BJP British Journal of Pharmacology

DOI:10.1111/bph.12982
www.brjpharmacol.org

RESEARCH PAPER

Indoline-3-propionate and 3-aminopropyl carbamates reduce lung injury and pro-inflammatory cytokines induced in mice by LPS

E Finkin-Groner[1], D Moradov[1], H Shifrin[1], C Bejar[1], A Nudelman[2] and M Weinstock[1]

[1]*Institute of Drug Research, The Hebrew University of Jerusalem, Jerusalem, Israel, and*
[2]*Department of Chemistry, Bar Ilan University, Ramat Gan, Israel*

Correspondence
Marta Weinstock, Institute of Drug Research, The Hebrew University of Jerusalem, Israel.
E-mail: martar@ekmd.huji.ac.il

Received
6 August 2014
Revised
1 October 2014
Accepted
8 October 2014

BACKGROUND AND PURPOSE

In the search for safer and effective anti-inflammatory agents, we investigated the effect of methyl indoline-3-propionate and indoline-3-(3-aminopropyl) carbamates on LPS-induced lung injury and pro-inflammatory cytokines in mice. Their mechanism of action was determined in murine peritoneal macrophages.

EXPERIMENTAL APPROACH

Lung injury was induced by intratracheal infusion of LPS and assessed by the change in lung weight and structure by light microscopy after staining by haematoxylin and eosin. In LPS-activated macrophages, MAPK proteins and IκBα were measured by Western blotting and the transcription factors, AP-1 and NF-κB by electromobility shift assay. Cytokines in the plasma and spleen of mice injected with LPS were measured by ELISA-based assay.

KEY RESULTS

AN917 and AN680 (1–10 pM) decreased TNF-α protein in macrophages by inhibiting phosphorylation of p38 MAPK, IκBα degradation and activation of AP-1 and NF-κB without affecting cell viability. In vivo, these compounds (10 μmol·kg^{-1}) markedly decreased lung injury induced by LPS and the elevation of TNF-α and IL-6 in lung, plasma and spleen. Activation of α-7nACh receptors contributed to the reduction of TNF-α by AN917, which inhibited AChE in the spleen by 35%.

CONCLUSION AND IMPLICATIONS

Indoline carbamates are potent inhibitors of pro-inflammatory mediators in murine macrophages and in mice injected with LPS, acting via the p38 MAPK, AP-1 and NF-κB cascades. Indirect α-7nACh receptor activation by AN917, through inhibition of AChE, contributes to its anti-inflammatory effect. Indoline carbamates may have therapeutic potential for lung injury and other diseases associated with chronic inflammation without causing immunosuppression.

Abbreviations

AP-1, activator protein 1; BALF, broncho-alveolar lavage fluid; iNOS, inducible NOS; IκBα, inhibitor of NF-κB; MPO, myeloperoxidase; α-7nACh receptor, α-7 nicotinic ACh receptor

Tables of Links

TARGETS
Ligand-gated ion channel[a]
α-7nAChR, nicotinic cholinergic receptor
Enzymes[b]
AChE, acetyl cholinesterase
BuChE, butyryl cholinesterase
iNOS, inducible NOS

LIGANDS
Budesonide
Dexamethasone
IL-6
Mecamylamine
Rivastigmine
TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Inflammation is an adaptive response to infection and tissue injury (Medzhitov, 2008) that involves activation of inducible NOS (iNOS), release of reactive nitrogen and oxygen species and of pro-inflammatory cytokines for example TNF-α, IL-6, IL-1β (Watters *et al.*, 2002). Chronic or excessive activation of the immune system may increase susceptibility to infections and cause ulcerative colitis (Podolsky, 2002), rheumatoid arthritis (McInnes and Schett, 2007), diabetes (DeFuria *et al.*, 2013) and cancer (Reuter *et al.*, 2010). Pro-inflammatory cytokines also play a critical role in the aetiology of the acute respiratory distress syndrome, which can be caused by Gram-negative bacteria (Ulich *et al.*, 1994). This syndrome comprises pulmonary oedema, low lung compliance and widespread capillary leakage and fibrosis (Levitt and Matthay, 2006). Mortality rates are 40–70% (Rubenfeld *et al.*, 2005), and therapeutic options are limited. Although drugs that block the action of TNF-α are very effective in the treatment of several types of chronic inflammatory conditions (Magro and Portela, 2010; Gonzalez-Juanatey *et al.*, 2012; Sorrentino, 2013), their long-term use can increase the risk of serious adverse effects (Bongartz *et al.*, 2006; Adler *et al.*, 2013; van Dartel *et al.*, 2013; Raaschou *et al.*, 2013; Seror *et al.*, 2013). Therefore, there is a need for safer drugs that can reduce the excessive release of pro-inflammatory cytokines without preventing any essential activity of these cytokines.

The afferent and efferent limbs of the vagus nerve constitute the cholinergic anti-inflammatory pathway (Tracey, 2002; Pavlov *et al.*, 2003), which acts as an interface between the immune system and the CNS. Most immune cells contain AChE and α-7 nicotinic ACh receptors (α-7nAChR) (Kawashima *et al.*, 2007), the activation of which can reduce the release of pro-inflammatory cytokines (Gallowitsch-Puerta and Pavlov, 2007). By preserving concentrations of ACh in immune cells and at vagal nerve endings the AChE inhibitor, physostigmine, lowered TNF-α in plasma and reduce the circulatory disturbance caused in rats by injection of LPS, a component of the outer membrane of Gram-negative bacteria. Another AChE inhibitor, rivastigmine, decreased cytokines in the colon and macrophages in a mouse model of colitis (Shifrin *et al.*, 2013).

LPS interacts with Toll-like receptor 4 (TLR4) in immune cells initiating a cascade that includes activation of MAPKs, ERK, p38 or JNK (Watters *et al.*, 2002). This in turn, leads to the activation of transcription factors activator protein 1 (AP-1) and NF-κB resulting in the expression of predominantly pro-inflammatory cytokines such as TNF-α and IL-6. Also, via activation of TLR4, LPS promotes serine phosphorylation thereby dissociating NF-κB from its binding protein IκB and facilitating its binding to DNA (Moynagh, 2005). This is believed to be part of the mechanism employed by LPS to activate iNOS and the production cytokines.

We have previously reported that carbamate derivatives of methyl indoline-3-propionate inhibit AChE and decrease cytotoxicity induced by oxidative stress at much lower concentrations than their indole analogues or melatonin (Yanovsky *et al.*, 2012). They also decrease NO, TNF-α and IL-6 in LPS-activated macrophages (Furman *et al.*, 2014). In the current study, we examined in more detail the mechanism of anti-inflammatory activity of the most active compounds of this series. When injected into mice, LPS activates macrophages primarily in the spleen and liver, inducing the formation and release of cytokines (Ge *et al.*, 1997). Therefore, we also assessed the effect of the indoline derivatives on the levels of pro-inflammatory cytokines in the spleen and circulation of mice after injection of LPS. As some, but not all the carbamates, inhibit AChE at concentrations that reduce TNF-α, we assessed the contribution of indirect α-7nACh receptor activation via AChE inhibition on their anti-inflammatory effect. Lastly, we examined the effect of two compounds on lung injury induced by intratracheal administration of LPS.

We found that indoline carbamates reduced pro-inflammatory mediators in LPS-activated murine macrophages by inhibiting p38 MAPK, reducing the degradation of IκBα and the nuclear translocation of NF-κB and AP-1. In mice, AN680 and AN917 (10 μmoles·kg^{-1}) almost completely abolished LPS-induced lung injury and significantly decreased the levels of TNF-α and IL-6 in the lung, spleen and plasma elevated by the endotoxin. These compounds may

have therapeutic potential in diseases associated with chronic inflammation.

Methods

Animals

All animal care and experimental procedures complied with the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985) and were performed according to protocols approved by the Ethics Committee of the Hebrew University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 355 animals were used in the experiments described here.

Male Bagg albino (Balb)/c mice and female C57BL/6 mice (8 weeks old), provided by Harlan (Jerusalem, Israel), were used for these experiments. The mice were housed in groups of five per cage in a pathogen-free unit under controlled 12 h light/12 h dark cycle (lights on at 0700 h and lights off at 1900 h) ambient temperature 21 ± 1°C humidity 40–50% with free access to standard rodent chow (Harlan) and water. The mice were acclimatized to the Animal House for at least 7 days before the experiment.

