH 7.2, in a concentration of 0.2 mg/mL. In previous articles, we published results from the application of U-74389G, which did not differ statistically from the controls [21, 22].

Assessment of the severity of experimental colitis

Several parameters were measured from day 0 to day 6: body weight, food, and water intake, stool consistency (0=normal, 1=loose stool, 3=diarrhoea), presence of blood in the stool (0=negative, 1=positive, 3=gross bleeding).

On day 6, the animals were sacrificed, and the colonic segment samples were obtained. The assessment of the severity of intestinal inflammation was performed macroscopically and histologically, following the criteria reported by Antonioli et al. [22]. The criteria for macroscopic scoring of colon damage were as follows: the presence of adhesions between the colon and other organs (0 – none, 1 – small, and 2 – significant adhesions); the presence of ulceration (0 – none, 1 – hyperemia, 2 – ulceration without inflammatory reaction, 3 – wound with inflammation at one site, 4 –ulcers and inflammation at two sites, 5 – large lesions, extending 1-2 cm along the length of the colon, 6 – large lesions, more than 2 cm in length) and the result was increased with 1 for each millimeter of colon wall thickness. The length of the colon was measured. All parameters were evaluated independently by two individuals. Microscopic examination of the inflamed distal colon areas was performed by light microscopy on hematoxylin and eosin-stained slides. The histological criteria included mucosal architecture changes, cellular infiltration, presence of crypt abscess, and goblet cell depletion.

Immunological tests

The ELISA assay was performed according to the manufacturer's instructions. Each sample was double tested.

Statistical analysis

The results were presented as mean values±S.E.M. and were tested by one-way ANOVA, followed by Fisher's least significant difference procedure as a post-hoc test. A *P*-value lower than 0.05 was considered significant. Analyses were performed using STATGRAPHICS® Centurion XV statistical software.

Results

Changes in body weight, food intake, and diarrhoeal status

Changes in body weight, food intake, and diarrhoeal status were accepted as indicators of disease activity. During the five days after inducing colitis, DNBS-treated rats showed a significant decrease in body weight, most pronounced on day 3 ($P<0.05$). The food consumption was significantly less than that of control, U-74389G- and SS-treated rats over the initial three days after DNBS insertion (Table 1).

The diarrhoea status was assessed based on the consistency of the stool and the presence of blood in it (Figure 1). Changes in the diarrhoea index were observed in all colitis-induced animals. The highest diarrhoeal index was

Table 1. Changes in body weight and food intake

Groups	Bodyweight (g)			Food intake (g/100 g body weight)		
	Day 1	Day 3	Day 6	Day 1	Day 3	Day 5
Control group	235.63±5.93	249.37±3.59	255.00±4.23	5.02±0.09	5.12±0.07	5.27±0.08
DNBS group	237.00±2.52	207.80±4.75*	212.50±6.25*	5.05±0.08	2.15±0.17*	3.82±0.19*
U-74389G group	243.37±4.62	221.75±3.65*†	237.37±5.25*†	5.1±0.09	4.46± 0.04†	4.54± 0.22*†
SS group	233.13±4.90	209.13±3.97*	221.37±2.42*	5.04±0.07	4.19±0.07* †	4.26±0.12*

Each result is given as a mean value for eight rats ± SEM. (*) shows a significant difference compared to control animals; (†) shows a significant difference compared to DNBS-treated animals.

