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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