Measurement of cytokines in cultures of murine peritoneal macrophages

Peritoneal macrophages were elicited from C57BL/6 mice by i.p. injection of 1.5 mL aged sterile thioglycolate solution (3% w/v: Becton Dickinson, Franklin Lakes, NJ, USA) as described by Quntar *et al.*, (2007). Responses of macrophages from female C57BL/6 were found to be more reproducible than those of males of the same strain or from Balb/C mice. The cells were seeded in 96-well culture plates (NUNC, Thermo Scientific, Roskilde, Denmark) at a density of 1.5×10^5 cells per well in DMEM. Budesonide (as positive control) and all the compounds mentioned earlier or their metabolites (Figure 1) were added to the cells at concentrations ranging from 0.01 pM–1.0 nM. After 2 h, the macrophages were treated with complete DMEM or DMEM containing 1 $\mu g \cdot mL^{-1}$ of LPS with or without the compounds at the concentrations mentioned earlier. Supernatants were collected after 6 h for TNF-α and after 24 h for IL-6. Cytokine detection was performed using an ELISA kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. All tissue culture reagents were purchased from Biological Industries (Beit HaEmek, Israel).

Measurement of nitrite production in RAW 264.7 cell cultures

RAW 264.7 macrophages purchased from the American Type Culture Collection (Manassas, VA, USA) were used for these experiments because we were unable to detect NO in peritoneal macrophages activated by LPS. RAW 264.7 macrophages were cultured in complete DMEM at a density of 0.5×10^5 cells per well in 96-well culture plates (NUNC). The experiment was performed as described in the preceding section, but LPS was added at a concentration of 5 $\mu g \cdot mL^{-1}$. Supernatants were collected after 24 h and NO released into the medium was measured in the form of $NaNO_2$ using Griess reagent reaction (Horvath *et al.*, 2008).

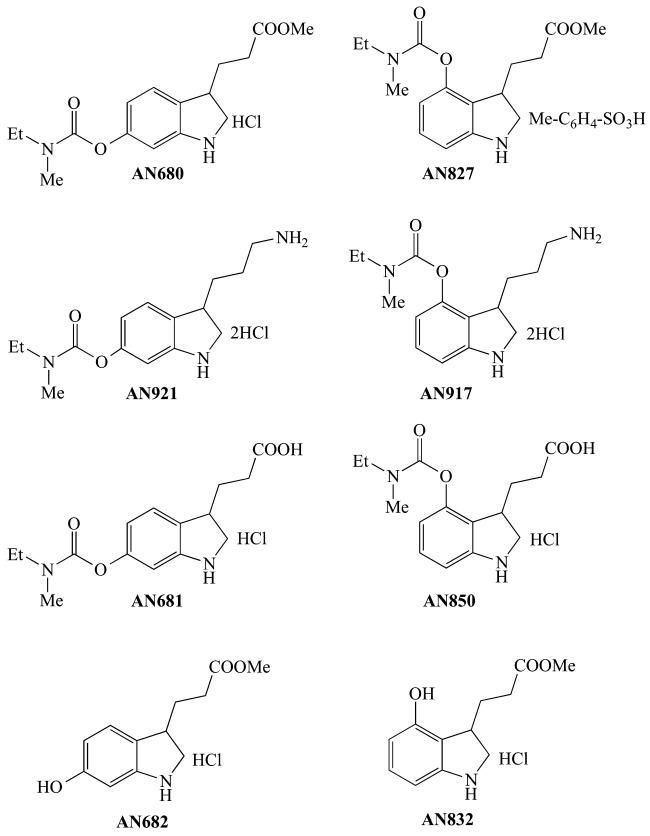

Figure 1
Chemical structures of indoline carbamates and some of their metabolites.

Cell viability

RAW 264.7 cells and peritoneal macrophages were treated as described in the preceding sections. After 24 h, with complete DMEM or DMEM containing LPS (1 or 5 $\mu g \cdot mL^{-1}$ for peritoneal macrophages or RAW 264.7, respectively) together with the compounds at concentrations ranging from 1 pM to 1 μM, MTT solution was added to each well to a final concentration of 0.5 $mg \cdot mL^{-1}$ diluted in complete medium. Cell viability was measured as described by Mosmann (1983).

Isolation of proteins and immunoblotting detection

The experiments were performed on peritoneal macrophages to see whether the compounds AN917 and AN680 acted in a similar or different manner to that of AN827 (Furman *et al.*, 2014). Macrophages were seeded in six-well culture plates [5×10^6 cells per well (NUNC)], and incubated in complete DMEM for 2–3 h. The medium was aspirated and the cells were rinsed twice with 2 mL of PBS to remove non-adherent cells. PBS was replaced with medium containing AN917 or AN680 (10 pM). After 2 h, the cells were treated with complete DMEM or DMEM containing LPS (1 $\mu g \cdot mL^{-1}$) with or

without the compounds for 15 min for the determination of IκBα degradation and for 30 min for phosphorylation of p38, JNK and ERK. Cells were harvested and centrifuged at 17 000 g at 4°C for 5 min. Cell pellets were incubated for 30 min with shaking on ice in radioimmuno-precipitation assay lysis buffer (Beit HaEmek, Israel) containing phosphatase inhibitor and protease inhibitor cocktails (1%) purchased from Sigma. After centrifugation at 13 000× *g* at 4°C for 5 min, the supernatants were collected and stored at (–80°C) until use. Protein samples (20 µg) were separated on 10% SDS polyacrylamide gels with 4.5% SDS stacking gel. Samples were electro-transferred onto nitrocellulose membranes (0.45 µm; Schleicher, Dassel, Germany) and blocked with 4% non-fat dry milk (Bio-Rad, Hercules, CA, USA). Blots were probed with antibodies against IκBα (1:500), ERK (1:500), phosphorylated ERK (*p*-ERK) (1:650), p38 (1:1000), phosphorylated p38 (*p*-p38) (1:600) and tubulin (1:1000), JNK (1:700) and phosphorylated JNK (*p*-JNK) (1:700) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The membranes were incubated overnight at 4°C with appropriate primary antibodies and then incubated (1 h at room temperature) with appropriate IRDye conjugated fluorescent secondary antibodies: donkey anti-mouse 800 (1:8000, Rockland Immunochemicals, Limerick, PA, USA) and alexa-Fluor goat anti-rabbit 680 (1:8000, Molecular Probes, Grand Island, NY, USA). IRDye conjugates, all optimized for the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Blots were quantified using TINA software (version 2.07d; Raytest, Straubenhardt, Germany) and results were presented as OD of IκBα relative to that of tubulin for the same band. For all other proteins, the OD of the phosphorylated form was expressed relative to that of the non-phosphorylated form for the same band.

Electromobility shift assay (EMSA)

Murine peritoneal macrophages were seeded in 40–50 mL culture flasks (NUNC) at a density of 1×10^7 cells per well. The experiment was performed as described for immunoblot detection except that activation of NF-κB was measured after 30 min and that of AP-1 after 60 min. Nuclear proteins were isolated from peritoneal macrophages using Cayman Nuclear Extraction Kit (Ann Arbor, MI, USA) and total protein concentration was determined as described earlier. Double-stranded NF-κB IRDye 700 and AP-1 IRDye 700 labelled oligonucleotide corresponding to NF-κB and AP-1 specific consensus sequence were used for the binding reaction at a concentration of 50 nM per reaction (LI-COR Biosciences). Oligonucleotides corresponding to the downstream NF-κB (5′-GTTGAGGGGACTTTCCCAGGC-3′), AP-1 (5′-GCTGATGACTCAGCCGG AA-3′) binding sequences were synthesized, annealed and end-labelled with Biotin 3′ End DNA Labelling Kit. The unlabelled oligonucleotide with the similar sequence (cold probe) was used as control for specificity of the binding reaction. The binding reaction was performed using an Odyssey EMSA buffer kit (LI-COR Biosciences) with 50 µg of total nuclear protein per each reaction. The reaction took place during the 30 min in the dark at 25°C, and 2 µL of orange loading dye X10 was added to each vial before loading to the gel. A 4% polyacrylamide Tris/Borate/EDTA gel was prepared and pre-run at 70 mV for 30 min before the samples were loaded. Protein–DNA complexes (20 µL) were resolved by electrophoresis for 1 h at 70 mV in the dark. The gels were scanned at 700 nm using LI-COR Odyssey Imaging system. Blots were quantified using TINA software.

