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

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Abstract

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 methylprednisolone on the histological abnormalities and serum levels of tumor necrosis factor-α (TNF-α) in experimental colitis produced by 2,4,6-trinitrobenzenesulfonic acid (TNB). A total number of 24 rats were randomly assigned to one of four groups of six 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 of the extent of tissue damage and of certain histological features (edema, inflammatory infiltration) ($P < 0.05$). Compound A also resulted in a statistically significant reduction of the levels of serum TNF-α, compared to those of controls ($P < 0.005$). The beneficial effect of this compound was probably due to the combination, on a single molecule, of anti-inflammatory and antioxidant properties as well as to its basic character. The reduction of the serum TNF-α levels could be one of the possible mechanism(s) of action of the compound. Further studies are necessary to establish the direct mechanism of action(s) of the drug and to evaluate its long-term efficacy and safety.

Keywords: NSAID (non-steroidal-antiinflammatory agent, basic character); Antioxidant; Experimental colitis; Treatment; TNF-α (tumor necrosis factor-α); Methylprednisolone

1. Introduction

Ulcerative colitis is a chronic relapsing inflammatory process of the large bowel and is 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 (Nassif et al., 1996). 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 (Boughton-Smith and Pettipher, 1990). 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 for evaluating the effects of different therapeutic agents (Buch et al., 1995).

Compound A {5-(2-hydroxy-ethylamino)-1-cyclohexyl-2-pentanone} has been synthesized as an anti-inflammatory agent with basic character (Andreadou et al., 1997). This

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compound has been demonstrated to have potent anti-inflammatory activity in the carraggeenan-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 interact with the 1,1-diphenyl-2-picrylhydarzyl stable free radical (DPPH) (Andreadou et al., 1997).

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-α (TNF-α) in order to find a possible mechanism of action, and to compare the effects found with those of methylprednisolone, a drug widely used for the treatment of ulcerative colitis.

Table 1
Results of treatment with compound A and methylprednisolone on the extent of tissue damage (percentage)

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 ± SDV	66.7 ± 20.4	0 ± 0	33.3 ± 13.3[a]	13.7 ± 8.6[a]

[a] Statistically significant difference among groups 1, 3 and 4 at the level of $P < 0.05$ (Bonferroni correction and Duncan test).

2. Materials and methods

2.1. Materials

The synthesis of compound A was previously described in detailed (Andreadou et al., 1997). The chemical structure of A is shown in Fig. 1. TNB was 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 TNB. When coupled to high-molecular-weight substances, such as tissue proteins, TNB induces an immunologic response (Morris et al., 1989).

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

A total of 24 rats were used and randomly assigned to one of four 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 methylprednisolone administration and group 4 ($n = 6$): induction of experimental colitis plus administration of compound A. All rats were killed 1 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 Greek Law 160, A-64, May, 1991.

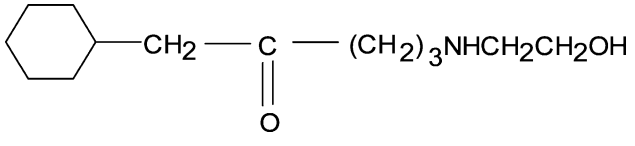

Fig. 1. Chemical structure of compound A.

2.2.2. Induction of experimental colitis

Distal colitis was induced by intracolonic instillation of 25 mg of 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. everyday for 7 days.
Group 4: Compound A, 0.6 mmol/kg, i.v. everyday for 7 days.

The dose of compound A used (0.6 mmol/kg) was the same dose that we used in the carraggeenan experiments (determination of the anti-inflammatory activity). The LD_{50} of the compound A is 0.75 mmol/kg intraperitoneally (Andreadou et al., 1997).

2.2.4. Histology

After 8 days, all rats were killed and the colon was removed. Specimens were fixed in 10% buffered formalin and examined "blind" by light microscopy. Tissues were assessed for the presence and activity of colitis as well as for the extent of tissue damage, using a large number of serial sections. Diagnosis of colitis was based on the presence of

