Epithelial barrier and dendritic cell function in the intestinal mucosa
Verstege, M.I.

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Chapter 7.

Selective α7 nicotinic acetylcholine receptor agonists worsen disease in experimental colitis

Susanne A. Snoek1, Marleen I. Verstege1, Esmerij P. van der Zanden1, Nigel Deeks2, David C Bulmer2, Michael Skynner2, Kevin Lee2, Anje A. te Velde1, Guy E. Boeckxstaens1,3, and Wouter J. de Jonge1

1. Laboratory of Gastroenterology and Hepatology, Academic Medical Centre, The Netherlands,

2. Immuno-Inflammation CEDD, GlaxoSmithKline Stevenage UK.

3. Catholic University of Leuven, Belgium.
Abstract

Introduction. In various models vagus nerve activation has been shown to ameliorate intestinal inflammation, via nicotinic acetylcholine receptors (nAchRs) expressed on immune cells. As the α7 nAchR has been put forward to mediate this effect, we studied the effect of nicotine and two selective α7 nAchR agonists (AR-R17779, (-)-spiro[1-azabicyclo[2.2.2] octane-3,5′-oxazolidin-2′-one and GSK1345038A) on disease severity in two mouse models of experimental colitis.

Materials and methods: Colitis was induced by administration of 1.5% dextran sodium sulphate (DSS) in drinking water or 2 mg 2,4,6-trinitrobenzene sulphonic acid (TNBS) intrarectally. Nicotine (0.04 and 0.4 mg/kg), AR-R17779 (0.1-5 mg/kg) or GSK1345038A (3-60 mg/kg) was administered daily by i.p. injection. After 7 (DSS) or 5 (TNBS) days clinical parameters and colonic inflammation were scored.

Results. Nicotine and both α7 nAchR agonists reduced the activation of NF-κB and pro-inflammatory cytokines in whole blood and macrophage cultures. In DSS colitis, nicotine treatment reduced colonic cytokine production, but failed to reduce disease parameters. Reciprocally, treatment with AR-R17779 or GSK1345038A worsened disease and led to increased colonic pro-inflammatory cytokine levels in DSS colitis. The highest doses of GSK1345038A (60 mg/kg) and AR-R17779 (5 mg/kg) ameliorated clinical parameters, without affecting colonic inflammation. Neither agonist ameliorated TNBS-induced colitis.

Discussion. Although nicotine reduced cytokine responses in vitro, both selective α7 nAchR agonists worsened the effects of DSS-induced colitis or were ineffective in those of TNBS-induced colitis. Our data indicate the need for caution in evaluating α7 nAchR as a drug target in colitis.
Selective α7 nAchR agonists worsen disease in experimental colitis

Introduction

Genetic association studies and functional evidence have increased the recognition that intestinal macrophages play an important role in initiation and progression of inflammatory bowel disease (IBD). In several studies it was demonstrated that resident macrophages in mucosal samples of active ulcerative colitis (UC) and Crohn’s disease (CD) patients phenotypically and functionally differ from healthy controls. Similarly, data obtained from mouse models of colitis imply a crucial role for macrophages: in IL-10 deficient mice that develop colitis spontaneously, intestinal inflammation is prevented by the use of antagonists of chemokine receptors that are generally expressed by macrophages, or by elimination of tissue macrophages. Furthermore, colitis can still be induced in the absence of T and B cells. Recently, it has been shown that macrophage-derived IL-10 is crucial for the induction of regulatory T-cells thereby controlling intestinal inflammation in colitis.

Recently, the parasympathetic system, in particular the vagus nerve, has been shown to negatively regulate macrophage immune responses via the peripheral release of acetylcholine. Activation of the so-called ‘cholinergic anti-inflammatory pathway’ has been shown to ameliorate disease in various models of inflammatory disease including, sepsis, ischemia reperfusion, haemorrhage, and postoperative ileus. In mouse models of IBD and postoperative ileus, enhanced parasympathetic output is involved in the negative regulation of intestinal inflammation via efferent activity of the vagus nerve. Ghia et al. have recently demonstrated the eminent role for cholinergic inflammatory control in two experimental colitis models. In these studies it was shown that chemical as well as surgical blockade of vagus nerve signalling significantly worsens colitis and enhances colonic inflammatory mediators. The anti-inflammatory effect of the vagus nerve most likely involves activation of the nicotinic acetylcholine receptors (nAchRs) on immune cells such as macrophages or dendritic cells. This notion is supported by clinical observations that smoking, and the
administration of nicotine (i.e. via patches) may have a protecting effect on colonic inflammation in UC, even though results are generally disappointing due to the significant toxic adverse-events \textsuperscript{21}.