Cholinesterase (ChE) inhibition

The source of AChE in mouse spleen is T lymphocytes as there are no parasympathetic nerve terminals in this organ (Nieto-Ceron *et al.*, 2004). In mouse plasma about 90% of the enzyme is butyrylcholinesterase (BuChE). For measurement of total ChE activity, compounds or saline were injected into groups of six male mice, which were killed by decapitation 60 min later when ChE inhibition was shown to reach its peak (Finkin-Groner, 2013). Blood was collected into heparinized Eppendorf tubes and centrifuged at 17 000× *g* to obtain plasma. The spleen was rapidly removed, 100 mg·mL^{-1} cold phosphate buffer pH 7 containing 0.1% Triton was added and the tissue was homogenized in Ultra-TURRAX® homogenizer (IKA®, Staufen, Germany) at a speed of 24 000 r.p.m. and centrifuged at 17 000× *g* at 25°C for 4 min. ChE activity in the supernatants was determined as previously described (Yanovsky *et al.*, 2012), and the effect of the compounds was expressed as a percentage reduction of the enzyme activity in control mice injected with saline. Protein concentration was determined by means of a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA).

A 50-fold higher dose of AN827 was needed to cause significant ChE inhibition in plasma and spleen of mice than was predicted from its enzyme inhibitory activity *in vitro* and compared with that of other indoline carbamates (Table 1). It was considered that the loss in enzyme inhibitory activity was due to binding of AN827 to a constituent of plasma. Using BuChE as an indicator of enzyme inhibitory activity, the compounds, were incubated with BuChE for 120 min in the absence or presence of 40 mg·mL^{-1} human serum albumin (Sigma) or human plasma. The latter was obtained from the hospital blood bank and, like mouse plasma, con-

Table 1

Inhibition of cholinesterase in plasma and spleen after administration of compounds to mice

Compound	Dose (µmol kg^{-1})	Plasma	Spleen
AN680	100	11 ± 5	7 ± 3
	200	30 ± 1*	16 ± 6
	400	90 ± 2**	25 ± 3*
AN827	200	16 ± 7	1.2 ± 6.6
	400	57 ± 7**	24 ± 2*
AN917	10	37 ± 9*	35 ± 5*
Rivastigmine	3.3	42 ± 4**	46 ± 3**

AN compounds were injected i.p.; rivastigmine was given by gavage. Values represent % inhibition ± SEM of total ChE, 60 min after drug administration. Most of ChE in plasma is BuChE and in spleen, it is AChE. *$P < 0.05$, **$P < 0.01$, significant inhibition.

tains mainly BuChE. For separation of serum protein by ultrafiltration we used the 'Centristart® I' column (MW 5, 10 and 100 KD cut-off; Sartorius Stedim Biotech, Goettingen, Germany).

Measurement of LPS-induced cytokine elevation in mouse spleen

Balb/c mice were injected s.c. with saline (10 mL·kg^{-1}), dexamethasone (6 μmoles·kg^{-1} as a reference drug) or different doses of the compounds, followed 15 min or 2 h later by LPS (10 mg·kg^{-1}) injected i.p. The mice were anaesthetized with CO_2, and decapitated 4 h later (optimal time for difference between levels of cytokines in control and LPS-treated mice (Finkin-Groner, 2013). In other mice, mecamylamine was injected s.c. (eight per group) followed 10 min later by rivastigmine (3.3 μmoles·kg^{-1}), by gavage or saline (10 mL·kg^{-1}), AN917 or AN680 (10 μmoles·kg^{-1}) s.c. and LPS were given as indicated earlier. Blood and spleen were collected for cytokine measurements. Blood was centrifuged at 14 000× *g*, spleens were rapidly removed, snap frozen in liquid nitrogen and stored at (–80°C) until use. Spleen tissue was diluted with PBS solution (100 mg·mL^{-1}) containing NaCl, (0.8%), $NaHPO_4$ (0.144%), KH_2PO_4 (0.024%) and protease inhibitor cocktail (1%), homogenized in an Ultra-TURRAX® homogenizer (IKA®) at a speed of 24 000 r.p.m. and centrifuged at 14 000× *g* at 4°C for 15 min. Cytokine detection was performed by ELISA kits, (Biolegend) and protein concentrations were determined as described earlier.

Induction of acute lung injury in mice

AN680 and AN917 (10 μmoles·kg^{-1}) or 10 mL·kg^{-1} of saline was injected s.c. into groups of seven Balb/c mice. One hour later, the mice were anaesthetized with xylazine (0.7 g per mouse) and ketamine (29.75 mg per mouse). LPS (10 mg·kg^{-1}) or saline was instilled into the trachea as described by Rayamajhi *et al*. (2011) and Yu *et al*. (2008). A second dose of the compounds or saline was injected 9 h later. Twenty-four hours after administration of LPS, the mice were killed by injection of pentobarbitone sodium (50 mg·kg^{-1}). The lungs were carefully removed and the right side weighed and used for measurement of oedema that was depicted as the ratio of wet to dry weight after it was dried in an oven at 75°C for 24 h. The left lung was used for microscopic analysis of pathological changes. It was fixed in 4% buffered formalin for 24 h, dehydrated, embedded in paraffin, sectioned and stained with haematoxylin and eosin for analysis by means of an Olympus BX53 microscope (Olympus, Tokyo, Japan). From each slide, five fields containing 400 alveoli (×200 magnification) were chosen, and the degree of lung injury was evaluated by an investigator unaware of the treatments. The severity of microscopic injury was graded from 0 (normal) to 3 (severe) as described in Matute-Bello *et al*., (2001) based on the degree or amount of (i) congestion of alveolar septae; (ii) alveolar haemorrhage; (iii) intra-alveolar fibrin; and (iv) intra-alveolar infiltrates. The total injury score made up of four components was computed for each mouse. In other groups of six to eight mice treated with the AN compounds, saline and LPS as described earlier, broncho-alveolar lavage fluid (BALF) was collected by injection and withdrawal of three aliquots of 1 mL of saline at the end of the experiment via a polyethylene cannula placed into the trachea as previously described (Berkman *et al*., 2001). The BALF was centrifuged (10 min, 120x *g*, 4°C) and supernatant was collected for cytokine and total protein evaluation. The left lung was homogenized in 0.02 M phosphate buffer (pH 7.4) and neutrophil infiltration was monitored by measuring myeloperoxidase (MPO) activity according to the protocol described by Bradley *et al*. (1982). In the BALF, TNF-α and IL-6 and proteins were measured as described in the preceding section.

Data analysis

All doses and concentrations of compounds are expressed in terms of their respective salts. The results are presented as the mean ± SEM unless otherwise stated. Differences between data from cells treated with LPS alone and those with LPS plus compounds, and from mice treated with LPS with and without compounds were analysed by ANOVA using IBM SPSS Statistics Version 19 followed by Duncan's *post hoc* test. A P value of <0.05 was considered to be significant. All experiments on cells were repeated 2–3 times and contained at least 6 measures.

Materials

Dexamethasone phosphate was from Mylan, Inc., (Canonsburg, PA, USA); rivastigmine hemitartarate from Novartis (Basle, Switzerland) and mecamylamine hydrochloride from Waterstone Pharmaceuticals, Inc., (Indianapolis, IN, USA), AN827 mesylate, AN680 tosylate, AN917 dihydrochloride, and AN921 dihydrochloride, AN681, AN850, AN832, and AN682 all as hydrochlorides (Furman *et al*., 2014). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), LPS (from *Escherichia coli*, serotype 0111:B4) and budesonide all purchased from Sigma (St. Louis, MO, USA).

Results

Inhibition by indoline derivatives of LPS induced production of NO and cytokines in macrophages

All the concentrations of budesonide and AN compounds that are shown in Figure 2A–C significantly reduced TNF-α ($P < 0.01$) and, with the exception of AN680 (0.001 pM), also reduced IL-6 ($P < 0.01$). For TNF-α, none of the compounds reached the same magnitude of reduction of 70% as budesonide (1 nM). A peak reduction of 50–55% was achieved with AN827 0.01 pM; AN680, AN917 and AN921 1 pM. For IL-6, budesonide, AN827 and AN917 (10 nM) produced similar maximal reductions of 70–75% (Figure 2B). The peak reduction in NO of 48% produced by AN827 (10 pM) was greater than that of budesonide (10 nM) and of any concentration of the other AN compounds (Figure 2C).

