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Original article

Novel indoline derivatives prevent inflammation and ulceration in dinitro-benzene sulfonic acid-induced colitis in rats

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ABSTRACT

Background: In search of safer treatments for inflammatory bowel disease in subjects not responding to, or showing adverse effects to TNF-α antagonists, we tested three novel indoline carbamates in the 2,4-dinitrobenzene sulfonic acid (DNBS) model of colitis in rats. The compounds have anti-inflammatory activity in other disease models in mice.

Methods: AN827 (3-(2-(methoxy carbonyl) ethyl) indolin-4-ylethyl methyl) carbamate (0.1 or 1 mg/kg), AN680 (3-(2-(methoxy carbonyl) ethyl) indolin-6-ylethyl methyl) carbamate (1.25 or 2.5 mg/kg) and AN917 (3-(3-amino propyl) indolin-4-ylethyl methyl) carbamate (1 or 2 mg/kg), 5-aminosalycilic acid (5-ASA) (1 or 100 mg/kg) or saline (1 ml/kg) were administered rectally 1 h after intracolonic administration of DNBS, (35 mg/kg in 30% alcohol). Disease severity was assessed four days after DNBS administration by change in body weight, colon weight, area of ulceration, myeloid peroxidase (MPO) activity, colonic TNF-α, IL-6 and IL-1β levels. Histopathological scoring was performed after staining colon sections with hematoxylin and eosin and with antibodies to CD68 and CD11b.

Results: AN827 (0.1 and 1 mg/kg), AN680 (2.5 mg/kg) and AN917 (2.0 mg/kg) significantly reduced all macroscopic and microscopic parameters of colitis, colonic pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 and MPO activity by about 80%.

Conclusions: The indoline derivatives largely prevented the symptoms of colitis and were 500–50 times more potent and more effective than 5-ASA. It may be worth evaluating them in models of established colitis. Since AN827 is strongly bound by plasma proteins no adverse effects are expected if compound is absorbed into the circulation after rectal administration.

Introduction

Inflammatory bowel disease (IBD) is subdivided into ulcerative colitis (UC) and Crohn's disease (CD). Although the precise etiology of IBD is not known, it is probably facilitated by defects in the barrier function of the intestinal epithelium and hyper-responsiveness of the local immune system [1,2]. In IBD, there is a pronounced infiltration into the lamina propria of innate immune cells (neutrophils, monocytes, macrophages, eosinophils and natural killer T cells) and adaptive immune cells (B cells and T cells). These cells produce pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β [3,4], which induce the expression of adhesion molecules, selectin and ICAM-1 in the vascular endothelium, thereby enabling the invasion of inflammatory cells into the mucosal layer [5]. CD and UC have distinct profiles of cytokine production. In CD, IFN-γ and TNF-α predominate, while in UC, IL-5, IL-6 and IL-13 appear to play a greater role [6,7].

TNF-α antagonists are particularly effective in the treatment of CD [8] because of the important role played by the cytokine in its etiology [9]. However, approximately 33% of patients either do not respond to them or lose response over time within the first 12 months of therapy [10], possibly because of the presence of neutralizing antibodies raised against the biological drug. TNF-α antagonists may also cause allergic reactions like myalgias and arthralgias and increase the incidence of infections and lymphomas [11]. Although switching to another TNF-α antibody may be beneficial in some subjects there are still those who have adverse effects or do not respond [12,13]. An alternative treatment strategy could be the use of small molecules that do not induce allergic or immune reactions but lower the levels of TNF-α and other pro-inflammatory cytokines.

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We have described a series of novel indoline carbamates that reduce nitric oxide, TNF-α and IL-6 in peritoneal macrophages after their elevation by the endotoxin, lipopolysaccharide (LPS). They act by inhibiting phosphorylation of mitogen-activated protein kinase p38, degradation of IκBα and the activation of the transcription factors, activator protein 1 and nuclear factor κB [14]. AN827 (3-(2-(methoxy carbonyl) ethyl) indolin-4-ylethyl methylcarbamate mesylate showed the greatest anti-inflammatory activity in LPS-activated macrophages, but was ineffective after systemic administration because it binds strongly to a relatively high molecular weight protein in plasma [15]. The two other compounds, 3-(2-(methoxy carbonyl) ethyl) indolin-6-ylethyl methyl carbamate tosylate (AN680) and 3-(3-amino propyl) indolin-4-yl ethyl methyl carbamate dihydrochloride (AN917), do not bind to plasma proteins. AN680 (3.5 mg/kg) and AN917 (3.0 mg/kg) respectively, reduced TNF-α and IL-6 levels in the plasma and spleen in a model of LPS-induced sepsis in mice and also prevented cellular infiltration and lung damage after intra-tracheal installation of LPS [15].

