

Beneficial effect of a novel nonsteroidal anti-inflammatory agent with basic character and antioxidant properties on experimental colitis in rats

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SUMMARY

Ulcerative colitis is a chronic disorder of unknown etiology. Conservative treatment remains empirical, even today. The aim of this study was to test the efficacy of a novel non-steroidal anti-inflammatory agent {5-(2-hydroxy-ethylamino)-1-cyclohexyl-2-pentanone} (compound A), with basic character and antioxidant properties on an experimental model of ulcerative colitis in rats. The effect of this compound was compared with that of methyl-prednisolone on the histological abnormalities and serum levels of Tumor Necrosis Factor- α (TNF- α) of experimental colitis produced by 2,4,6-trinitrobenzenesulfonic acid (TNB). A total number of 24 rats were used, randomly assigned to one of four groups of 6 rats each. Group 1: colitis without treatment (disease control), group 2: normal animals (control), group 3: induction of experimental colitis treated with methylprednisolone (5.3×10^{-3} mmol/kg i.v., every day for 7 days) and group 4: induction of experimental colitis plus administration of compound A (0.6 mmol/kg i.v., every day for 7 days). The administration of compound A resulted in a statistically significant reduction ($p < 0.05$) of the histological damage (score) and the levels of serum TNF- α , compared

to controls. This beneficial effect was probably due to the combination in a single molecule of the anti-inflammatory, antioxidant properties and the basic character of this compound. These results indicate that this novel non-steroidal anti-inflammatory agent can be a possible potent therapeutic agent for the treatment of ulcerative colitis.

Key words: basic anti-inflammatory agent, antioxidant, experimental colitis, treatment, non-steroidal-antiinflammatory drugs, methylprednisolone

INTRODUCTION

Ulcerative colitis is a chronic relapsing inflammatory process of the large bowel of unknown etiology. It is widely believed that clinical manifestations represent an imbalance of the immune response, resulting in inflammation and clinical symptoms. A trigger, most likely an antigen, activates T-lymphocytes that release cytokines, thereby recruiting large numbers of neutrophils and mononuclear cells in the mucosa. Subsequent activation of these cells causes a self-augmenting cycle of cytokine production, cell recruitment and inflammation.¹ In addition to cytokines, leukotrienes, thromboxane, platelet-activating factor, nitric oxide and reactive oxygen species are released from activated mucosal cells – predominantly from neutrophils and macrophages.² The difficulties encountered in attempting to determine these mediators in the mucosa of patients with ulcerative colitis have led to the development of experimental models for investigating the inflammatory mechanisms involved, and evaluating the effects of different therapeutic agents.³

Compound A {5-(2-hydroxy-ethylamino)-1-cy-

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clohexyl-2-pentanone} has been synthesized as an anti-inflammatory agent with basic character.⁴ This compound has been demonstrated to have a potent anti-inflammatory activity in the carrageenan induced rat paw edema model. It has also been shown to be an antioxidant agent, as determined by its hydroxyl radical scavenging activity and to its ability to interact with the 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH).⁴

The aim of this study was to investigate the effect of this agent on the histological features induced by 2,4,6-trinitrobenzenesulfonic acid (TNB) in a model of experimental colitis in rats, assess its influence on the serum levels of Tumor Necrosis Factor – alpha, and compare the effect with that of methyl-prednisolone, a drug widely used for the treatment of ulcerative colitis.

MATERIALS AND METHODS

2.1. Materials

The synthesis of compound A has been previously described in detailed.⁴ The chemical structure of compound A is shown in Figure 1. 2, 4, 6-trinitrobenzenesulfonic acid (TNB) obtained from Sigma-USA.

2.2. Methods

2.2.1. General preparation

A model of chronic inflammatory bowel disease was developed in the rat using intraluminal instillation of the hapten 2, 4, 6-trinitrobenzenesulfonic acid (TNB). When coupled with high-molecular-weight substances, such as tissue proteins, TNB induces an immunologic response.⁵

The following experimental procedure was followed: Adult male Wistar rats weighting 200-240 g, were acclimatized to our laboratory conditions for 1 week prior to the experiments. They were housed individually in cages at a constant temperature (29°C) and 12-h day/night cycle and they had free access to food and water.