The metabolites of the ester derivatives reduced both cytokines significantly ($P < 0.01$) at concentrations ranging from 0.1 to 100 pM (Figure 3A and B). The maximum reduction was achieved at a concentration of 10 pM. Both decarbamoylated metabolites with an ester group were more effective inhibitors of TNF-α than their respective

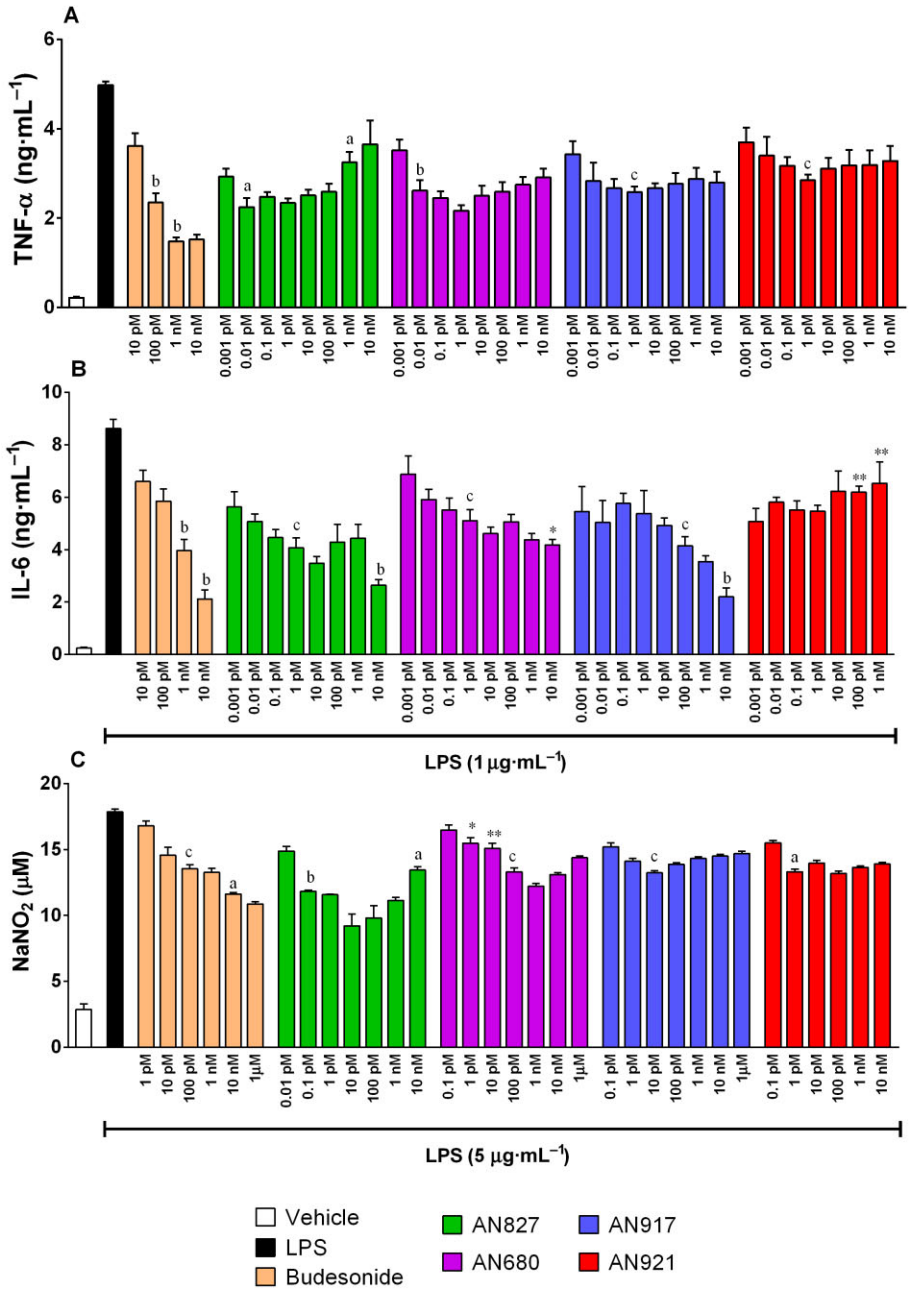

Figure 2

Reduction of LPS-induced cytokines in peritoneal macrophages and NO in RAW 264.7 cells by budesonide and indoline carbamates. (A) TNF-α. Compounds were administered 2 h prior to exposure to LPS (1 μg·mL^{-1}) and measurements made after 6 h. Each bar represents the mean ± SEM from six measurements per compound in three independent experiments. Medium for these experiments and those in Figure 3 was DMEM. ANOVA of the effect of all concentrations of budesonide and four compounds on TNF-α levels in peritoneal macrophages was ($F_{37,684}$ = 22.5 P < 0.0001). All concentrations shown caused a significant (P < 0.01) reduction of TNF-α. For specific data sets: a, P < 0.05; b, P < 0.01, significantly different from the 10-fold lower concentration; c, P < 0.05, significantly different from the lowest concentration. (B) IL-6. Compounds were administered 2 h prior to exposure to LPS (1 μg·mL^{-1}) and measurements made after 24 h. ANOVA for budesonide and compounds was ($F_{37,688}$ = 29.14, P < 0.0001). For specific data sets: b, P < 0.01, significantly different from the 10-fold lower concentration; c, P < 0.05, significantly different from the lowest concentration; *P < 0.05, significantly different from value for the same concentration of AN827; ** P < 0.01, significantly different from value for the same concentration of AN917. (C) NO. Compounds were administered 2 h prior to exposure of RAW 264.7 cells to LPS (5 μg·mL^{-1}) and measurements of NO (as $NaNO_2$) were made after 24 h. ANOVA for budesonide and compounds was ($F_{34,612}$ = 45.14, P < 0.0001). All concentrations shown except budesonide (1 pM) and AN680 (0.1 pM) caused a significant (P < 0.01) reduction of NO, For specific data sets: a, P < 0.05; b, P < 0.01; significantly different from the 10-fold lower concentration; c, P < 0.05; significantly different from the lowest concentration.. *P < 0.05; **P < 0.01, significantly different from value for same concentration of AN827.

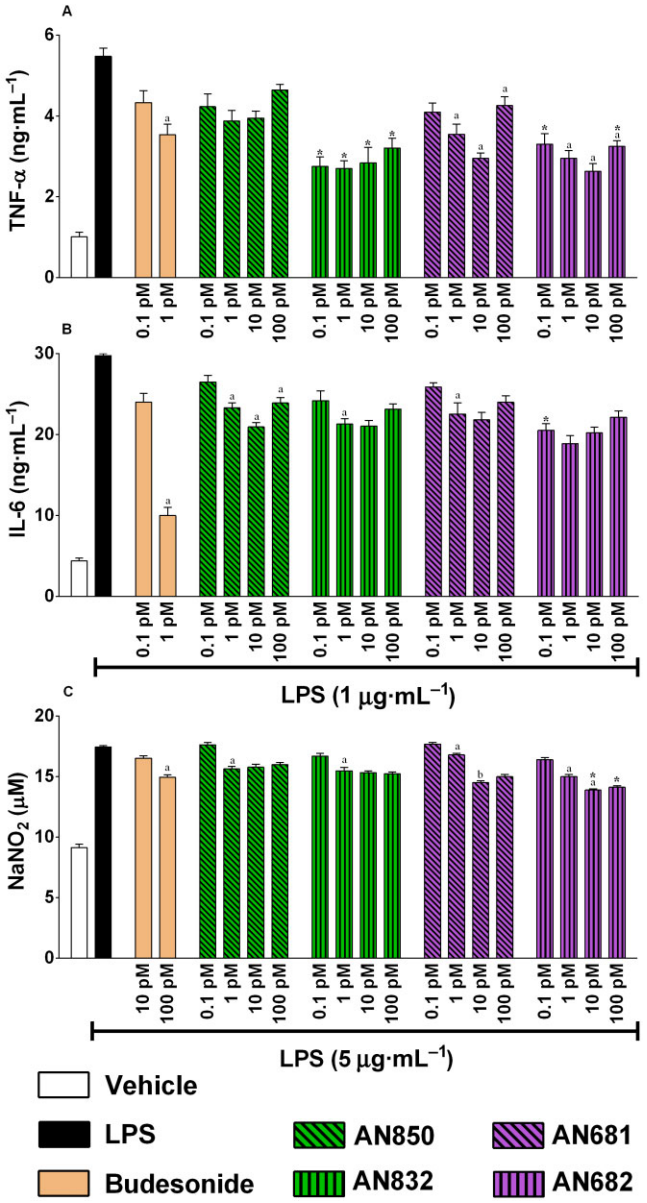

Figure 3

Reduction of LPS-induced cytokines in peritoneal macrophages and NO in RAW 264.7 cells by budesonide and metabolites of AN680 and AN827. (A) TNF-α. (B) IL-6. (C) NO. LPS and compounds were administered as described for parent compounds. ANOVA for budesonide and metabolites AN681, AN850, AN682 and AN832 for measures of TNF-α was ($F_{20,333}$ = 21.19 P < 0.0001) P < 0.0001); IL-6, ($F_{20,250}$ = 50.63 P < 0.0001); NO. ($F_{20,325}$ = 31.25, P < 0.0001). For TNF-α and IL-6, all concentrations shown caused a significant reduction P < 0.01. For NO, all concentrations except 1 nM of AN 850 and AN681 caused a significant reduction, P < 0.05. For specific data sets, a, P < 0.05; b, P < 0.01; significantly different from the 10-fold lower concentration. *P < 0.05, significantly different from value for same concentration of the respective acid.

carbamoylated acids. All the compounds significantly reduced NO (P < 0.01) at concentrations ranging from 0.1 to 100 pM, with the greatest effect seen at a concentration of 100 pM (Figure 3C).