The aim of the current study was to determine whether we could demonstrate an anti-inflammatory activity of AN827 *in vivo* by administering the drug rectally. We chose for this test the dinitrobenzene sulfonic acid (DNBS) model of ulcerative colitis in order to enable the detection of a local effect of AN827 in the colon, since if any would be absorbed into the circulation, it would lack activity because of its binding to plasma proteins. The effect of AN827 was compared to those of AN680 and AN917. The study was designed to provide preliminary information about the potential anti-inflammatory activity of the compounds in rats. Any compound showing significant activity could then be further evaluated in models of established colitis using appropriate routes of administration, oral for AN680 and AN917 and rectal or colonic delivery for AN827.

Material and methods

Animals

Male Wistar rats weighing 260–280 g were purchased from Harlan, Jerusalem and housed in a pathogen free animal facility under controlled 12 h light/12 h dark cycle. The experiments were performed according to the guidelines set forth by the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985). The protocol for induction of colitis was reviewed by the Institutional Committee on the care and use of experimental animals of the Hebrew University.

Compounds and reagents

2,4-Dinitrobenzene sulfonic acid (Sigma Ltd.), ketamine (Ketaset, USA), heparin (Trima, Israel), 5-aminosalycilic acid, (Rafa, Israel). AN917 dihydrochloride, AN827 mesylate and AN680 tosylate have an asymmetric center and were tested as racemic mixtures.

Induction of colitis

Rats were housed up to three per cage with free access to food and water for one week in the Animal facility before the experiment. They were fasted for 12 h prior to the induction of colitis but allowed free access to water. Colitis was induced under light isoflurane anesthesia by intra-colonic administration of 35 mg DNBS dissolved in ethanol 30% (v/v), which was found in preliminary experiments to induce reproducible colitis without signs of undue suffering in the rats. A perforated Foley catheter was inserted rectally for 5–8 cm from the anus and removed immediately after administration of DNBS. The rats were left in a head down position for additional 30 s. As in other studies in this model [16,17], the compounds were given 1 h after DNBS. The compounds were administered twice daily for four days since in preliminary experiments this was found to be more effective than once daily when given by this route. The doses of AN680 tosylate and AN917 dihydrochloride contain the equivalent amount of base of each compound and all regimens are shown in Table 1. A control group of rats was anesthetized, given saline by enema instead of DNBS, and saline rectally (1 ml/kg) twice daily and other rats were given 5 amino salicylic acid (5-ASA) (1 or 100 mg/kg) once daily as a positive control.

Body weight loss and rectal bleeding were monitored daily. When these occurred in the majority of untreated rats on the fourth day, they were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg). The colon was carefully excised, opened longitudinally, rinsed with ice-cold PBS, pH 7.4, blotted dry, weighed and examined for ulcerated and inflamed regions. The number of ulcers was counted, their area measured. The colon was sectioned into 1 cm portions, frozen in liquid nitrogen and stored at −80 °C.

Cholinesterase inhibition by AN827, AN680 and AN917

Subcutaneous injection of AN917 (3.0 mg/kg) in mice inhibited cholinesterase (ChE) in plasma and spleen by 35–37%, but this contributed to less than 50% of the anti-inflammatory effect. AN680 (3.5 mg/kg) did not inhibit ChE in spleen or plasma [15]. To determine whether rectal administration of the compounds inhibited ChE in plasma or colon the rats were anesthetized 60 min after drug administration on the 4th day. Blood samples were taken from the vena cava prior to sacrifice by puncturing the chest wall, added to heparin-containing Eppendorf tubes, centrifuged 5 min at 17,000*g* and the plasma was frozen in liquid nitrogen and stored at −80 °C until assay. ChE activity in the colon and plasma was measured [18] and changes in activity expressed as a percent of that in tissues of rats given saline.