A total of 24 rats were used and randomly assigned to one of 4 groups. Group 1 (n=6): induction of experimental colitis without further treatment, Group 2 (n=6): normal animals, Group 3 (n=6): induction of experimental colitis plus methyl-prednisolone administration and Group 4 (n=6): induction of experimental colitis plus administration of the compound A. All rats were euthanised one week after the induction of experimental colitis. All experimental procedures described below were approved by the Animal Care Committee according to the European Union Act and the Greek law 160, A-64, May, 1991.

2.2.2. Induction of experimental colitis:

Distal colitis was induced by intracolonic instillation of 25 mg of 2,4,6-trinitrobenzenesulfonic acid (TNB) dissolved in 0,25 ml of 50% ethanol (vol/vol). The solution was injected into the colon, 8 cm proximal to the anus with a PE-50 cannula. In order to ensure that TNB-ethanol solution was not immediately expelled by the rat, the cannula was left in place for 15 s prior to its removal.

2.2.3. Drug administration:

Group 1: Colitis without treatment.

Group 2: Normal animals.

Group 3: Methyl-prednisolone, 5.3×10^{-3} mmol/kg, i.v., every day, for 7 days.

Group 4: Compound A, 0.6 mmol/kg, i.v., every day, for 7 days.

2.2.4. Histology

After 8 days all rats were sacrificed and the colon was removed. Specimens were fixed in 10% buffered formalin and examined blindly by light microscopy. Tissues were assessed for the presence and activity of colitis and for the extent of tissue damage by performing a large number of serial sections. Diagnosis of colitis was based on the presence of changes of mucosal architecture, lamina propria cellularity, neutrophil polymorph infiltration and epithelial abnormality.⁶ Disease activity was assessed by a grading system using six grades: 0: normal, 1: structural changes only, 2: chronic inflammation, 3: neutrophils in epithelium, 4: crypt distortion, 5: erosions or ulcers. According to the above-mentioned grading, the mucosa was characterized as normal (grade 0), colitis in remission (grades 1&2), active colitis (grades 3,4,5).⁷ The extent of tissue damage was expressed as a percentage using a semiquantitative method (number of sections with the above lesions/number of sections examined).

2.2.5. Determination of serum TNF- α

The level of serum TNF- α was determined using ELISA method. In order to avoid alteration of the results if human antibody was used against TNF- α , a special antibody was used (antirat, DIACLONE Research).

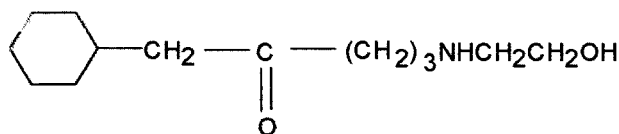


Figure 1. Chemical structure of compound A.

2.2.6. Data analysis and statistics

All results are presented as mean \pm standard deviation (SDV). Data were compared by one way analysis of variance (ANOVA) with Bonferroni and with Duncan post hoc analysis. Statistical significance was set up at a value of $p < 0.05$.

RESULTS

The extent of tissue damage ranged as follows: *Group 1*: 100% (7/7) to 50% (3/6), *Group 2*: 0% (0/0) to 0% (0/0), *Group 3*: 50% (3/6) to 20% (1/5) and *Group 4*: 25% (2/8) to 0% (0/0). The results of treatment with the tested compounds on the extent of tissue damage are shown in Table 1. A statistically significant difference in favor of group 4 was found after comparison of group 1 (colitis without treatment) with group 4 (compound A). The same observation was noted when we compare group 1 with group 3 (methyl-prednisolone administration). Moreover, when we compare group 3 with group 4, a statistically significant difference in favor of group 4 was found on Duncan test. That means that compound A resulted in less tissue damage along the colon compared

with that of methylprednisolone. This can also be seen from the percentage of the determination of the histological damage (33.3 for group 3 versus 13.7 for group 4).

The influence of treatment with compound A and methylprednisolone on individual histological lesions is shown in Table 2. Treatment with compound A resulted in the disappearance of inflammatory infiltration and edema. Statistically significant differences in favor of group 4 were observed, after comparison of group 1 and group 3 with group 4.