Effect of indoline derivatives on cell viability and NO production

None of the compounds or the metabolites tested for anti-inflammatory activity had any significant effect on cell viability in RAW 264.7 cells or peritoneal macrophages at concentrations ranging from 0.1 pM to 1.0 μM as measured by the MTT assay (data not shown).

Effect of AN680 and AN917 on MAPks, IkBα, NF-κB and AP-1

As previously shown for AN827 (Furman *et al.*, 2014), AN680 and AN917 (10 pM) decreased the phosphorylation of p38 (Figure 4B), but not that of ERK or JNK (Figure 4A and B). The compounds (10 pM) also decreased the degradation of IκBα in comparison with that in cells treated with LPS alone (Figure 4A). In the EMSA analysis, AN680 and AN917 reduced the levels of NF-κB (Figure 4C) and AP-1 (Figure 4D) at a concentration of 10 pM.

ChE inhibitory activity of indolines after administration to mice

In addition to AN827, AN680 and AN917 were selected for these experiments because they showed the largest differences in their ability to inhibit AChE *in vitro* (Furman *et al.*, 2014). AN917 (IC_{50} of 1.8 μM), reduced ChE activity significantly at a dose of 10 μmol·kg^{-1} both in plasma and spleen. AN680, which has 1/30th of the AChE inhibitory activity of AN917, reduced enzyme activity only at a dose of 400 μmol·kg^{-1}. Unexpectedly, although 7.5 times more potent as an AChE inhibitor than AN680, AN827 also reduced ChE activity in the spleen only at a dose of 400 μmol·kg^{-1} (Table 1). AN827 and AN680 are rapidly hydrolysed in plasma to their respective acids, AN850 and AN681, which are two to three times less potent as AChE inhibitors than the esters, but show similar potency against BuChE (Yanovsky *et al.*, 2012). Although this could explain the somewhat lower AChE inhibition *in vivo* of AN680 than predicted from its activity *in vitro* it does not account for the more than 50-fold reduction in the inhibitory activity of AN827.

Effect of plasma or serum albumin on BuChE inhibitory activity

In the presence of human plasma, BuChE inhibitory activity of AN827 was reduced more than 50-fold, but that of AN680 and AN917 was unaffected (Table 2). This reduction remained after dialysis of the plasma, indicating that it was due to the presence of a protein. This was not albumin as very little decrease in activity occurred in its presence. Ultrafiltration of plasma showed that the fraction that inhibited the activity of AN827 contained proteins that are larger than 100 KD.

Reduction of cytokines by indoline derivatives in LPS-injected mice

AN680 and AN917 (1 and 10 μmol·kg^{-1}) significantly reduced TNF-α in the spleen of mice, but AN827 was only effective at concentrations of 50 and 100 μmol·kg^{-1} (Figure 5A). The loss of activity of AN827 can be explained by binding to a protein constituent of plasma, as shown in the preceding section. All

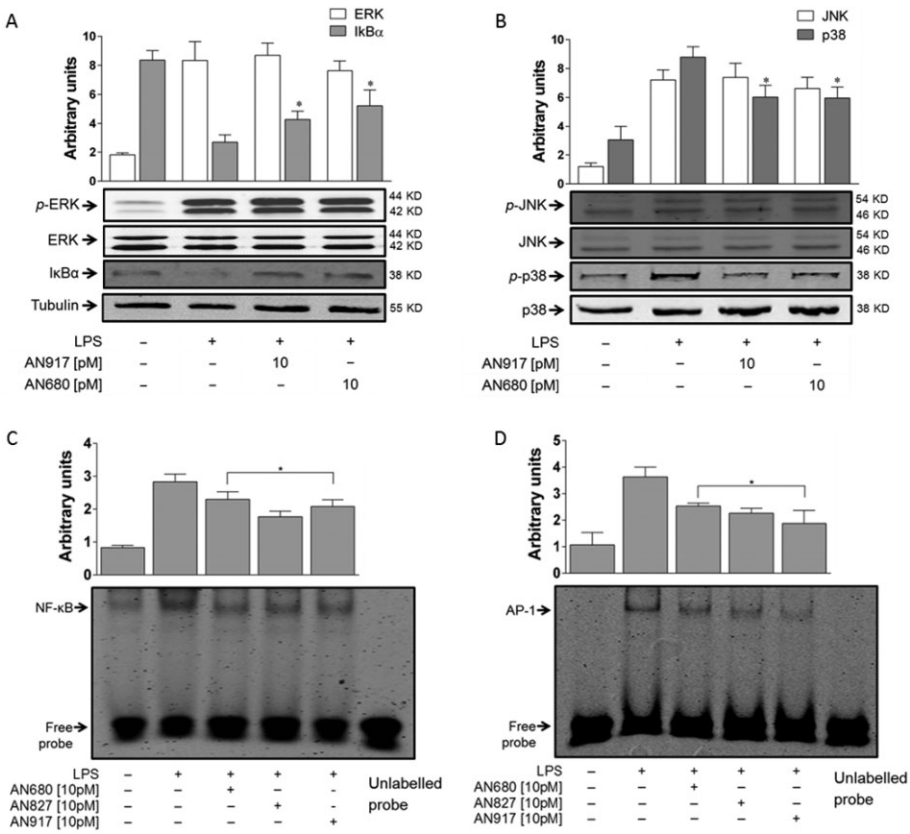

Figure 4

Reduction by AN680 and AN917 of phosphorylation of p38, IκBα, NF-κB and AP-1 levels in peritoneal macrophages. Peritoneal macrophages were treated with AN680 and AN917 and LPS as described in Figure 2. Each bar represents the mean ± SD from three to five measurements. (A) Shows the reduction by 10 pM compounds of the degradation of IκBα, but not phosphorylation of ERK. (B) Shows the reduction by 10 pM compounds of the phosphorylation of p38, but not of JNK. (C) Shows the reduction by the compounds of NF-κB. (D) Shows the reduction by the compounds of AP-1. *$P < 0.05$; **$P < 0.01$, significantly different from LPS alone.

Table 2

Effect of human plasma or human serum albumin on BuChE inhibitory activity of indoline derivatives

	Incubated with			Ratios	
Compound	**Buffer**	**Human plasma**	**High HSA**	**Human plasma/buffer**	**High HSA/buffer**
AN680	3.91 ± 0.20	4.30 ± 0.12	6.0 ± 0.2	1.1	1.5
AN827	0.30 ± 0.04	18.6 ± 0.6	0.40 ± 0.01	62	1.3
AN917	1.20 ± 0.04	1.00 ± 0.1	1.20 ± 0.03	0.8	1

Values shown are IC_{50} (μM) ± SEM (obtained after 120 min pre-incubation). HSA, human serum albumin (40 mg·mL^{-1}).

the compounds, except AN827 (10 μmol·kg^{-1}), also reduced IL-6 ($P < 0.01$) (Figure 5B).

AN680 and AN917 (10 μmol·kg^{-1}) reduced TNF-α ($P < 0.01$) in plasma when given 2 h before LPS, but were less effective, or ineffective (AN917) when given 15 min before LPS (Figure 6A). On the other hand, the compounds only reduced IL-6 significantly when given 15 min before LPS (Figure 6B). Dexamethasone reduced both cytokines when given 0.5 h before LPS ($P < 0.01$).