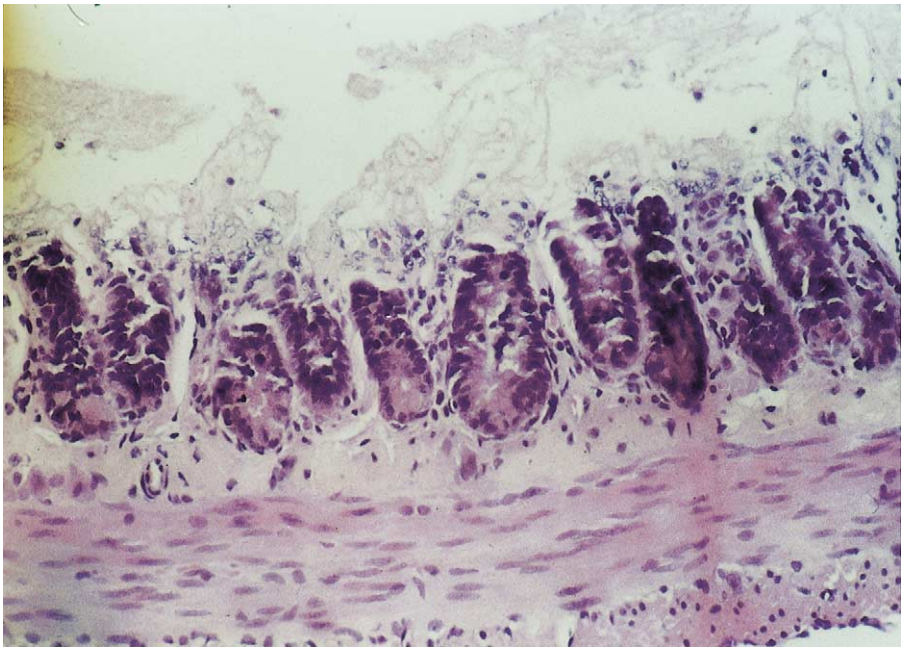

Fig. 2. Gross ulceration and other histological lesions, after induction of chemical colitis.

changes in mucosal architecture, lamina propria cellularity, neutrophil polymorph infiltration, and epithelial abnormality (Jenkins et al., 1997). Disease activity was assessed according to 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 and 2), active colitis (grades 3, 4 and 5) (Geboes et al., 2000). The extent of tissue damage was expressed as a percentage using a semiquantitive method (number of sections with the above lesions divided by the number of sections examined).

2.2.5. Determination of serum TNF-α

The level of serum TNF-α was determined using Enzyme-Linked ImmunoSorbent Assay (ELISA) method. In order to avoid errors in the results if human antibody was

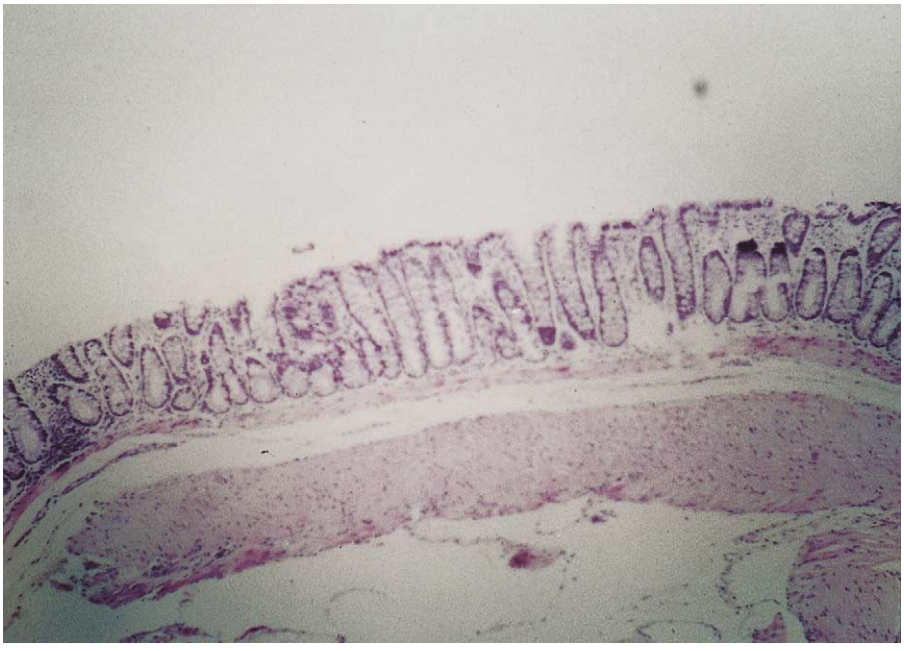

Fig. 3. Mild histopathological lesions seen in the group of animals treated with methylprednisolone.