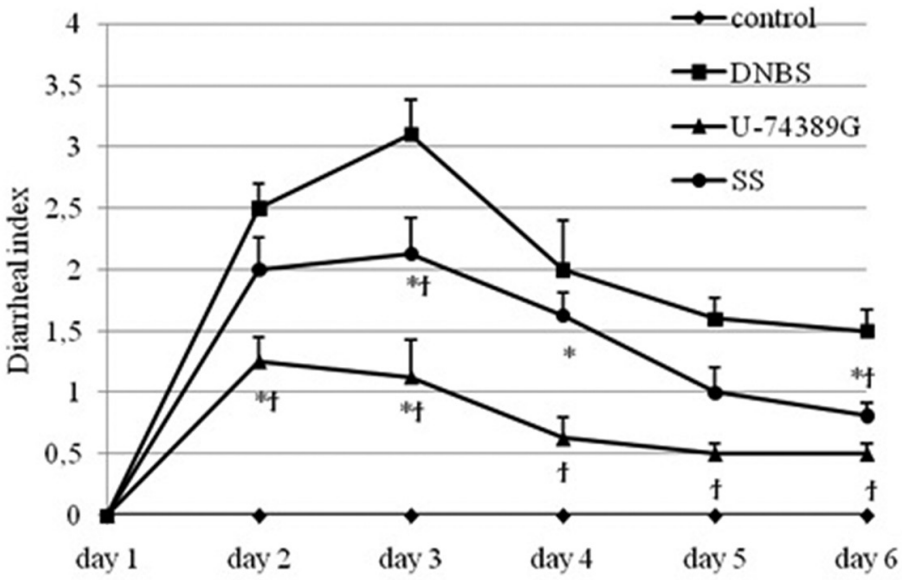

Figure 1. Daily diarrheal status during six days after induction of colitis. Each result is given as a mean value of eight rats ± SEM. (*) shows a significant difference compared to control animals; (ŧ) shows a significant difference compared to DNBS-treated animals

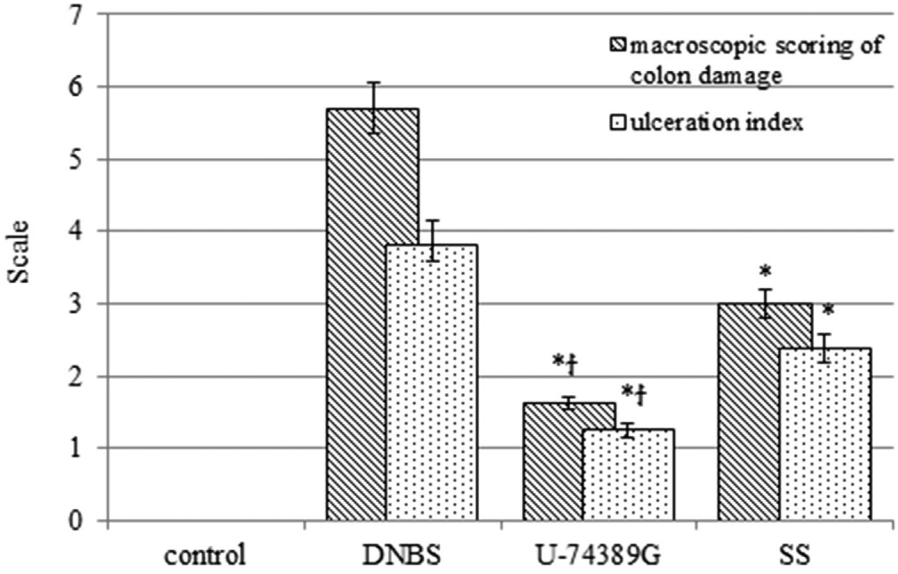

Figure 2. Macroscopic damage score estimated at day six after induction of colitis with DNBS. Each column represents mean value ± SEM (n=). The sham and the ethanol control groups showed a zero score as no disease was developed. (*) shows a significant difference compared to DNBS-treated animals; (ŧ) shows a significant difference compared to SS-treated animals

assessed at day 3 in all experimental groups (DNBS-, U-74389G-, and SS-rats) with a value of 3.1 in the DNBS group. No significant difference was seen in diarrhoeal status and food intake at day six between control groups and rats with colitis, treated with U-74389G at a dose of 5 mg/kg.

Macroscopic examination

DNBS treatment produced severe inflammation and ulceration in the colitis rats (Figures 2 and 3), expressed by ulceration index 3.87±0.61 and macroscopic scoring of colon damage 5.7±0.74 (presence of adhesions, ulcerations, and colon wall thickening). Macroscopic evaluation of tissues obtained from U-74389G-treated animals revealed a significant beneficial effect (P<0.05) of IBD symptoms (ulceration index 1.25±0.25 and macroscopic scoring of colon damage