Contribution of activation of the α-7nAChR to the anti-inflammatory effect

The contribution of activated α-7nAChRs to the anti-inflammatory effect of AN680 and AN917 in LPS-injected mice was only assessed on cytokine levels in the spleen because their reduction in plasma was too small to detect a significant effect of mecamylamine. In isolated macrophages, unlike the indoline derivatives, rivastigmine only shows anti-

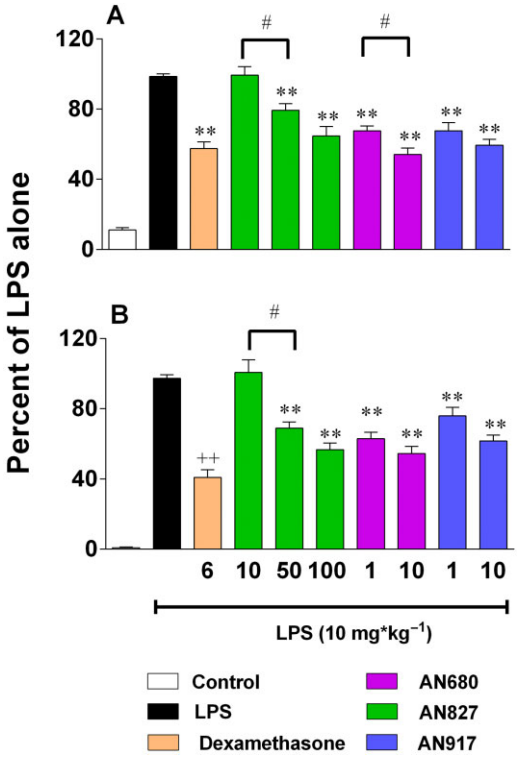

Figure 5

Reduction by compounds of TNF-α and IL-6 in spleen of mice injected with LPS. Measurements of cytokines in the spleen were made after s.c. injection of the drugs and i.p. injection of LPS. All doses are in μmoles·kg^{-1}. (A) TNF-α. Each bar represents the mean ± SEM from measurements in six to 12 mice per dose per compound. ANOVA of the measure of TNF-α in the spleen was ($F_{9,98}$ = 71.6, P < 0.0001). For specific data sets: **P < 0.01, significantly different from LPS alone; #P < 0.05, significantly different from lower dose. (B) IL-6. Each bar represents the mean ± SEM from measurements in six to 10 mice per compound. ANOVA, ($F_{9,85}$ = 59.8, P < 0.0001). For specific data sets: **P < 0.01, significantly different from LPS alone; #P < 0.05, significantly different from lower dose.

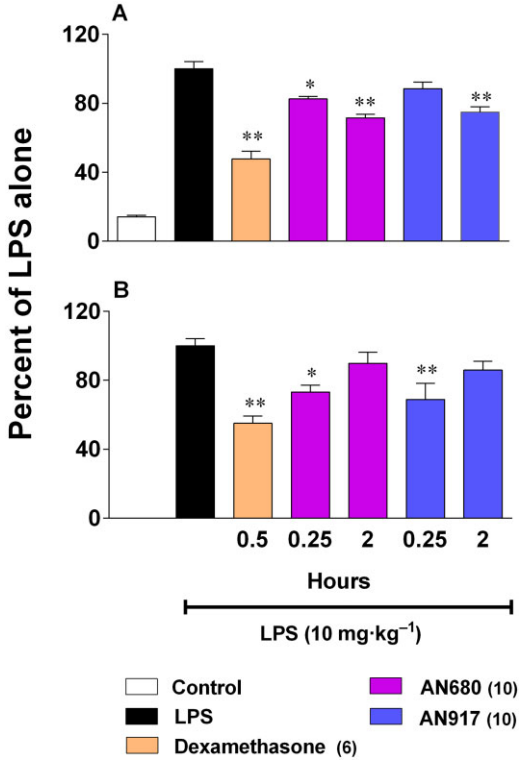

Figure 6

Effect of compounds on cytokines in plasma after their elevation by LPS in mice. Compounds were injected either 15 min or 2 h before LPS. Each bar represents the mean ± SEM from measurements in groups of five to nine mice of: A TNF-α; B IL-6. ANOVA for measures of TNF-α ($F_{6,51}$ = 43.9, P < 0.0001), IL-6, ($F_{6,51}$ = 22.7, P < 0.0001). For specific data sets: *P < 0.05; **P < 0.01, significantly different from LPS alone.

inflammatory activity if given together with a choline ester (Shifrin *et al.*, 2013). In mice, rivastigmine (3.3 μmol·kg^{-1}) inhibited AChE by 46% (Table 1) and reduced TNF-α in the spleen by 23%. Mecamylamine (10 mg·kg^{-1}) completely blocked the reduction in TNF-α induced by rivastigmine, partly reduced that of AN917 (10 μmol·kg^{-1}), but had no effect on that of AN680 (10 μmol·kg^{-1}), which did not inhibit AChE at this dose (Figure 7).

Reduction by AN680 and AN917 in lung injury induced by intratracheal injection of LPS in mice

Intratracheal injection of LPS (10 mg·kg^{-1}) did not induce the symptoms of endotoxaemia that are seen after i.p. administration in mice, but increased alveolar wall thickness, neutrophil infiltration and alveolar congestion (Figure 8A). LPS also increased the MPO activity in lung tissue, together with protein content and the levels of TNF-α and IL-6 in the BALF (Figure 8D–G). The change in lung weight wet/dry ratio induced by LPS 24 h after its injection was relatively small and was only significantly reduced by AN680 (Figure 8B). The degree of the lung tissue damage, cell infiltration, protein content and MPO activity were all significantly attenuated (P < 0.01) by AN680 and AN917 (Figure 8C–F). The reduction by AN680 in TNF-α and IL-6 in BALF was greater than that produced by AN917.

Discussion

The findings in this study confirm and extend those in our previous report (Furman *et al.*, 2014). They show that concentrations of 0.01 pM (or lower) of indoline-3-propionate ester and indoline-3-(3-aminopropyl) compounds with an *N*-ethyl, *N*-methyl-carbamate substituent can reduce TNF-α and IL-6 in murine peritoneal macrophages and NO in RAW 264.7 cells activated by LPS. The reduction in TNF-α peaked at 1 pM and that of NO at 1–100 pM then lessened with increasing concentration or remained unchanged. However, the reduction in IL-6 increased with increasing concentration for all compounds except AN921. These differences arise from the timing and magnitude of the interactions of the individual compounds with proteins and enzymes in the cascade

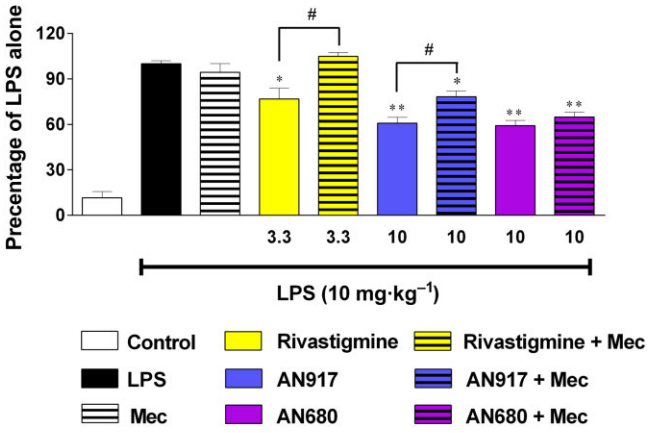

Figure 7

Effect of mecamylamine on reduction by rivastigmine, AN680 and AN917 in levels of TNF-α in spleen of mice injected with LPS. Mec, mecamylamine. Doses of rivastigmine and compounds (μmol·kg^{-1}); mecamylamine (10 mg·kg^{-1}). Cytokines in the spleen were measured 4 h after injection of mecamylamine, rivastigmine, AN680 and AN917, with and without mecamylamine, in groups of six to eight mice per treatment. ANOVA for the different drug treatment groups was ($F_{9,76}$ = 37.6, P < 0.0001). Mecamylamine completely blocked the reduction in TNF-α caused by rivastigmine, partly antagonized that caused by AN917 and had no effect on that of AN680. *P < 0.05; **P < 0.01, significantly different from LPS alone. #P < 0.05, significant effect of mecamylamine treatment.