Cytokine detection in colonic tissues

TNF-α and IL-6 were measured in a colon segment by means of a BioLegend ELISA Rat kit and IL-1β by a Cloud-Clone Rat kit as described in [18]. Total protein concentration in the tissue supernatant was measured by means of a bicinchoninic acid (BCA) protein assay kit. Levels of cytokines are expressed as ng or pg/mg total tissue protein.

Tissue myeloperoxidase activity

Neutrophil infiltration was monitored by measuring myeloperoxidase (MPO) activity in colon segments containing evidence of macroscopic damage [19]. Tissues were homogenized in 0.5 ml solution containing hexadecyltrimethyl ammonium bromide

Table 1
Doses of compounds administered.

Treatment	Dose (mg/kg)	No of rats
Saline	1	14
5-ASA	1	8
5-ASA	100	8
AN827	0.1	10
AN827	1	10
AN680	1.25	10
AN680	2.5	10
AN917	1	10
AN917	2	10

(0.5%) in 50 mM phosphate buffer (pH 6.0). Homogenates were frozen and thawed twice, sonicated and centrifuged for 15 min at 6800*g*. Supernatants (10 μl) were transferred to a 96-well micro-titer plates containing 290 μl of o-dianisidine dihydrochloride reagent (Sigma) consisting of 0.17 mg/ml *o*-dianisidine (2HCl) in 50 mM phosphate buffer (pH 6.0). MPO activity was expressed as units per gm total protein, measured by BCA as described above.

Histological and immunohistochemical analyses in colon

Gut specimens from the inflamed region were excised, clean from fecal material, blotted on filter paper, and weighed and their length measured. They were fixed in 4% formalin (Sigma, St. Louis, MO, USA), and embedded in paraffin. For histological observations, the sections were stained with hematoxylin and eosin (H&E) and scored for damage by a pathologist blind to the animal treatment. A score from zero to four was given as described in [20].

Immunohistochemical staining of colon segments was performed as described in [21]. Sections were blocked with avidin and biotin from Avidin/Biotin blocking kit (Vector Laboratories, Inc, Burlingame, CA, USA), washed and incubated overnight at 4 °C with primary mouse anti rat CD68 1:30 (Serotec, USA) and OX-42 (CD11b/c) 1:500 (Merck Millipore, USA). Tissues were rinsed with PBS, incubated with biotinylated sheep anti rat secondary antibody 1:200 (Boehringer, Germany) for 1 h and stored at 4 °C overnight. Streptavidin conjugated with Cy3 as a secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) at dilution 1:200 was applied for 1 h at room temperature, followed by 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, USA) and counterstaining (blue) for 5 min. Finally, samples were washed in PBS and mounted with Immu-Mount medium (Thermo Scientific, USA). Images were processed with a Nikon-TL microscope. For quantification of infiltration of CD68 expressing cells three fields measuring 250 × 250 μm were sampled from each rat. Using image analysis software (Image J) pink (co-localized) cells that have a range of ovoid and crescent shapes were counted [22].

Statistical analysis

Doses of the compounds are expressed in terms of mg/kg of their respective salts. The results are presented as the mean ± SEM. Differences between data from groups of rats in which more than one treatment was given together with DNBS were analyzed by one way Analysis of Variance using IBM SPSS Statistics Version 20 followed by Duncan's *post hoc* test. A p value of <0.05 was considered to be significant.

Results

Effect of indoline derivatives on macroscopic parameters of acute colitis

One rat given DNBS alone, two, DNBS + 5-ASA (100 mg/kg) and one, AN680 (1.25 mg/kg) died during the study because of rectal perforation. In the remaining rats, DNBS and saline caused a significant loss in body weight, diarrhea and damage to colon tissue, indicated by hyperemia and ulceration 4 days after its instillation. ANOVA for% change in body weight was; $F_{6,85} = 8.29$, $p < 0.0001$; colon weight $F_{6,857} = 11.2$, $p < 0.0001$; ratio of colon weight to body weight $F_{6,85} = 12.8$, $p < 0.0001$ and area of ulceration, $F_{6,85} = 13.1$, $p < 0.0001$. The effect of the lower doses of 5-ASA, AN827 AN680 and AN917 on body weight loss, colon weight, area of ulceration is shown in Table 2. 5-ASA (1 mg/kg) did not significantly affect any of these parameters. We therefore increased the dose to 100 mg/kg that is equivalent to the amount given on a daily basis to human subjects and used in other studies in this model [23–25]. Of the three indoline derivatives tested, only AN827 (0.1 mg/kg) significantly reduced colon weight and its ratio to body weight and decreased the area of ulceration by more than 80%.