The cellular pathways of nicotinic inhibition of macrophage activation involve the activation of anti-inflammatory Stat3/Socs3 signalling pathways \textsuperscript{14} and inhibition of NF-κB signalling \textsuperscript{22}. Earlier studies indicate that the anti-inflammatory effect of acetylcholine is mediated through the \( α \)7 nicotinic acetylcholine receptor (\( α \)7 nAChR) \textsuperscript{11,18} expressed by human \textsuperscript{18,22} and mouse macrophages \textsuperscript{18,20,22}. Given the purported role of \( α \)7 nAChRs in mediating the cholinergic anti-inflammatory pathway \textsuperscript{14,18,23}, selective \( α \)7 nAChR agonists may bear more therapeutic potential in ameliorating disease compared to nicotine. Therefore, we explored the potential of pharmacological activation of the cholinergic anti-inflammatory pathway by treatment with nicotine and two \( α \)7 nAChR selective agonists in two mouse models of colitis. In dextran sodium sulphate (DSS) induced colitis, our results show that nicotine does not affect disease severity. Both selective \( α \)7 nAChR agonists AR-R17779 and GSK1345038A affect disease severity in a bell-shaped response curve; low doses aggravate disease, while high doses ameliorate disease. In 2,4,6-trinitrobenzene sulphonic acid (TNBS) colitis, treatment with GSK1345038A was ineffective. These data have important repercussions on the therapeutic potential of \( α \)7 nAChR specific agonists in colitis.
Material and Methods

Animals

Female C57BL/6 mice (8-10 weeks old and weighing 20-25 g; Charles River) were housed and maintained under standard conditions at our animal facility. Food and water were given ad libitum. All animal experiments were performed according to the guidelines of the Animal Research Ethics Committee of the University of Amsterdam.

Induction of colitis

To induce DSS colitis, 1.5% (w/v) DSS (TdB Consultancy, Uppsala, Sweden) was administered in the drinking water of the mice during 7 days. Body weight was recorded daily and weight loss as on day 7 as compared to day 0 was calculated. Animals were killed on day 7 of DSS administration. Hapten-induced colitis was induced by rectal administration of one dose of 2 mg TNBS (Sigma Chemical Co, St Louis, MO) in 40% ethanol (Merck, Darmstadt, Germany), using a vinyl catheter that was positioned 3 cm from the anus. During the instillation, the mice were anaesthetised using isoflurane (1-chloro-2,2,2-trifluoroethyl-isoflurane-difluoromethyl-ether; Abbott Laboratories Ltd., Queenborough, Kent, UK), and after the instillation mice were kept vertically for 30 seconds. Five days after TNBS instillation, mice were killed. Mice received a daily intraperitoneal (i.p.) injection with nicotine (0.04 or 0.4 mg/kg, or 0.25 or 2.5 μmol/kg respectively) (Sigma-Aldrich, Zwijndrecht, the Netherlands); AR-R17779 (0.1, 0.3, 1, 3 or 5 mg/kg; or 0.6-30 μmol/kg) (kindly provided by Critical Therapeutics, Lexington, MA) or GSK1345038 (3, 10, 30 or 60 mg/kg or 6-120 μmol/kg) (kindly provided by Glaxo SmithKline, Stevenage, UK) in 1% methylcellulose. The treatment with agonists was started at the first day of DSS or TNBS administration.
**GSK1345038A pharmacokinetics**

GSK1345038A, 60 or 120 μmol/kg, was administered i.p. to C57Bl/6 mice, and blood samples were taken at time points 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 12 h. Blood samples were analysed for the free base of GSK1345038A using a method based on protein precipitation and HPLC-MS/MS analysis. Acetonitrile: ammonium acetate (10mM) (8:2, 250 µL) containing an appropriate internal standard was added to the samples of blood (50 µL diluted with 50 µL with water). Samples were mixed thoroughly (mechanical shaking for 20 minutes), and then centrifuged (2465 x g for 15 minutes at room temperature). An aliquot of the resulting supernatant was analysed for GSK1345038A by reverse phase HPLC-MS/MS using a heat-assisted electrospray interface in positive ion mode (Sciex API 4000) and an ACE-3 C18 column (50 x 4.6mm ID, 3μm; Hichrom). The mobile phase was delivered as a linear gradient of 20% to 95% acetonitrile : ammonium acetate (1mM containing 0.1%v/v formic acid) over 0.8 minutes. The final composition was held for 0.8 minutes before return to initial composition. Nominal MRM transition for GSK1345038A was 454 to 123. Concentration range for the assay was; 0.011 to 22.0 μM with a lower limit of quantification (LLQ) of 0.011 μM.