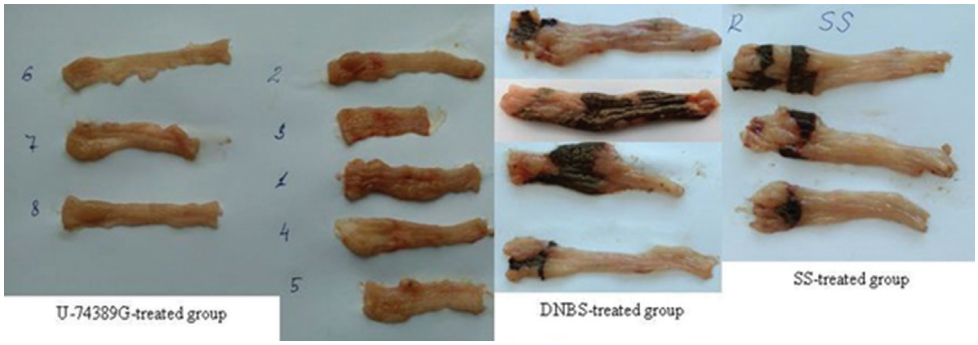

Figure 3. Macroscopic changes in the colon on day six after induction of colitis from U-74389G-, DNBS- and SS-treated animals

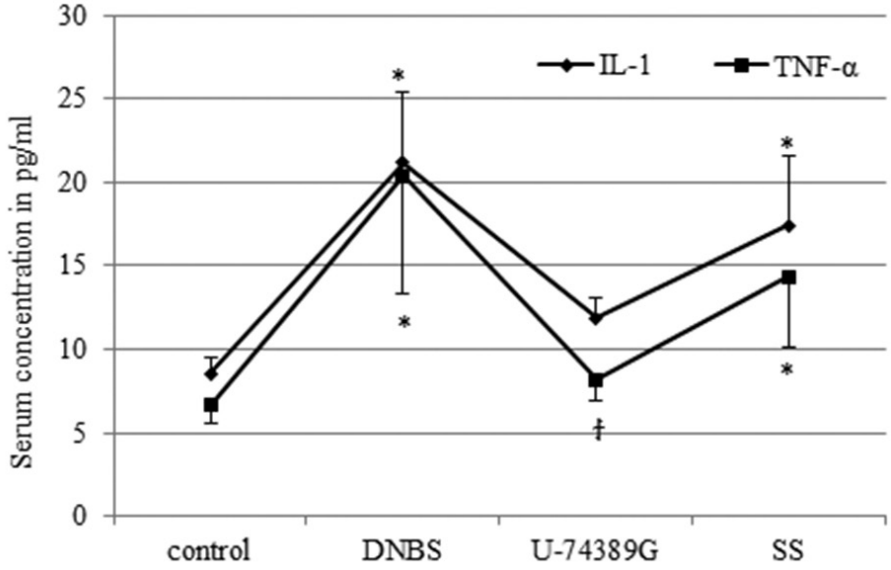

Figure 4. Serum concentrations of inflammatory cytokines IL-1 and TNF-α at day six after induction of colitis with DNBS. Each result is given as a mean value of eight rats ± SEM. (*) shows a significant difference compared to control animals; (ŧ) shows a significant difference compared to DNBS-treated animals