Figure 2 shows the histological grading in the four groups of animals tested. No difference between group 1 and 3 was observed. However, a statistically significant difference between group 1 (colitis) and 4 (compound A) was noticed as far as the different elements of the grading system used (normal histology, colitis in remission and active colitis) was concerned. ($P < 0.05$).

Serum TNF- α levels in the four groups of animals tested is shown in Table 3. A statistically significant difference is seen when we compare group 1, (colitis without treatment) with group 4 (compound A).

Table 1. Results of treatment with compound A and methyl-prednisolone on the extent (percentage) of tissue damage.

Animals	Group 1 (colitis without treatment)	Group 2 (control)	Group 3 (colitis plus methyl-prednisolone)	Group 4 (colitis plus compound A)
1	75	0	25	15
2	75	0	50	25
3	100	0	20	25
4	50	0	25	0
5	50	0	50	1
6	50	0	25	10
Mean Value \pm SDV	66.7 \pm 20.4	0 \pm 0	33.3 \pm 13.3*	13.7 \pm 8.6*

*Statistically significant difference between Groups 1 and 3 and 4 at the level of $p < 0.05$ (Bonferroni test and Duncan test).

Table 2. Influence of treatment with compound A and methylprednisolone on individual histological features.

Group	Histological features					
	Ulcer	Abscess	Epithelial damage	Distortion of glands	Inflammatory infiltration	Edema
1	5	1	5	4	5	4
2	0	0	0	0	0	0
3	5	1	3	2	5	2
4	2	0	4	3	0*	0**

*Statistically significant difference between Groups 1 and 3 with Group 4 at the level of $p < 0.05$ (Bonferroni test and Duncan test).

** Statistically significant difference between Groups 1 and 4 at the level of $p < 0.05$ (Bonferroni test and Duncan test).

DISCUSSION

It is well known that the major limitation concerning the use of non-steroidal anti-inflammatory drugs (NSAIDs) in various medical disorders is their adverse effects on gastrointestinal mucosa. It has been proposed that neutrophils significantly contribute to the ulcerative process of gastrointestinal mucosa produced by NSAIDs through the release of tissue damaging proteases and reactive oxygen metabolites.⁸

In recent years, basic anti-inflammatory agents are increasing in interest because they possess better pharmacokinetic properties and cause less gastric irritation compared to the acidic agents and several modifications in their formulation have been introduced to reduce their toxicity. Highly selective cyclooxygenase-2 (COX-2) inhibitors, NSAIDs containing nitric oxide, and several other compounds are being developed, including NSAIDs associated with zwitterionic phospholipids, chi-

ral NSAIDs, basic fibroblast growth factor, and trefoil peptides. Although initial studies indicate that some of these compounds may have limited gastrointestinal toxicity compared to traditional NSAIDs, their safety has not been clearly established.⁹

It is well accepted that NSAIDs can induce inflammatory bowel disease or exacerbate a pre-existing one. Previous observations in experimentally produced colitis in rats using mainly COX-1 inhibitors such as indomethacin and naproxen showed that these agents can markedly exacerbate colitis and that this effect is unrelated to alterations in colonic leukotriene B4 synthesis.¹⁰

Compound A was designed and synthesized as a basic anti-inflammatory agent. It has previously been shown that it has a mean inhibition of the carrageenan induced rat paw edema by 77.6% compared to controls. This compound has also significant antioxidant properties as shown by its ability to scavenge hydroxyl radicals and to interact with the DPPH stable free radical.⁴

Ulcerative colitis induced by TNBS is accompanied by a shift in the antioxidant enzyme activities, and low levels of glutathione.¹⁰ In the present study we showed that treatment with compound A resulted in a significantly beneficial effect on TNBS induced experimental colitis by reducing the histological damage. Compound A resulted in statistically significantly less tissue damage along the rat colon compared with that of the untreated ulcerative colitis group and with that of rats treated with methylprednisolone.

Moreover, treatment with compound A resulted in the disappearance of inflammatory infiltration, edema and ulcers of the mucosa compared to all other groups.