**Assessment of colitis**

Faecal blood, diarrhoea and disease activity index (DAI) were scored as described in table 1. The wet weight of each colon was recorded and used as an index of disease-related intestinal wall thickening. The total length of the colon was measured and colon shortening as a consequence of DSS-induced colitis was used as a disease parameter. Subsequently, the colons were separated from mesentery and fat and longitudinally divided into two parts for histological examination and measurement of cytokines.
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Table 1. Scoring of the Disease Activity Index (DAI). To determine the DAI, scores of a., b. and c. were combined and divided by three. Bodyweight loss was calculated as the percentage difference between the body weight on day 0 and the body weight on day 7 of the experiment. The appearance of diarrhoea is defined as mucus/faecal material adherent to anal fur.

<table>
<thead>
<tr>
<th>a. weight loss</th>
<th>b. stool consistency</th>
<th>c. bleeding</th>
</tr>
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<tbody>
<tr>
<td>0: &lt; 1%</td>
<td>0: normal</td>
<td>0: negative</td>
</tr>
<tr>
<td>1: 1-5%</td>
<td>2: loose stools</td>
<td>2: positive</td>
</tr>
<tr>
<td>2: 5-10%</td>
<td>4: diarrhoea</td>
<td>4: gross bleeding</td>
</tr>
<tr>
<td>3: 10-15%</td>
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<td>4: &gt;15%</td>
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Histological examination

The longitudinally divided colons were fixed in 4% formalin and embedded in paraffin for routine histology. An experienced pathologist microscopically evaluated formalin-fixed haematoxylin tissue sections in a blinded fashion. Rolled colon was evaluated, and graded from 0 to 26 points as indicator of incidence and severity of inflammatory lesions based on the extent of the involved area, the number of follicle aggregates, oedema, fibrosis, hyperplasia, erosion/ulceration, crypt loss, and infiltration of granulocytes and mononuclear cells (see table 2).

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>Area involved</td>
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<td>Folllicles</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Oedema</td>
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<td>Erosion/ulceration</td>
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<td>Fibrosis</td>
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<tr>
<td>Hyperplasia</td>
<td></td>
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<tr>
<td>Crypt loss</td>
<td></td>
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<tr>
<td>Granulocytes</td>
<td></td>
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<tr>
<td>Mononuclear cells</td>
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</table>

Table 2. Histopathology score
Colonic cytokine production

For cytokine measurements, colons were diluted 1:9 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, pepstatin A, leupeptin, and aprotinin (all 20 ng/ml; pH 7.4), and incubated at 4°C for 30 minutes. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until analyses. TNF, IL-6 and IL-17 in supernatants were analysed by mouse ELISA Duoset kits (R&D Systems, Minneapolis, MN). Assays were performed according to the manufacturer’s instructions.

Whole blood stimulation assays

Whole blood was taken via heart puncture following anaesthesia. Aliquots of 50 µl were divided onto round bottom 96 wells plates and treated with appropriate concentrations of nicotinic agonists diluted in 50uL of RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (FCS; Gibco-BRL, Breda, The Netherlands), 2 mM L-glutamine, 1000 U/ml penicillin, 1000 µg/ml streptomycin, 250 ng/ml amphotericin B (Gibco) for 15 minutes 37°C. Subsequently, heat killed E. coli (1x10⁴/well) or lipopolysaccharide (LPS) (Sigma) at a final concentration of 100 ng/ml was added to the wells. After 3 hours of stimulation, plates were centrifuged, supernatants were collected and levels of TNF, IL-6 and IL-17 were analysed by ELISA (R&D Systems).

NF-κB activity assay

Immortalised peritoneal macrophages RAW264.7 were stably transfected with a NF-κB luciferase reporter construct (Clontech, Mountain View, CA) in which a PDNA3.1(+) derived neomycin resistance TK cassette was inserted (referred to as pNF-κBneo-luc). Transfection was performed using Nucleofactor V (Lonza, Cologne, Germany). Briefly, 0.5 µg per 10⁶ cells of constructs pNF-κBneo-luc was suspended in 75 µl of 150mM sterile NaCl solution. The transfection was allowed to proceed
for 16 hours, and the medium refreshed. Twenty-four hours after transfection, neomycin resistant clones were selected and subcloned. For assay, cells were pretreated with nicotinic agonists at the concentration indicated for 20 minutes, washed and subsequently stimulated with LPS (100ng/mL; Sigma) for 6 hours. After treatment, the medium was removed; the cells were washed three times with ice-cold PBS and lysed with Passive Lysis Buffer supplied in the Luciferase™ Reporter Assay Kit (Promega Corporation, Madison, WI) and the lysate was assayed for luciferase activity according to the manufacturer’s instructions.