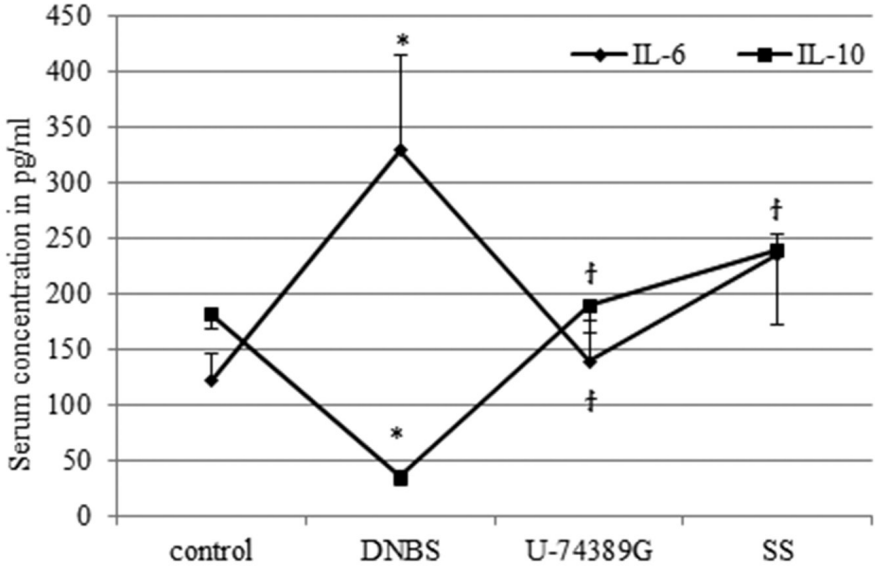

Figure 5. Serum concentrations of inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 at day six after induction of colitis with DNBS. Each result is given as a mean value of eight rats ± SEM. (*) shows a significant difference compared to control animals; (ŧ) shows a significant difference compared to DNBS-treated animals

1.625±0.41). The effect of sulfasalazine on the studied markers was less pronounced than the effect of U-74389G, as evident in Figures 2 and 3.

Immunological assessment of cytokine levels

The serum levels of inflammatory cytokines IL-1, IL-6, and tumour necrosis factor-alpha (TNF-α) in the U-74389G-treated group were significantly lower than those of the DNBS group. In contrast, the level of anti-inflammatory IL-10 was significantly elevated by U-74389G (Figures 4 and 5).

Microscopic examination

The histological assessment of the colon from the DNBS group showed extensive damage with ulcers in some areas, transmural infiltration with neutrophils and lymphocytes, mucus depletion, hemorrhage, and edema. The DNBS group had a greater extent of injury, followed by the SS group, while the U-74389G showed significantly decreased hemorrhage, infiltration of inflammatory cells, and tissue necrosis.

Discussion

In this study, we evaluated the protective effect of 21-aminosteroid U-74389G in DNBS-induced colitis in rats. Our data showed that U-74389G improved clinical symptoms and macroscopic and histological damage of the colon.

IBD is considered a multifactorial disease due to the interaction between the immune system, genetic predisposition, and responses to environmental and microbial factors [23]. In the literature, data prove the significant role of free radicals and free radical-stimulated pathways in the development of IBD and as pharmacological targets for anti-IBD drugs [24]. Oxidative dysbalance induces and worsens the disease in two ways, including oxidative damage of intestinal mucosal cells and increased formation of inflammatory cytokines. A model of hapten-induced DNBS experimental colitis was used. The aggressive agent dissolves in alcohol, which improves the penetration of DNBS into the lamina propria [25]. DNBS then haptenizes the local microbial proteins; they become immunogenic and provoke immune responses of the host, infiltration of neutrophils, macrophages, and T lymphocytes.

DNBS-hapten-induced inflammation involves specific cells, including polymorphonuclear neutrophils, lymphocytes, plasma cells, and eosinophils [5]. Inflammatory cells can cause tissue damage by releasing oxygen free radicals, proteases, and inflammatory mediators such as cytokines and chemokines.

Our study results demonstrated that intrarectal administration of DNBS at a dose of 30 mg dissolved in 0.25 ml of 50% ethanol-induced inflammation resembling IBD. The clinical course, immunology, and histopathology of the changes all made us assume that the model was suitable for studying the effect of potential therapeutic agents.

We found that DNBS caused significant weight loss, decreased food intake, and significant stool consistency changes, including blood in all the colitis rats, without mortality among the animals. During the five days after the induction of colitis, the DNBS-treated rats showed a significant decrease in body weight and food consumption, most pronounced on day 3 ($P<0.05$). At the same time, these indicators were favorably affected by U-74389G, and their values were significantly increased compared to the DNBS group. On day three, after inserting the aggressive agent, loose stools were seen in 33% of the animals, diarrhoea - in 67%, and blood in the stool (gross bleeding) in 37.5% of the rats with colitis. Only one rat in the group treated with U-74389G had diarrhoea and was positive for the presence of blood in the stool on day 3.

The macroscopic evaluation of tissues obtained from the U-74389G-treated animals showed that there was hyperemia in 50% (n=4) of the animals, and in 3 rats, there were ulcerations without inflammatory reaction. Significantly more severe were the changes in the animals with colitis: in 7 out of 8 animals, there were ulcerative changes with inflammation and large lesions.