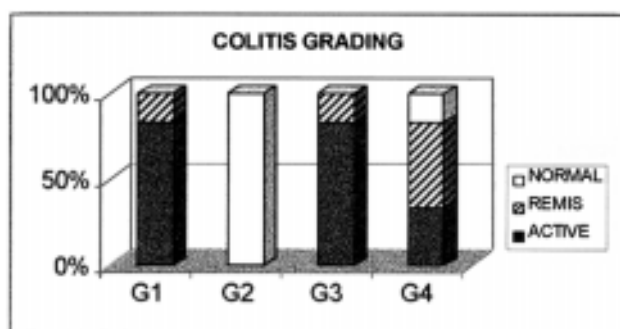


Figure 2: Grading of colitis in the four groups of animals tested: Statistically significant differences between group 1 and 4 were found ($P < 0.05$).

Table 3. Serum TNF- α levels in the four groups of animals tested.

Animals	Group 1 (colitis without treatment)	Group 2 (control)	Group 3 (colitis plus methyl-prednisolone)	Group 4 (colitis plus compound A)
1	0.43	0	0.29	0
2	1.6	0	0.32	0.14
3	0.51	0	0.38	0.27
4	0.57	0	0.26	0.13
5	0.64	0	0.30	0.13
6	0.16	0	0.24	0.11
Mean \pm SDV	0.65 \pm 0.49	0 \pm 0	0.30 \pm 0.05	0.13 \pm 0.08*

Serum TNF- α U/L, U = 50 pg/mL.

0 = not detectable levels

*Statistically significant difference between Groups 1 and 4 at the level of $p < 0.05$ (Bonferroni test and Duncan test)

The reduction in the number of ulcers was an important element of the overall response, indicating a more causative therapeutic effect of compound A on this model of colitis.

Initial trials relating to the efficacy of highly selective COX-2 inhibitors on the TNBS model of colitis in rats produced negative results,¹¹ as they did not show any beneficial effect despite their potent anti-inflammatory activity. However, two very recently published studies described completely different results. Thus, Guo et al¹² showed that pretreatment with COX-2 inhibitors protected against the development of 2,4,6-trinitrobenzenesulfonic acid-induced colitis, indicating that the highly induced COX-2 expression plays a significant role on the pathogenesis of TNBS induced colitis. Similar results were obtained by Karmeli et al,¹³ again using COX-2 inhibitors in two models of ulcerative colitis in rat, a result similar to that found in our study. It is of interest that compound A produced better results compared to corticosteroids, a result similar to that observed by Guo et al,¹² and Karmeli et al.¹³ This is not a curious finding as other substances administered rectally (such as L-glutamine) have also been shown to reduce mucosal injury - both macroscopically and biochemically - a significantly higher degree compared to corticosteroids.¹⁴

It is well established that TNBS-induced colitis is an Interleukin-12-driven, Th1 T cell-mediated colitis. In this model of colitis the levels of serum Interferon-gamma are also significantly increased.¹⁵ Moreover, upregulation of ICAM-1 and P-selectin as well as high tissues of malondialdehyde have recently been shown to be associated with marked neutrophil infiltration of the mucosa.¹⁶

Tumor Necrosis Factor-alpha is a pro-inflammatory cytokine, which has been shown to be one of the most significant of those participating in the inflammatory process of the bowel in patients with inflammatory bowel disease, especially in patients with Crohn's disease.¹⁷ A recently published study showed that the administration of avian tumor necrosis factor antibodies effectively treated experimental colitis in rat.¹⁸ It is possible, that TNF- α plays an important role for the development of colitis in rats treated with TNBS. In our study we have shown that compound A significantly reduced the levels of serum TNF- α . Although the same reduction of serum TNF- α was observed in the group of rats treated with methylprednisolone, the beneficial effect produced by methylprednisolone was lower compared to that of compound A, indicating that this novel substance has marked anti-inflammatory properties due to its basic character

and antioxidant properties.¹⁹ Other antioxidant substances, such as water-soluble Vitamin E derivative, have been shown to be effective in TNBS model of colitis in rat.²⁰

The results of the present study support the assumption that it may be important to further investigate whether a series of non-steroidal anti-inflammatory agents with basic character and antioxidant properties combined in a single molecule could be of value in an effort to treat ulcerative colitis patients. Since the optimal mode of treatment for this important disease remains unsolved, structures like that of compound A with potent antioxidant properties may be used as possible leads for the development of a novel class of therapeutic agents for inflammatory bowel disease in man.

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