**Statistics**

The values of the groups without DSS and TNBS groups treated with nicotine and α7 nAchR agonists are relative values (%) as compared to the DSS and TNBS groups treated with vehicle. Differences between groups were analysed using the nonparametric Mann-Whitney U test. \( P<0.05 \) was considered significant. All analyses were performed using SPSS (SPSS Inc. Chicago, Ill, USA).
**Results**

**Macrophage activation is modulated by nicotine, and α7nAchR agonists GSK1345028A and AR-R17779**

Initially, we reproduced experiments showing that nicotine, AR-R17779 \(^{14,24}\), and GSK1345028A reduced TNF and IL-6 release *in-vitro* in Biogel elicited peritoneal macrophages stimulated with heat-killed *E. coli* or LPS in a 0-1000nM concentration range \(^{24}\) (and data not shown). In line with these previous observations, AR-R17779 and GSK1345028A, as specific α7 nAchR agonists were less potent in reducing peritoneal macrophage cytokine release as compared to nicotine \(^{24}\) (data not shown). In whole blood cell preparations (see figure 1), nicotine or the α7 nAchR agonists, AR-R17779 and GSK1345028A, significantly reduced TNF and IL-6 production in LPS-or heat-killed *E. coli* activated whole blood cell preparations, albeit the potency to reduce cytokine production was less pronounced. None of the three agonists significantly reduced IL-6 production after stimulation with LPS (see figure 1). The values for these inflammatory substances in unstimulated cells were below levels of detection (data not shown).

The potential of nicotinic agonists to reduce cytokine production has previously been associated with inhibition of NF-κB activity \(^{23,24}\). We explored the potency of GSK1345028A and AR-R17779 to reduce NF-κB transcriptional activity in activated macrophages. To this end, we investigated the effect of nicotine, AR-R17779 and GSK1345028A on NF-κB activation induced by LPS in a reporter assay using the macrophage cell line RAW264.7, which was stably transfected with a NF-κB reporter construct. As shown in figure 2, LPS-induced NF-κB transcriptional activity that was significantly reduced by nicotine, AR-R17779, as well as GSK1345038A.
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Figure 1. Cytokine production in mouse whole blood induced by LPS and *E. coli*. Whole blood from healthy female C57BL/6 mice was stimulated *ex vivo* with nicotine (0-1000 nM), AR-R17779 (0-10,000 nM), GSK1345038A (0-10,000 nM) and subsequently incubated with LPS (100 ng/ml) (left panels) or *E. coli*. IL-6 (upper panels) and TNF (lower panels) Values are indicated as compared to vehicle. Data represent three independent experiments. Asterisks indicate significant differences (*= p<0.05), bars indicate mean ± SEM.

Figure 2. Effects of nicotine, AR-R17779 and GSK1345038A on NF-κB activation in RAW264.7 cells. RAW264.7 cells stably transfected with NF-κB luciferase reporter constructs were pre-treated with different concentrations of nicotine (0-1000 nM), AR-R17779 (0-10,000 nM) or GSK1345038A (0-10,000 nM) and stimulated with LPS (100 ng/ml). Values are relative as compared to vehicle. EV= empty vector, NS = not stimulated. Data represent three independent experiments. Asterisks indicate significant differences (*= p<0.05, **= p<0.01) as compared to vehicle. Bars represent mean ± SEM.
Treatment with nicotine does not affect clinical parameters in DSS-induced colitis

Given the reported potential of the vagus nerve to reduce disease in various mice models, including colitis, and the positive association of smoking and the course of UC, we next tested whether treatment with nicotine affected the disease course of DSS–induced colitis. Daily treatment with nicotine did not alter weight loss (see figure 3a) or DAI (see figure 3b) as compared with the vehicle-treated group. Only colon weight, which represents thickening of the colon by oedema, was significantly reduced by treatment with both 0.04 and 0.4 mg/kg nicotine (see figure 3c) but colon shortening was not affected by nicotine administration (see figure 3d). To test the effect of nicotine on intestinal inflammation we measured the production of TNF, IL-6 and IL-17 in colon homogenates. Although TNF levels were not altered, colonic IL-6 and IL-17 levels were significantly reduced by nicotine treatment (see figure 3e). However, this reduced cytokine production was not reflected in a decreased histopathology score (see table 3).
Figure 3. Effects of nicotine on DSS-induced colitis. a. Percentage body weight on day 7 as compared with body weight on day 0 of the experiment. b. Disease activity index (DAI) as described in material and methods. c. Colon length. d. Colon weight per centimetre colon. e. TNF, IL-6 and IL-17 levels in colon homogenates. Data are expressed as % of mice receiving DSS and treated with vehicle. Asterisks indicate significant differences (*p<0.05, **p<0.01) as compared to DSS group treated with vehicle. n= 10 per group. Columns indicate mean ± SEM.
Treatment with α7nAchR agonists GSK1345038A and AR-R17779 worsened the clinical parameters of colitis