The effect of the higher doses of the compounds is shown in Fig. 1. Although 5-ASA (100 mg/kg) did not prevent body weight loss or increase in colon weight, it significantly reduced the area of ulceration ($p < 0.01$). AN827 (1 mg/kg) and AN917 (2 mg/kg) significantly prevented body weight loss, ($p < 0.05$). All the indoline derivatives significantly reduced colon weight, ratio of colon to body weight and the area of ulceration.

Tissue MPO activity and cytokine levels

MPO and cytokines were measured in the rats given DNBS and saline and the doses of 5-ASA and the indoline derivatives that had shown significant effects on the macroscopic effects of DNBS-alcohol on the colon, 5-ASA (100 mg/kg) AN827 (0.1 and 1 mg/kg), AN680 (2.5 mg/kg) and AN917 (2 mg/kg). ANOVA for MPO activity was $F_{6,56} = 35.1$, $p < 0.0001$; TNF-α, $F_{6,56} = 16.6$, $p < 0.0001$; IL-6, $F_{6,56} = 16.0$, $p < 0.0001$; IL-1β, $F_{6,56} = 23.4$, $p < 0.0001$. The data are shown in Fig. 2. DNBS increased MPO activity in untreated rats 15-fold. AN827 (0.1 mg/kg and 1 mg/kg) and AN680 (2.5 mg/kg) reduced MPO activity by at least 50%, while 5-ASA (100 mg/kg), AN827 (1 mg/kg) and AN917 (2 mg/kg) reduced it by 75–80%. 5-ASA and AN827 (0.1 mg/kg) were less effective than the other treatments in preventing the increase in TNF-α. AN827 (1 mg/kg), AN680 and AN917 all significantly reduced IL-1β, but 5-ASA and

Table 2
Effect of lower doses of 5-ASA and indoline derivatives on macroscopic measures of DNBS-induced ulcerative colitis.

Treatment (mg/kg)	Change in body weight (%)	Colon weight (g)	Colon weight/body weight (%)	Area of Ulceration (cm^2)
Saline control	8.06 ± 0.89	1.14 ± 0.07	0.4 ± 0.02	0 ± 0
With DNBS + ethanol				
Saline	−7.20 ± 2.11$^{\#\#}$	2.12 ± 0.08$^{\#\#}$	0.85 ± 0.04$^{\#\#}$	4.72 ± 0.66$^{\#\#}$
5-ASA (1)	−7.60 ± 2.56$^{\#\#}$	2.10 ± 0.09$^{\#\#}$	0.85 ± 0.04$^{\#\#}$	3.18 ± 0.64$^{\#}$
AN827 (0.1)	−5.14 ± 1.70$^{\#\#}$	1.52 ± 0.07$^{\#**}$	0.58 ± 0.02$^{\#**}$	0.37 ± 0.15**
AN680 (1.25)	−8.46 ± 1.66$^{\#\#}$	2.05 ± 0.11$^{\#\#}$	0.86 ± 0.05$^{\#\#}$	2.03 ± 0.57$^{\#**}$
AN917 (1)	−5.78 ± 1.74$^{\#\#}$	1.94 ± 0.20$^{\#\#}$	0.73 ± 0.07$^{\#\#*}$	3.32 ± 0.91$^{\#}$

5-ASA = 5 aminosalicylic acid. Data represent mean and SEM from 8 to 10 rats per group. Significantly different from DNBS untreated, $^{*}p < 0.05$, $^{**}p < 0.01$; significantly different from control # $p < 0.05$; ## $p < 0.01$.