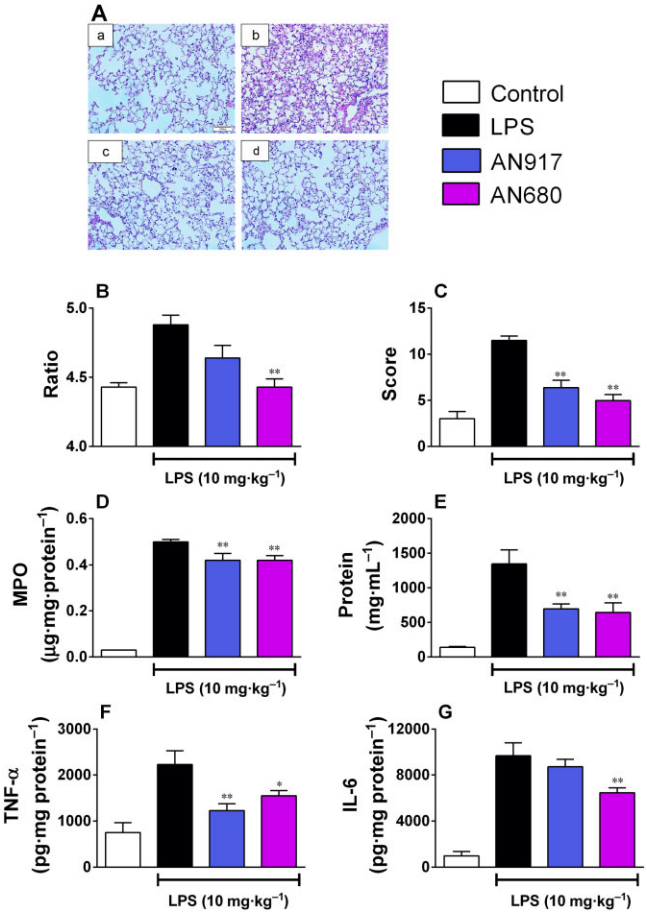

Figure 8

Reduction by AN680 and AN917 of pathological changes in lungs of mice, induced by intratracheal injection of LPS. (A) Lung sections stained with haematoxylin-eosin 24 h after LPS administration revealed pulmonary pathological changes. Magnification ×200. (a) Control group: normal structure. (b) LPS + saline showing alveolar wall thickening, haemorrhage, alveolar collapse and inflammatory cell infiltration. (c) LPS + AN680, (d) LPS + AN917. Pathological changes in the treated groups are virtually indistinguishable from those in control mice. (B) Ratio of wet to dry weight of the right lung as a measure of lung oedema. ANOVA ($F_{3,24}$ = 7.00, P < 0.005). Only AN680 showed a significant difference from LPS injected controls. **P < 0.01, significantly different from LPS alone. (C) Score of alveolar damage in left lung. ANOVA for the injury score was ($F_{3,24}$ = 30.1, P < 0.0001). Both compounds significantly reduced lung damage induced by LPS, **P < 0.01, significantly different from LPS alone.. (D) MPO activity in lung tissue. ANOVA ($F_{3,26}$ = 127.3 P < 0.0001). Both compounds significantly reduced MPO activity, **P < 0.01, significantly different from LPS alone.. (E) Protein content in BALF. ANOVA ($F_{3,26}$ = 14.9 P < 0.001). Both compounds significantly reduced MPO activity, **P < 0.01, significantly different from LPS alone.. (F) TNF-α in BALF. ANOVA ($F_{3,26}$ = 10.9 P < 0.001). Both compounds significantly reduced TNF-α . *P < 0.05; **P < 0.01, significantly different from LPS alone.. (G) IL-6 in BALF. ANOVA ($F_{3,24}$ = 30.4 P < 0.001). Only AN680 significantly reduced IL-6, **P < 0.01, significantly different from LPS alone.

leading to cytokine release. In general, the indolines with a carbamate in position 4 produced greater reductions in IL-6 and NO than their 6- N-methyl, *N*-ethyl analogues. The maximal reduction in cytokines by all the compounds was greater, and the concentrations were 10–100-fold lower, than we found previously when we allowed a longer incubation time between the compounds and the macrophages before adding LPS. Like AN827, AN680 and AN917 produce their anti-inflammatory activity by decreasing the phosphorylation of p38 and the activation of AP-1. In addition, they prevent the degradation of IκBα and the nuclear translocation of NF-κB.

Although the indoline-3-propionates are hydrolysed by enzymes in plasma, the resulting acids, and the hydroxy derivatives formed by hydrolysis of the carbamate moiety by AChE, retain the anti-inflammatory activity of the parent molecules. Although AN827 consistently showed greater anti-inflammatory activity than the other compounds *in vitro*, its activity *in vivo* was strongly restricted by binding to a protein constituent of plasma. Thus, AN827 only inhibited AChE and reduced cytokines in LPS-injected mice at doses more than 50 times larger than would be predicted from its activity *in vitro*. Substitution of the carbamate moiety in position 6 (AN680) or exchange of the propionic ester for propylamine (AN917) prevented the protein binding.

In response to endotoxaemia induced by LPS, TNF-α is synthesized in the spleen and liver and released into the circulation (Ge *et al.*, 1997). The reduction by AN680 and AN917 (10 μmoles·kg^{-1}) in splenic TNF-α and IL-6 induced by LPS was similar to that of dexamethasone (6 μmoles·kg^{-1}), which in previous studies, gave an optimal effect on this measure (Wang *et al.*, 2008). The reduction in plasma levels of each cytokine 4 h after drug injection was lower than in the spleen and depended on the amount of time before LPS the compound was injected.

The formation of TNF-α in the spleen is reduced by stimulation of the vagus nerve and enhanced by vagotomy (Huston *et al.*, 2006). This is accomplished by neurotransmitters from peripheral autonomic nerves that promote the release of ACh from CD4 positive T-cells. ACh interacts with α-7nAChRs on macrophages to inhibit the production of cytokines and other inflammatory proteins (Tracey, 2007; Rosas-Ballina *et al.*, 2011). In the current study, the ChE inhibitor rivastigmine, was used to demonstrate the effect of indirect stimulation of α-7nAChRs. Rivastigmine significantly reduced the levels of TNF-α in the spleen and its effect was completely blocked by the nicotinic receptor antagonist mecamylamine (10 mg·kg^{-1}). Mecamylamine is not a selective antagonist of α7nAChR, nevertheless, like vagotomy, it has the opposite effect to that of the selective α7nAChR agonist GTS-21 on pancreatitis in mice (van Westerloo *et al.*, 2006). In LPS-activated RAW 264.7 macrophages, the reduction in NO by rivastigmine was antagonized by the selective α7nAChR antagonist α-bungarotoxin (Shifrin *et al.*, 2013). Therefore, it may be concluded that the reduction in TNF-α by rivastigmine in mouse spleen was also mediated by indirect activation of α7nAChR.

Although AN680 and AN917 (10 μmol·kg^{-1}) caused a similar reduction in TNF-α in mouse spleen, mecamylamine had no effect on that of AN680, but partly antagonized the reduction produced by AN917, in keeping with the 35% ChE inhibition induced in the spleen by this dose. These data support the suggestion that the anti-inflammatory effect of AN917 results from a combination of a direct action through p38, AP-1 and NF-κB on splenic lymphocytes and an indirect action via preservation of ACh released from vagal nerve endings. Activation of α7nAChR reduces cytokine production in LPS-activated macrophages by preventing STAT3 tyrosine phosphorylation and inhibiting NF-κB signalling (Filippini *et al.*, 2012). Reduction of STAT3 tyrosine phosphorylation does not increase the overall anti-inflammatory effect of AN917 in comparison with that of AN680, which does not activate α7nAChR indirectly but acts only by reducing phosphorylation of p38, NF-κB and AP-1 signalling.

Endotoxin or LPS is an important cause of acute respiratory distress syndrome in which there is recruitment of inflammatory cells into the lung, an increase in capillary permeability and alveolar oedema (Worthen *et al.*, 1987). Experimental lung injury caused by intratracheal injection of LPS is mediated by pro-inflammatory cytokines, such as TNF-α, which are expressed in lung tissue (Park *et al.*, 2001; Goodman *et al.*, 2003). Treatment of mice with AN680 and AN917 (10 μmol·kg^{-1}) almost normalized the histological signs of lung injury, including infiltration of inflammatory cells and alveolar oedema. AN680 and AN917 also reduced protein content and MPO activity indicating a reduction in neutrophil infiltration in the lung (Bradley *et al.*, 1982). They also reduced TNF-α in the BALF, but only AN680 significantly decreased IL-6.

In conclusion, four novel indoline carbamate derivatives reduced NO, TNF-α and IL-6 proteins in macrophages activated by LPS at concentrations of 0.001–1.0 nM, which are at least 10^{-8}-fold lower than those inhibiting AChE. The anti-inflammatory effect was mediated by reduction of the phosphorylation of p38, IκBα degradation and a decrease in the activation of both transcription factors, AP-1 and NF-κB. Compounds AN680 and AN917, which unlike AN827, did not bind to plasma protein, reduced pro-inflammatory cytokines elevated in the spleen of mice by the injection of LPS. They also markedly reduced microscopic evidence of lung injury, together with cellular infiltration and pro-inflammatory cytokines in BALF. The potent anti-inflammatory activity of indoline carbamate esters and amines support a role for their use in the treatment of chronic inflammatory conditions.