We next questioned whether nicotine treatment failed to reduce disease severity in DSS-induced colitis because nicotine does not selectively target the α7 nAchR. In separate experiments, we therefore tested the efficacy of AR-R17779 and GSK1345038A to ameliorate DSS-induced colitis. A dose of 6 μmol/kg of AR-R17779 results in a C\text{max} of 4.6 μM and a half life of approximately 150 min (G.R., personal communication), and should thus reach the effective concentration range to reduce cytokine release in macrophages \textsuperscript{24} and whole blood (see figures 1 and 2). Hence, we administered AR-R17779 at a 0.1-5 mg/kg dose daily. Daily i.p. injection with 0.3, 1 and 3 mg/kg of AR-R17779 aggravated weight loss (see figure 4a). In contrast, in the group treated with a highest dose of AR-R17779 (5 mg/kg) weight loss was prevented (see figure 4a).

Table 3. The effect of nicotine on colonic inflammation in DSS-induced colitis. C57BL/6 mice were administered 1.5% DSS in drinking water and sacrificed at day 7. H&E stainings were performed on whole colons including rectum from groups treated with vehicle, 0.04 and 0.4 mg/kg nicotine and scored by an experienced pathologist. H&E stainings of colon and rectum showed inflammatory features including crypt damage, follicles, oedema, ulceration and influx of inflammatory cells. Mice per group: n=10. Data represent mean ± SEM.
Selective α7 nAchR agonists worsen disease in experimental colitis

To confirm these data, we next tested another specific α7 nAchR agonist GSK1345038A in the same model of DSS-induced colitis. Similar to AR-R17779, we first assessed the optimal dosage range for GSK1345038A by measurement of the blood concentration of GSK1345038A. The pharmacokinetics indicated that GSK1345038A has a half live of 2-3 hours, and reaches blood concentrations of 5-25 μM in a dosage range of 60-120 μmol/kg mouse (see figure 5), that is, the effective dose range to reduce cytokine release in our in vitro assays (see figures 1
and 2). Hence, to reach optimal circulation levels in vivo, we administered doses of 6, 20, 60, and 120 \( \mu \text{mol/kg} \) (3, 10, 30 and 60 mg/kg) i.p. daily.

![Graph showing blood concentration over time for GSK1345038A](image)

**Figure 5. The time course for the concentrations of GSK1345038A in the blood.**

Concentrations of GSK1345038A (60 [closed circles] and 120 [open circles] \( \mu \text{mol/kg} \)) in mouse blood was assessed as a function of time. Data shown are the mean ± SEM of triplicate measurements of 4 mice.

In line with the results obtained using AR-R17779, weight loss was significantly enhanced by daily injection with 3, 10 or 30 mg/kg GSK1345038A (see figure 4a). In accord with the effect of the highest dose of AR-R17779 on the course of colitis, weight loss was prevented by daily treatment with the highest dose of GSK1345038A (60 mg/kg) tested (see figure 4a).

The effects of both \( \alpha 7 \)nAchR agonists on the DAI paralleled those of the effects on weight loss as treatment by AR-R17779 significantly enhanced DAI (see figure 4b). In contrast, DAI was significantly reduced after treatment with the highest dose of 5 mg/kg AR-R17779 (see figure 4b). Similar results were obtained by treatment with GSK1345038A in that it significantly worsened disease, as reflected in DAI, except with the highest dose of 60 mg/kg GSK1345038A, that ameliorated DAI compared with the vehicle control (see figure 4b). In contrast to nicotine treatment, the increase of colon weight was unaffected by either of the \( \alpha 7 \) nAchR agonists (see figure 4c), while the DSS-induced decrease in colon length was further reduced by AR-R17779 and treatment with GSK1345038A was ineffective (see figure 4d).
The effect of the α7nAchR agonists GSK1345038A and AR-R17779 on colonic inflammation in DSS-induced colitis

We next measured the effect of α7 nAchR agonists on colonic cytokine production after 7 days of DSS administration. In line with the augmented disease outcome, nicotine treatment (see figure 3e), but neither of the α7 nAchR agonists reduced colonic TNF and IL-6 (see figure 6). In contrast, colonic TNF, IL-6, and IL-17 were significantly elevated after treatment with AR-R17779, but not GSK1345038A (see figure 6).