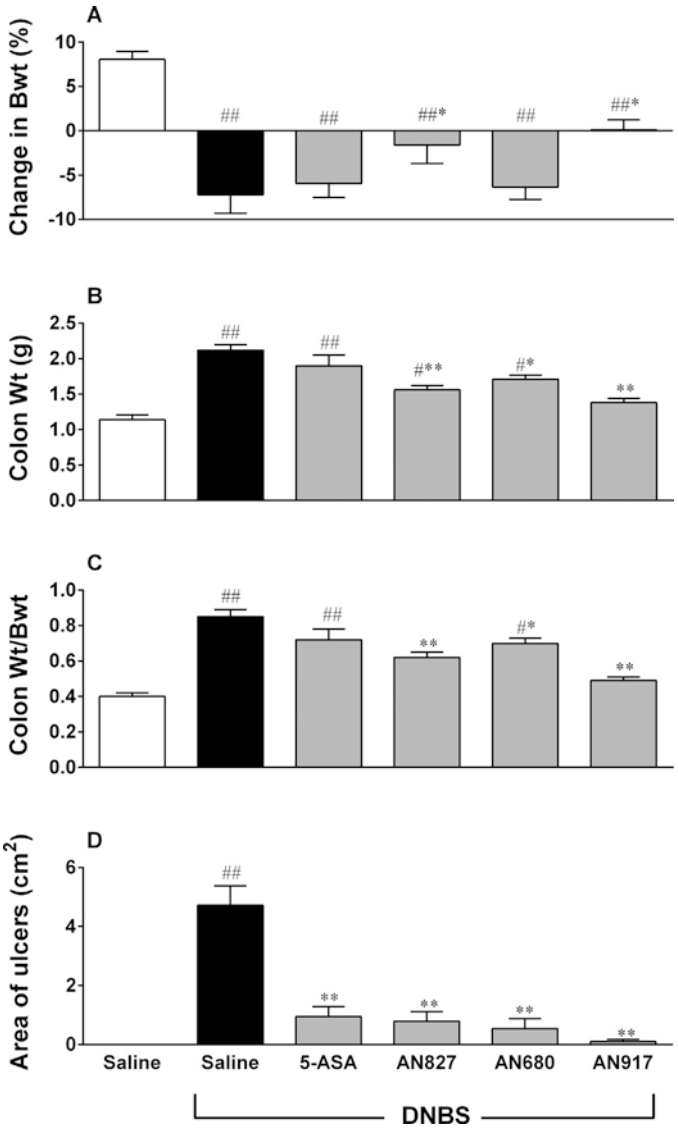

Fig. 1. Effect of compounds on macroscopic measures of DNBS-induced ulcerative colitis. A). Change in body weight. B). Colon weight. C). Colon weight/body weight. D). Area of ulcers. Each column represents the mean ± SEM (n = 6–12). 5-ASA = 5-aminosalycilic acid. Significantly different from DNBS untreated, $^{*}p < 0.05$, $^{**}p < 0.01$; significantly different from control, # $p < 0.05$; ##, $p < 0.01$.