Cytokines are essential in the pathogenesis of IBD, and the regulation of their synthesis successfully reduces the severity of the disease and maintains remission. Activated immune cells secrete several cytokines that actively regulate the inflammatory response in UC and CD. Once secreted by the antigen-presenting cells, these cytokines trigger and differentiate

many T-cells activating the adaptive immune response. TNF-<UNK> expression of TNF-α in macrophages has been found in colon tissue, and serum levels of TNF-α correlate with clinical and laboratory indicators of intestinal disease activity [26]. In addition to TNF-α, IL-1 and IL-6 have pronounced proinflammatory activity. Different cell types produce them by initiating cyclooxygenase type 2, phospholipase A, and inducible nitric oxide synthase and are involved in immunological reactions in IBD development. IL-10 has anti-inflammatory and immunosuppressive properties. It reduces antigens' presentation and the subsequent release of proinflammatory cytokines, thus attenuating mucosal inflammation. An elevation in serum concentrations of inflammatory cytokines TNF-α, IL-1, and IL-6 and a decreased level of anti-inflammatory cytokine IL-10 on day six after induction of colitis was found in the DNBS-treated rats. U-74389G significantly influenced the concentration of the cytokines, supporting the assumption that this aminosteroid produces anti-inflammatory effects.

In our study, we compared the effects of U-74389G with the effects of sulfasalazine (SS) on markers of disease activity, macroscopic scoring of colon damage, immunological parameters, and histological changes. Sulfasalazine has been studied in IBD and proved effective in inducing remission in mild-to-moderate disease and maintaining remission [27]. SS has anti-inflammatory, immunosuppressive, and antibacterial actions, mainly attributed to its sulfapyridine and 5-aminosalicylic acid breakdown. The effects of SS on the studied markers were less pronounced compared to those of U-74389G. These experimental findings can be explained by the fact that it may take weeks before the effects of SS occur.

The therapy of IBD aims to modulate the inflammatory response. Despite using different therapeutic groups, including biologics, not all patients receive satisfactory results from therapy [9]. Glucocorticosteroids are effective in the treatment of IBD by controlling the exacerbation of the disease and inducing remission. With their receptors, GCSs modulate the immune response. They inhibit the expression of adhesion molecules and the movement of inflammatory cells to target tissues, including the intestine [27, 28]. GCSs cannot be recommended to prevent disease recurrence because of their safety profile and uncertain data about the efficacy [12].

A family of 21-aminosteroids, analogues of methylprednisolone, has been developed, including compounds with free radical scavenging and membrane stabilizing activities and lack of glucocorticoid activity due to the substitution of the 11-b-hydroxy group. The 21-aminosteroids include tirilazad and U743 89G (16-desmethyl-tirilazad). They are tested for the ability to inhibit the peroxidation of lipids in tissue following various types of injuries [29-31]. U-74389G has been shown to have neuroprotective properties in a stroke model [32] and injury of the brain [33]. It also inhibited lipid peroxidation in cultured endothelial cells [34] and was protective in other models of multiple organ injury, such as in the lung, the heart, and in skeletal muscle reperfusion injury [35, 36]. The lazaroids have a high affinity for the membrane lipids, and by incorporating them into the lipid bilayer because of their lipophilicity, they produce cell membrane-stabilizing effects [15]. The protonated piperazine nitrogen interacts with negatively charged phosphate-containing head groups of the membrane lipid bilayer. This action limits the movement of lipid peroxyl radicals within the membrane, thus reducing their interaction with fatty acids and inhibiting lipid peroxidation.

U-74389 has been demonstrated to inhibit initial inflammation, including inflammatory cells' migration and activation [37]. New experimental results have shown that lazaroids are potent, time-dependent inhibitors of caspase-1 that convert the inactive IL-1β to active cytokine [38].

Conclusions

In conclusion, the intrarectal DNBS administration produced severe damages of the colon in rats, assessed by markers of disease activity, macroscopic scoring of colon damage, immunological parameters, and histological changes. Our study demonstrated that the beneficial effects of 21-aminosteroid U-74389G may be due to membrane stabilizing activity, anti-inflammatory, and antioxidant properties of the lazaroid.

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