In addition, histopathology scores were assessed for the doses of the α7 nAchR agonists with most pronounced effects on disease severity. As indicated in table 4, total histopathology scores after treatment with 5 mg/kg AR-R17779 and 60 mg/kg GSK1345038A did not parallel clinical scores as there was no significant difference between groups. Similar effects were observed by administration of lower doses of AR-R17779 (0.3 mg/kg) and GSK1345038A (60 mg/kg); clinical outcome was poorer, but total histopathology scores were not significantly different from vehicle controls, except for crypt loss that was significantly worsened by treatment with 0.3 mg/kg AR-R17779 and 60 mg/kg GSK1345038A dosage (see table 4).
Figure 6. TNF, IL-6 and IL-17 production in the colon. The effects of treatment with AR-R17779 and GSK1345038A at the indicated dose on colonic cytokine production. Asterisks indicate significant differences (*p<0.05) as compared to vehicle. Agonist treated groups: n = 10; vehicle groups and 60 mg/kg GSK1345038A: n= 18. Data are expressed as the mean ± SEM.
Selective α7 nAChR agonists worsen disease in experimental colitis

To investigate whether the effects of α7 nAChR agonists on colitis were specific for DSS-induced colitis, we tested GSK1345038A in another acute model of colitis, TNBS-induced colitis. The main read out for this model is colonic inflammation and not clinical parameters as the mice are allowed to recover after one dose of TNBS. As indicated in figure 7a, weight loss 5 days after instillation of TNBS was not significantly different between groups treated with GSK1345038A and vehicle. In addition, histopathology scores were not significantly altered by treatment with 30 mg/kg and 60 mg/kg GSK 1345038A (see table 5). GSK1345038A treatment did not alter colonic production of TNF but not of IL-17, while IL-6 production was below levels of detection (see figure 7b).

Table 4: The effect of α7 agonists AR-R17779 and GSK1345038A on colonic inflammation in DSS-induced colitis. C57BL/6 mice were administered 1.5% DSS in drinking water and sacrificed at day 7. H&E stainings were performed on whole colons including rectum from groups treated with vehicle, 0.3 and 5 mg/kg AR-R17779 or 30 and 60 mg/kg GSK1345038A and scored by an experienced pathologist. H&E stainings of colon and rectum showed inflammatory features including crypt damage, follicles, oedema, ulceration and influx of inflammatory cells. Mice per group: Vehicle: n=18; 0.3 mg/kg AR-R17779 and 30 mg/kg GSK1345038A n = 10; 60 mg/kg GSK1345038A: n = 18. Data represent mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>AR-R17779 (mg/kg)</th>
<th>GSK 1345038A (mg/kg)</th>
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<tr>
<td></td>
<td>vehicle 0.3 5</td>
<td>vehicle 30 60</td>
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<tr>
<td>Area involved</td>
<td>2.9 ± 0.10 2.6 ± 0.53 2.9 ± 0.1</td>
<td>3.6 ± 0.15 3.8 ± 0.15 3.0 ± 0.24</td>
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<tr>
<td>Follicles</td>
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<tr>
<td>Oedema</td>
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<td>Erosion/ulceration</td>
<td>1.8 ± 0.36 1.4 ± 0.22 1.9 ± 0.11</td>
<td>0.9 ± 0.22 1.3 ± 0.24 1.5 ± 0.21</td>
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<tr>
<td>Crypt loss</td>
<td>2.0 ± 0.15 2.3 ± 0.09* 2.1 ± 0.99</td>
<td>1.6 ± 0.15 2.4 ± 0.24 * 2.2 ± 0.19</td>
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<td>Granulocytes</td>
<td>1.1 ± 0.06 0.8 ± 0.13 1.2 ± 0.07</td>
<td>1.6 ± 0.18 1.2 ± 0.17 1.1 ± 0.17</td>
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<tr>
<td>Monocytes</td>
<td>0.7 ± 0.11 0.9 ± 0.10 0.8 ± 0.12</td>
<td>1.3 ± 0.15 1.7 ± 0.14 1.6 ± 0.15</td>
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<tr>
<td><strong>Total score</strong></td>
<td>11.1 ± 0.6 10.7 ± 2.2 11.0 ± 0.3</td>
<td>11.4 ± 1.25 12.6 ± 1.26 11.3 ± 0.8</td>
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Figure 7. Effect of 30 and 60 mg/kg α7nAChR agonist GSK1345038A on TNBS-induced colitis. 

a. Body weight is shown as percentage of body weight on day 0 of the experiment. 
b. Cytokine levels in colon homogenates. Asterisks indicate significant differences (*p<0.05) as compared to vehicle. Bars indicate mean ± SEM, n=5.
Selective α7 nAChR agonists worsen disease in experimental colitis