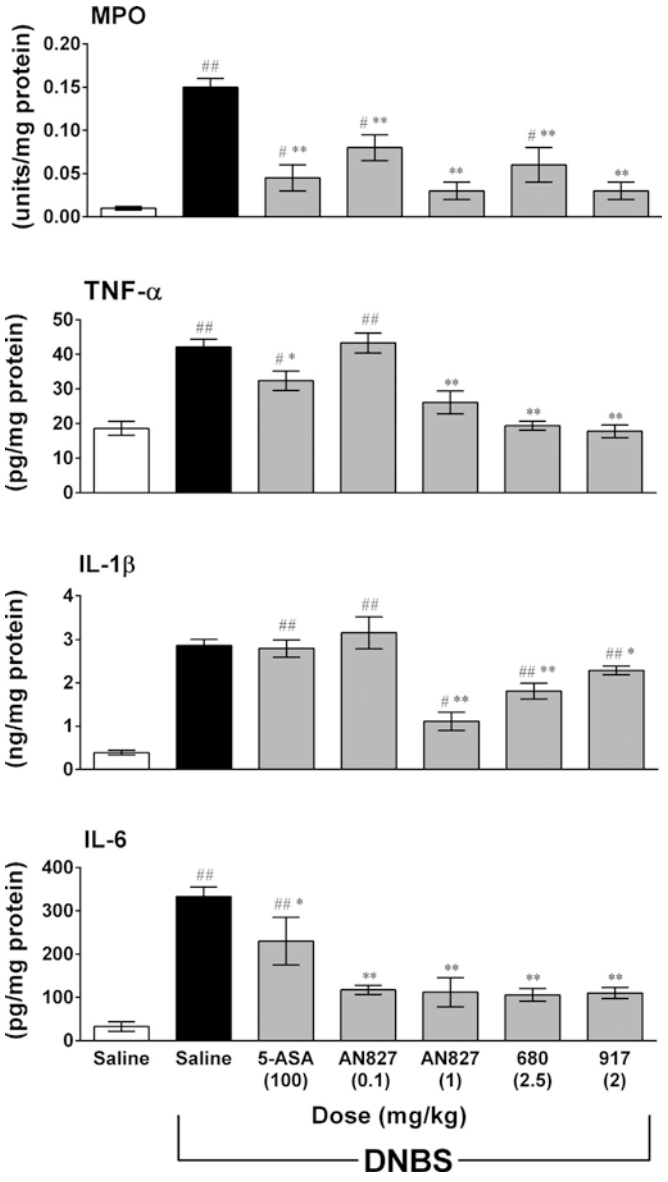

Fig. 2. Effect of compounds on MPO activity and cytokine levels in colonic tissue. MPO = myeloperoxidase activity. Each column represents the mean ± SEM (n = 6–10). Significantly different from DNBS untreated, $^{*}p < 0.05$, $^{**}p < 0.01$; significantly different from control, # $p < 0.05$; ##, $p < 0.01$.

AN827 (0.1 mg/kg) were ineffective. All the indoline derivatives also greatly reduced the elevation in IL-6 ($p < 0.01$).

Histological and immunohistochemical analyses in rat colon

The effect of drug treatment on colon histology was only performed on tissue of rats treated with the higher doses of each of the indoline derivatives. The colon of rats given DNBS and saline showed pronounced crypt loss and substantial infiltration of inflammatory cells into the submucosa (Fig. 3A). There was an almost 20-fold increase in cells stained with anti-CD68 (3B and 4B) and a 3-fold increase in cells labeled with anti-CD1lb (3C and 4C). AN827 (1 mg/kg) and AN680 (2.5 mg/kg) significantly decreased crypt damage and infiltration of inflammatory cells, and these were almost completely prevented by AN917 (2 mg/kg), as reflected in the reduction in the damage score (Fig. 4A) and MPO activity. While all the compounds were equally effective in preventing the increase in CD11b-labeled cells (3C and 4C), AN680 (2.5 mg/kg) and AN917 (2 mg/kg) were more effective than AN827 against those labeled with CD68 (3B and 4B).

None of the compounds significantly inhibited ChE in plasma at the doses tested and only AN917 (2 mg/kg) inhibited ChE by 13.6 ± 3.5% ($p < 0.05$).

Discussion

Rectal administration of DNBS in rats is a convenient, reproducible model of UC since it mimics acute neutrophil influx at disease onset, thought to play a causative role in inflammatory mucosal injury [26]. This model has been used in preclinical investigations to assay the anti-inflammatory properties of novel drugs with potential usefulness against intestinal inflammation in humans [27,28]. Release of pro-inflammatory cytokines including TNF-α and IL-6 among others, contribute to disease progression [29]. TNF-α regulates the expression of adhesion molecules and promotes infiltration of inflammatory cells [30].