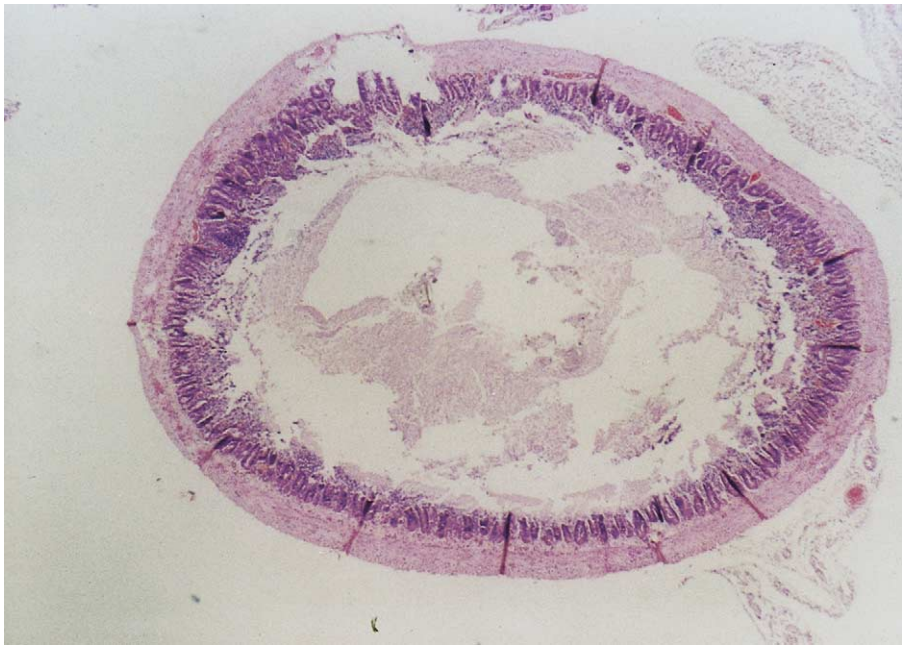

Fig. 4. Minor histopathological lesions seen in the group of animals treated with compound A.

used against TNF-α, a special rat antibody was used (antirat, DIACLONE Research).

2.2.6. Data analysis and statistics

All results are presented as means ± standard deviation (S.D.). Data were compared by one-way analysis of variance (ANOVA) with Bonferroni correction and with Duncan post hoc analysis. Statistical significance was set at a value of $P < 0.05$.

3. Results

The extent of tissue damage was ranked 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). Results of treatment with the compounds tested 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 was noted for group 1 vs. group 3 (methylprednisolone administration). Moreover, when we compare group 3 vs. group 4, showed significant difference in favor of group 4 in the Duncan test. That means that compound A resulted in less tissue damage along the colon than did methylprednisolone (Figs. 2–4). This can also be seen from the percentage for histological damage (33.3 for group 3 vs. 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.

Fig. 5 shows the histological grading for the four groups tested. No difference between group 1 and group 3 was observed. However, a statistically significant difference

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

Group	Histological features					
	Ulcers	Abscesses	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[a]	0[b]

[a] Groups 1 and 3 significantly different from group 4 at the level of $P < 0.05$ (Bonferroni correction and Duncan test).

[b] Statistically significant difference between groups 1 and 4 at the level of $P < 0.05$ (Bonferroni correction and Duncan test).

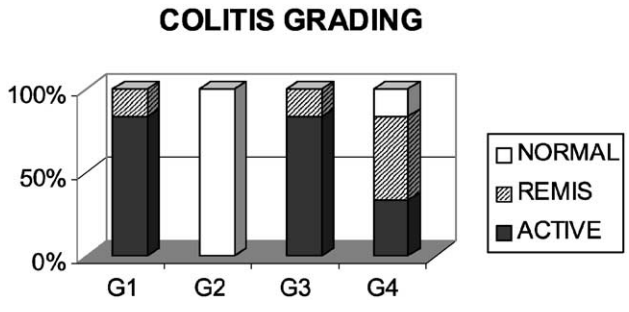

Fig. 5. Grading of colitis in the four groups of animals tested.

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 ± SDV	0.65 ± 0.49	0 ± 0	0.30 ± 0.05	0.13 ± 0.08[a]

Serum TNF-α U/l, $U = 50$ pg/ml.
0 = non-detectable.
[a] Statistically significant difference between groups 1 and 4 at the level of $P < 0.05$ (Bonferroni correction and Duncan test).

between group 1 (colitis) and group 4 (compound A) was noticed as far as the different elements of the grading system (normal histology, colitis in remission and active colitis) were concerned ($P < 0.05$) (Fig. 5).