<table>
<thead>
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<th>60</th>
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<td><strong>Area involved</strong></td>
<td>3.7 ± 0.19</td>
<td>3.7 ± 0.18</td>
<td>2.9 ± 0.26</td>
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<tr>
<td><strong>Follicles</strong></td>
<td>1.0 ± 0.24</td>
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<td>0.4 ± 0.20</td>
</tr>
<tr>
<td><strong>Oedema</strong></td>
<td>0</td>
<td>0.2 ± 0.14</td>
<td>0.3 ± 0.10</td>
</tr>
<tr>
<td><strong>Erosion/ulceration</strong></td>
<td>0.4 ± 0.19</td>
<td>0.6 ± 0.32</td>
<td>0.7 ± 0.36</td>
</tr>
<tr>
<td><strong>Fibrosis</strong></td>
<td>0.7 ± 0.20</td>
<td>0.6 ± 0.30</td>
<td>0.7 ± 0.21</td>
</tr>
<tr>
<td><strong>Hyperplasia</strong></td>
<td>3.7 ± 0.20</td>
<td>2.6 ± 0.69</td>
<td>3.1 ± 0.34</td>
</tr>
<tr>
<td><strong>Crypt loss</strong></td>
<td>0.8 ± 0.15</td>
<td>1.4 ± 0.48</td>
<td>0.5 ± 0.20</td>
</tr>
<tr>
<td><strong>Granulocytes</strong></td>
<td>0</td>
<td>0.6 ± 0.17 *</td>
<td>0.3 ± 0.14</td>
</tr>
<tr>
<td><strong>Monocytes</strong></td>
<td>0.8 ± 0.15</td>
<td>1.1 ± 0.13</td>
<td>0.9 ± 0.20</td>
</tr>
<tr>
<td><strong>Total score</strong></td>
<td>11.1 ± 1.3</td>
<td>11.9 ± 2.8</td>
<td>9.8 ± 2.0</td>
</tr>
</tbody>
</table>

Table 5. The effect of α7 agonists AR-R17779 and GSK1345038A on colonic inflammation in TNBS-induced colitis. Mice (n = 7 per group) received one dose of 2 mg TNBS in 30% ethanol intrarectally and were killed after 5 days. Vehicle, 30 or 60 mg/kg GSK1345038A was injected daily. H&E stainings were performed on whole colons including rectum and scored by an experienced pathologist. H&E stainings of colon and rectum showed inflammatory features including crypt damage, follicles, oedema, ulceration, hyperplasia and influx of inflammatory cells. Asterisks indicate significant differences (*P < 0.05) as compared with vehicle. Data represent mean ± SEM.
Chapter 7

Discussion

IBD patients suffer from chronic and relapsing intestinal inflammation, initiated by aberrant responses of the innate immune system. Recently, a number of animal studies demonstrate that innate immune responses are attenuated by stimulation of the efferent arm of the vagus nerve through its neurotransmitter acetylcholine that acts on nAchRs, in particular the α7 nAchR, on resident macrophages.

In various mouse models of inflammatory disease, we and others observed anti-inflammatory effects of vagus nerve stimulation, as well as pharmacological stimulation of the cholinergic system by administration of nicotine and α7 nAchR agonists. In the current study, we aimed to extend these studies by treating experimental colitis through targeting α7 nAchRs with nicotine, and two selective α7 nAchR agonists AR-R17779 and GSK1345038A. The agonists were tested in two mouse models of acute colitis induced by DSS or TNBS. In vitro, nicotine reduces macrophage NF-κB activity and cytokine release significantly. In addition, treatment of DSS-induced colitis with nicotine led to a significant reduction in colonic oedema and colonic IL-6 and IL-17 production. However, this reduction was not marked enough to be reflected in clinical parameters and histopathological scores. The histopathological scores are the end point of the inflammatory reaction and contribute greatly to the functionality of the colon and thus have a large influence on clinical outcome. In addition, reduced IL-17 levels do not strictly imply reduced disease activity and, recently, data have been obtained indicating that IL-17 might act as an anti-inflammatory cytokine in the gut.

UC Patients with a history of smoking usually acquire their disease after they have stopped smoking. Patients who smoke intermittently often experience improvement in their colitis symptoms during the periods when they smoke. Following this reasoning and given the previous reports on the positive effect of cholinergic activation in experimental models of DSS colitis, nicotine treatment may well be beneficial in UC. Indeed, in patient studies treatment with transdermal
nicotine was effective for the induction of disease remission in UC patients\textsuperscript{21}, but the number of patients that suffered from adverse effects was significantly higher in the nicotine-treated patient groups\textsuperscript{21} as compared with patients treated with standard therapy. It should be noted that smoking in CD patients worsens the disease.