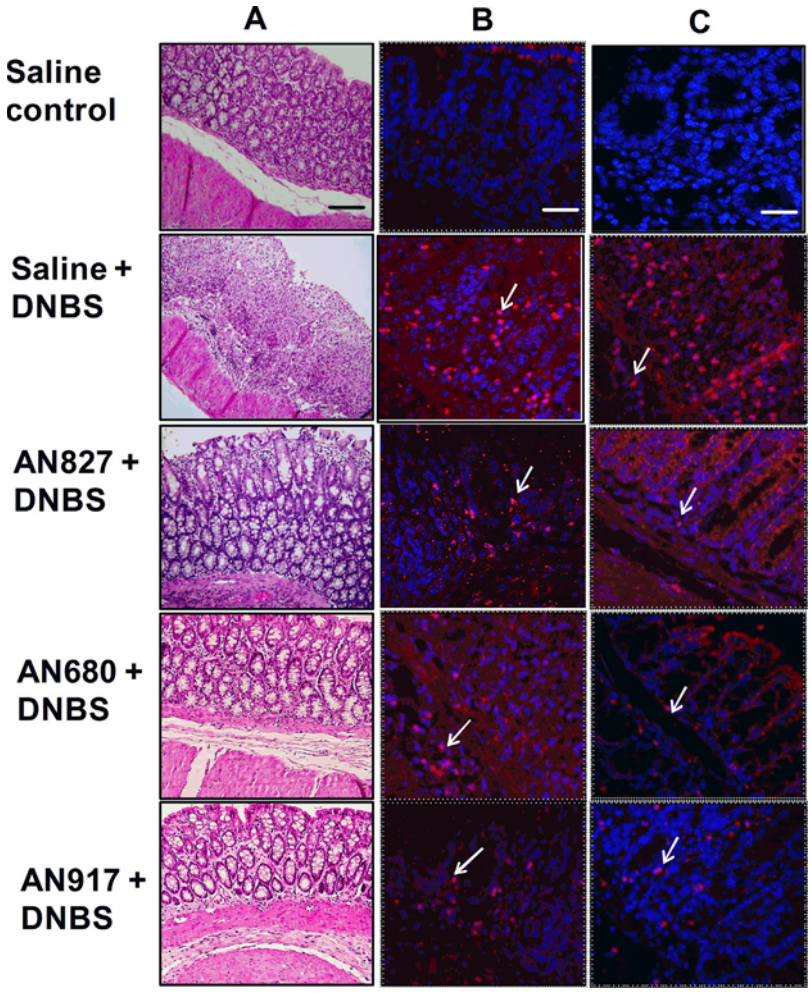

Fig. 3. Effect of AN827, AN680 and AN917 on histological characterization of DNBS-induced colitis in rats. A). Representative sections of colon stained with H&E from the normal control, DNBS treated with saline, AN827 (1 mg/kg), AN680 (2.5 mg/kg) and AN917 (2 mg/kg). Tissue from control rats shows intact epithelial cell layer, normal crypt formation and basal cells in lamina propria. In rats given DNBS and saline, the sub-mucosal layer is thickened with massive infiltration of inflammatory cells into the lamina propria visualized as dark purple nuclei. AN827, AN680 and AN917 all significantly improved crypt architecture and reduced infiltration of inflammatory cells. Scale bar 200 μM. B). Representative sections of colon showing immunofluorescence staining in normal control, DNBS-induced colitis untreated, and rats treated with the above doses of compounds and stained with a monoclonal antibody to CD68 (red) and DAPI (blue). White arrows indicate pink (co-localized) cells with ovoid and crescent shapes represent CD68 in the cell membrane. Scale bar 20 μM. C). Representative sections of colon showing immunofluorescence staining in normal control, DNBS-induced colitis untreated, and rats treated with the above doses of compounds and stained with a monoclonal antibody to CD11b (red) and DAPI (blue). White arrows indicate pink (co-localized) cells with ovoid and crescent shapes represent CD11b in cell membrane. Scale bar 20 μM.

As in previous studies in this model with different types of compounds that were used to prevent the symptoms of colitis [16,17,24,31], we observed body weight loss, diarrhea, ulceration, bleeding, neutrophil infiltration and elevation of cytokines, including TNF-α, IL-6 and IL-1β, four days after the colonic administration of DNBS (35 mg/kg, in 30% ethanol).

Although AN827 is 10 times more potent than AN680 and AN917 in reducing cytokines released from peritoneal macrophages activated by LPS [14], it has to be injected at much higher doses to produce an anti-inflammatory effect in mice because of strong protein binding [15]. After rectal administration, AN827 significantly reduced the macroscopic symptoms of colitis and associated increase in cytokine levels at less than 1/10th of the dose of AN680 and AN917, thereby confirming the results on macrophages. The indoline derivatives at doses ranging from 0.1–2.5 mg/kg, were also at least, if not more effective, than 5-ASA (100 mg/kg) in preventing the symptoms of colitis and associated macrophage infiltration.

CD68 is regarded as a selective marker for monocytes and macrophages and is widely used in human pathology studies to confirm recruitment of MPO-positive neutrophil granulocytes inflamed colonic mucosa. It was recently shown that the increased expression of MPO in injured rat and human liver is due to the presence of neutrophil granulocytes [32]. This finding contradicts the traditional view that the CD68 antigen is specific for macrophages. The antigen CD11b is known to be expressed on the surface of polymorphonuclear leucocytes, macrophages [33] and natural killer cells and is markedly increased in inflammation [34] and in the colon of DNBS-induced colitis in rats. In the current study, nearly twice the number of cells was labeled with an antibody to CD68 as with CD11b, as presumably, they also included other cells like neutrophil granulocytes. The reduction by the compounds in the number of such cells closely paralleled that of microscopic damage score and MPO activity. Cytokines TNF-α, IL1β and IL-6 are elevated in the colon of patients with UC and their levels are correlated with the endoscopic grade of inflammation [35]. In the colon of rats with DNBS-induced colitis, the concentrations of these cytokines were also increased and correlated well with measures of cellular infiltration and disease severity.