Serum TNF-α levels in the four groups of animals are shown in Table 3. A statistically significant difference is seen when we compare group 1 (colitis without treatment) with group 4 (compound A) ($P < 0.005$).

4. 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. In recent years, basic anti-inflammatory agents have become of increasing interest because they possess better pharmacokinetic properties and cause less gastric irritation than do the acidic agents (Andreadou et al., 1992). Moreover, several modifications in their formulation have been recently introduced to reduce their toxicity. Highly selective cyclooxygenase-2 inhibitors, NSAIDs containing nitric oxide, and several other compounds are being developed, including NSAIDs associated with zwitterionic phospholipids, chiral 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 (Wolfe et al., 1999).

Compound A was designed and synthesized as a basic anti-inflammatory agent. It was previously shown that it had a mean inhibition of the carraggenan-induced rat paw edema of 78% (0.6 mmol/kg, intraperitoneally) compared to the controls. Indomethacin was used as a reference compound (11 μmol/kg intraperitoneally) and produced a 50% edema reduction. This compound also had significant antioxidant properties as was shown by its ability to scavenge hydroxyl radicals and to interact with the DPPH stable free radical (Andreadou et al., 1997). Previous observations in experimentally produced colitis of rats, using indomethacin and naproxen, showed that these agents could markedly exacerbate colitis and that this effect was unrelated to alterations in colonic leukotriene B_4 synthesis (Nieto et al., 2000). It is now well accepted that NSAIDs can induce inflammatory bowel disease or exacerbate pre-existing disease.

In the present study, we showed that treatment with compound A resulted in a significantly beneficial effect on TNB-induced experimental colitis by reducing the histological damage. Moreover, compound A resulted in significantly less tissue damage along the rat colon compared with that of the untreated ulcerative colitis group and, more importantly, with that of rats treated with methylprednisolone. It is of particular interest that treatment with compound A resulted in a significantly better result concerning inflammatory infiltration, edema and ulcers of the mucosa, compared to all other groups. The reduction in the number of ulcers was also an important element of the overall response, indicating a more effective therapeutic action of compound A on this model of colitis. However, the lack of an effect of methylprednisolone on ulcer healing was the main reason for the absence of a difference in the histological score between group 1 and group 3 (Fig. 5) when the grading system proposed by Geboes et al. (2000) was used.

The aim of our study was not to investigate the mode of action of compound A. However, previous studies have shown that modifications of the structures of known NSAIDs that yield an antioxidant, neutral molecule or a molecule with greatly reduced acidic character, can reduce the gastrointestinal toxicity of some NSAIDs (Kourounakis et al., 2000). Compound A has a basic character, antioxidant properties and anti-inflammatory functions and these effects most probably contribute to its protective effect against the individual histological lesions. It is widely accepted that ulcerative colitis induced by TNB is accompanied by a shift in antioxidant enzyme activities, and low levels of glutathione (Nieto et al., 2000). Since reactive oxygen species are important contributors to tissue injury in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Halliwell and Gutteridge, 1998), the effect of compound A may also be due to its free radical scavenging properties.

TNF-α is a pro-inflammatory cytokine, which has been shown to be one of the most significant factors participating in the inflammatory process of the bowel of patients with inflammatory bowel disease (Louis, 2001; Blam et al., 2001). TNF-α induces the production in cascade of a number of other cytokines including adhesion molecules, arachidonic acid metabolites, and activation of immune and non-immune cells (Louis, 2001). A recently published study showed that the administration of avian tumor necrosis factor antibodies effectively treated experimental colitis in rats (Worledge et al., 2000). It is possible that TNF-α plays an important role for the development of colitis in rats treated with TNB. In our study, 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 of methylprednisolone was less than that of compound A, indicating that this novel substance has marked anti-inflammatory properties probably due to its antioxidant properties in addition to its basic character. Other antioxidant substances, such as a water-soluble vitamin E derivative, have been shown to be effective in the TNB model of colitis in rats (Yoshida et al., 1999). It is possible that this reduction in the levels of TNF-α could be part of the mechanism(s) of action of compound A.

In conclusion, 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 such as 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 the human. Further studies are needed to evaluate the exact mechanism(s) of action of this compound as well as its efficacy and safety under long-term conditions.

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