In the current study, besides nicotine, we tested two selective $\alpha_7$ nAChR receptor agonists, AR-R17779\textsuperscript{36} and GSK1345038A, in the mouse model of DSS and induced colitis. Treatment with the $\alpha_7$ nAChR agonists both displayed a bell-shaped dose-response curve; the highest doses of AR-R17779 and GSK1345038A significantly ameliorated clinical parameters, whereas lower doses of both compounds worsened or did not affect clinical parameters. Although our data confirm the capacity of AR-R17779 and GSK1345038A to reduce pro-inflammatory mediator release\textit{ in vitro} in macrophage cultures\textsuperscript{24} and whole blood, the reduction in cytokines by nicotine as well as both $\alpha_7$ agonists was around 20–40%, which proved not to affect disease outcome in the colitis models used in this study.

However, it is possible that at the highest dose the $\alpha_7$ nAChR agonists might exhibit off-target activity and lose their selectivity for the $\alpha_7$ nAChR, thereby affecting disease in an $\alpha_7$ nAChR-independent manner. This is also indicated by the marked dose–response relationship observed between colonic IL-17 levels and AR-R17779 treatment, which may be explained by concentration-dependent off-target activity of AR-R17779. It should be borne in mind that nAChRs are expressed peripherally as well as centrally and that activation of nAChR on neurones can have analgesic effects, or modify mucus production, gut motility and blood flow to the gut\textsuperscript{25,37}. In the DSS colitis model, these effects might control food intake and formation of stools thereby influencing disease activity parameters independently of the severity of colonic inflammation. Another effect of nAChR activation can be a change in muscle tone thereby reducing colon length. This might play a role in the significant reduction of colon length we observed on treatment with AR-R17779.
In addition, activation of nAchRs plays a role in regulating epithelial permeability 37,38 and bacterial clearance 39,40, important factors in the development of colitis that were not assessed in our experiments. Thus, nAchR activation can have a variety of effects on disease, independent of immune mediation, because of its widespread expression on different cell types as well as on different tissue types. Although we report here that treatment with nicotine, or selective α7 nAchR agonists, is not effective in experimental colitis, enhanced vagus nerve output has been shown to reduce inflammation in various mouse models 11,24,28,29,40,41. This cholinergic anti-inflammatory effect seems to rely on the expression of the α7 nAchR on innate immune cells 11,18. Reciprocally, in mouse models of colitis, it has been shown that vagotomy worsens colitis, an effect that was shown to be counteracted by nicotine administration 15,15,16,42. Of note in the interpretation of these studies is that the vagus nerve only marginally innervates the distal colon, making direct effects of Ach on colonic immune cells unlikely. The vagus nerve probably relays its immune modulatory effects to the colon in an indirect fashion, that is, via postganglionic activation or by targeting alternative cell types. Of interest in this respect is a more recent study in which vagotomy was shown to worsen DSS colitis due to an impaired potential of antigen presenting cells to induce regulatory T-cells 42. Notably, the physiological effects of vagus nerve stimulation or vagotomy as compared with pharmacological activation of acetylcholine receptors differ greatly, especially when taking into account the changes in sympathetic output. In addition, vagus nerve stimulation or vagotomy will not only target nAchRs, but also influence the release of a number of neurotransmitters in the gut that regulate immune functions, and gut functions such as permeability and blood flow that possibly influence disease outcome.

Irrespectively, however, nicotine administration ameliorated disease in previous studies of experimental colitis 15,43. We cannot explain why the effectiveness of nicotine to reduce disease parameters was less pronounced in our study. The nicotine dose used in this study had been shown to be effective at reducing inflammation in other models of inflammation 24,44, but possibly, in our
colitis experiments a higher dose is required. However, in mice, a higher dose would result in adverse effects because of activation of a broad range of receptors both peripherally and centrally. In addition, a large array of nAchRs subtypes are expressed, and previous studies point towards a role in modulation of intestinal inflammation for nAchRs containing α5 or α4β2. Thus, the nAchR subtype involved in the immunomodulatory properties of the vagus nerve remains to be established.

Alternatively, the outcome of animal experiments with nAchR agonists could be dependent on the model of inflammation studied, as expression of the nAchR might vary depending on tissues and cell types involved in disease development. Notably, in other studies, nicotine treatment worsened the course of jenunitis in rodent models and TNBS colitis. There are notable differences among colitis models, which might be important in the effectiveness of the administered agents. Thus, the effects of nicotine and α7 nAchR agonists may depend on many experimental factors such as the dose used, administration method, disease severity and disease model. We conclude that in developing a strategy for treating colitis using cholinoreceptor agonists we should keep in mind that the expression of nAchRs is extremely widespread both centrally and peripherally. In addition, the expression of the various nAchRs subtypes on a particular target cell should be carefully investigated before evaluating the effectiveness of α7 nAchRs as a drug target in colitis patients.
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Selective $\alpha_7$ nAChR agonists worsen disease in experimental colitis

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Selective α7 nAChR agonists worsen disease in experimental colitis


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