The beneficial effect of AN827 and AN680 was accomplished without any ChE inhibition in plasma or colon. AN917 which is

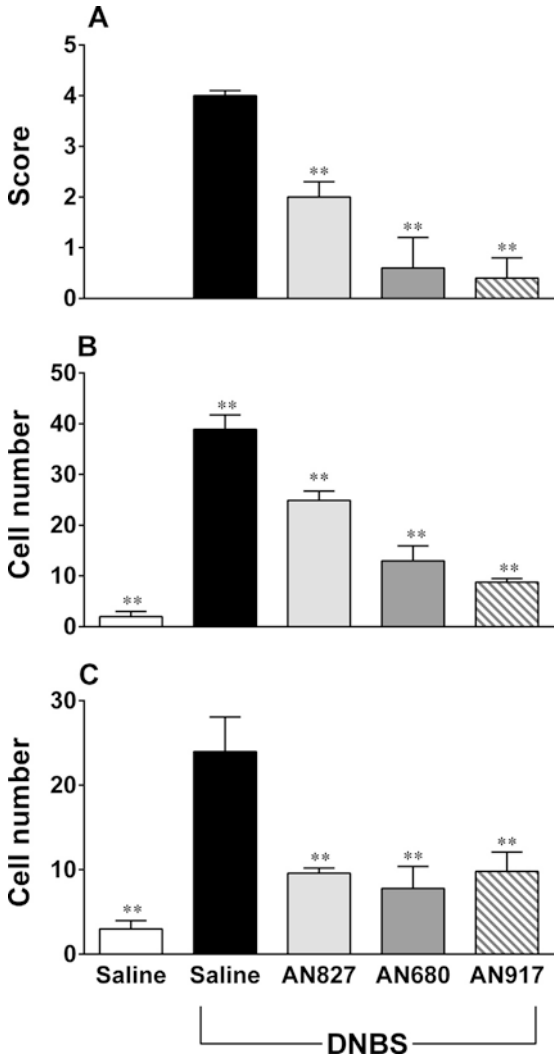

Fig. 4. Quantification of effect of AN827, AN680 and AN917 on histological characterization of DNBS-induced colitis in rats. A). Scores for colon damage from sections stained with H&E in rats with colitis treated with saline, AN827 (1 mg/kg) AN680 (2.5 mg/kg) or AN917 (2 mg/kg). B). Number of CD68 expressing cells in rats with colitis with and without treatment with the indoline derivatives. C). Number of CD11b expressing cells in rats with colitis with and without treatment with the indoline derivatives. Significantly different from DNBS untreated, $^{**}p < 0.01$.

6 times more potent as a ChE inhibitor than AN827 and 42 times more potent than AN680 [14] only caused 15% inhibition in the colon and none in plasma.

Although 5-ASA is considered a safe drug, it has been reported to cause acute pancreatitis [36] and possible renal damage [37]. As the indoline derivatives are small molecules, they are unlikely to cause allergic reactions, infections or lymphomas if given to subjects that do not respond to TNF-α antagonists, or in those that show allergic and immune reactions. AN827 appears to be effective over at least a 10-fold dose range. Like 5-ASA, it could be administered as an enema to human subjects and will not cause adverse effects if absorbed into the circulation because of protein binding. AN827 could also be prepared in a formulation that would be given orally and control its release in colon at the appropriate concentration during 24 h, thereby reducing its administration to once daily. Both AN680 and AN917 do not bind to plasma proteins and are absorbed after oral administration [38].

Conclusion: Because of their remarkable potency and efficacy in preventing ulceration and disruption of colon structure in this model, all three indoline derivatives could serve as potential replacements for 5-ASA in the treatment of UC or CD. Further studies could be performed to see whether AN827 and the other compounds can reverse inflammation and ulceration when given by appropriate routes of administration in models of these conditions after the disease is already present.

Conflict of interest

The authors declare no conflict of interest.

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