Author contributions

E. F.-G., D. M., H. S. and C. B. performed all the experiments in macrophages and mice. A. N. designed and supervised the synthesis of the compounds. M. W. designed the experiments and wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

References

Adler S, Kolev M, Varisco PA, Tham M, von Gunten M, Tappeiner C *et al.* (2013). Induction of severe systemic lupus erythematosus by TNF blockade and response to anti-IL-6 strategy. J Allergy Clin Immunol 131: 1235–1237, 1237 e1231.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL Spedding M *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Ligand-Gated Ion Channels. Br J Pharmacol 170: 1582–1606.

Berkman N, Kremer S, Or R, Lossos IS, Christensen TG, Goldstein RH *et al.* (2001). Human recombinant interferon-alpha2a and interferon-alphaA/D have different effects on bleomycin-induced lung injury. Respiration 68: 169–177.

Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V (2006). Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. JAMA 295: 2275–2285.

Bradley PP, Christensen RD, Rothstein G (1982). Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood 60: 618–622.

van Dartel SA, Fransen J, Kievit W, Dutmer EA, Brus HL, Houtman NM *et al.* (2013). Predictors for the 5-year risk of serious infections in patients with rheumatoid arthritis treated with anti-tumour necrosis factor therapy: a cohort study in the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry. Rheumatology (Oxford) 52: 1052–1057.

DeFuria J, Belkina AC, Jagannathan-Bogdan M, Snyder-Cappione J, Carr JD, Nersesova YR *et al.* (2013). B cells promote inflammation

in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. Proc Natl Acad Sci U S A 110: 5133–5138.

Filippini P, Cesario A, Fini M, Locatelli F, Rutella S (2012). The Yin and Yang of non-neuronal alpha7-nicotinic receptors in inflammation and autoimmunity. Curr Drug Targets 13: 644–655.

Finkin-Groner E (2013). Novel multi-functional drugs for the treatment of neurodegenerative diseases. PhD thesis, The Hebrew University of Jerusalem, Israel.

Furman S, Nissim-Bardugo E, Zeeli S, Weitman M, Nudelman A, Finkin-Groner E *et al*. (2014). Synthesis and *in vitro* evaluation of anti-inflammatory activity of ester and amine derivatives of indoline in RAW 264.7 and peritoneal macrophages. Bioorg Med Chem Lett 24: 2283–2287.

Gallowitsch-Puerta M, Pavlov VA (2007). Neuro-immune interactions via the cholinergic anti-inflammatory pathway. Life Sci 80: 2325–2329.

Ge Y, Ezzell RM, Clark BD, Loiselle PM, Amato SF, Warren HS (1997). Relationship of tissue and cellular interleukin-1 and lipopolysaccharide after endotoxemia and bacteremia. J Infect Dis 176: 1313–1321.

Gonzalez-Juanatey C, Vazquez-Rodriguez TR, Miranda-Filloy JA, Gomez-Acebo I, Testa A, Garcia-Porrua C *et al*. (2012). Anti-TNF-alpha-adalimumab therapy is associated with persistent improvement of endothelial function without progression of carotid intima-media wall thickness in patients with rheumatoid arthritis refractory to conventional therapy. Mediators Inflamm 2012: 674265.

Goodman RB, Pugin J, Lee JS, Matthay MA (2003). Cytokine-mediated inflammation in acute lung injury. Cytokine Growth Factor Rev 14: 523–535.

Horvath RJ, Nutile-McMenemy N, Alkaitis MS, Deleo JA (2008). Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures. J Neurochem 107: 557–569.

Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA *et al*. (2006). Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. J Exp Med 203: 1623–1628.

Kawashima K, Yoshikawa K, Fujii YX, Moriwaki Y, Misawa H (2007). Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci 80: 2314–2319.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Levitt JE, Matthay MA (2006). Treatment of acute lung injury: historical perspective and potential future therapies. Semin Respir Crit Care Med 27: 426–437.

Magro F, Portela F (2010). Management of inflammatory bowel disease with infliximab and other anti-tumor necrosis factor alpha therapies. Biodrugs 24 (Suppl. 1): 3–14.

Matute-Bello G, Winn RK, Jonas M, Chi EY, Martin TR, Liles WC (2001). Fas (CD95) induces alveolar epithelial cell apoptosis in vivo: implications for acute pulmonary inflammation. Am J Pathol 158: 153–161.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

McInnes IB, Schett G (2007). Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 7: 429–442.

Medzhitov R (2008). Origin and physiological roles of inflammation. Nature 454: 428–435.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Moynagh PN (2005). The NF-kappaB pathway. J Cell Sci 118: 4589–4592.

Nieto-Ceron S, Moral-Naranjo MT, Munoz-Delgado E, Vidal CJ, Campoy FJ (2004). Molecular properties of acetylcholinesterase in mouse spleen. Neurochem Int 45: 129–139.

Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella F 2nd, Park DR *et al*. (2001). Cytokine balance in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 164: 1896–1903.

Pavlov VA, Wang H, Czura CJ, Friedman SG, Tracey KJ (2003). The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol Med 9: 125–134.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al*.; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl. Acids Res. 42 (Database Issue): D1098–D1106.

Podolsky DK (2002). Inflammatory bowel disease. N Engl J Med 347: 417–429.

Quntar AA, Gallily R, Katzavian G, Srebnik M (2007). Potent anti-inflammatory activity of 3-aminovinylphosphonates as inhibitors of reactive oxygen intermediates, nitric oxides generation, and tumor necrosis factor-alpha release. Eur J Pharmacol 556: 9–13.

Raaschou P, Simard JF, Holmqvist M, Askling J, Group AS (2013). Rheumatoid arthritis, anti-tumour necrosis factor therapy, and risk of malignant melanoma: nationwide population based prospective cohort study from Sweden. BMJ 346: f1939.

Rayamajhi M, Redente EF, Condon TV, Gonzalez-Juarrero M, Riches DW, Lenz LL (2011). Non-surgical intratracheal instillation of mice with analysis of lungs and lung draining lymph nodes by flow cytometry. J Vis Exp 51: e2702.

Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010). Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med 49: 1603–1616.

Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C *et al*. (2011). Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science 334: 98–101.

Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M *et al*. (2005). Incidence and outcomes of acute lung injury. N Engl J Med 353: 1685–1693.

Seror R, Richez C, Sordet C, Rist S, Gossec L, Direz G *et al*. (2013). Pattern of demyelination occurring during anti-TNF-alpha therapy: a French national survey. Rheumatology (Oxford) 52: 868–874.

Shifrin H, Nadler-Milbauer M, Shoham S, Weinstock M (2013). Rivastigmine alleviates experimentally induced colitis in mice and rats by acting at central and peripheral sites to modulate immune responses. PLoS ONE 8: e57668.

Sorrentino D (2013). State-of-the-art medical prevention of postoperative recurrence of Crohn's disease. Nat Rev Gastroenterol Hepatol 10: 413–422.

Tracey KJ (2002). The inflammatory reflex. Nature 420: 853–859.

Tracey KJ (2007). Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest 117: 289–296.

Ulich TR, Fann MJ, Patterson PH, Williams JH, Samal B, Del Castillo J *et al.* (1994). Intratracheal injection of LPS and cytokines. V. LPS induces expression of LIF and LIF inhibits acute inflammation. Am J Physiol 267: L442–L446.

Wang X, Nelin LD, Kuhlman JR, Meng X, Welty SE, Liu Y (2008). The role of MAP kinase phosphatase-1 in the protective mechanism of dexamethasone against endotoxemia. Life Sci 83: 671–680.

Watters JJ, Sommer JA, Pfeiffer ZA, Prabhu U, Guerra AN, Bertics PJ (2002). A differential role for the mitogen-activated protein kinases in lipopolysaccharide signaling: the MEK/ERK pathway is not essential for nitric oxide and interleukin 1beta production. J Biol Chem 277: 9077–9087.

van Westerloo DJ, Giebelen IA, Florquin S, Bruno MJ, Larosa GJ, Ulloa L *et al.* (2006). The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. Gastroenterology 130: 1822–1830.

Worthen GS, Haslett C, Rees AJ, Gumbay RS, Henson JE, Henson PM (1987). Neutrophil-mediated pulmonary vascular injury. Synergistic effect of trace amounts of lipopolysaccharide and neutrophil stimuli on vascular permeability and neutrophil sequestration in the lung. Am Rev Respir Dis 136: 19–28.

Yanovsky I, Finkin-Groner E, Zaikin A, Lerman L, Shalom H, Zeeli S *et al.* (2012). Carbamate derivatives of indolines as cholinesterase inhibitors and antioxidants for the treatment of Alzheimer's disease. J Med Chem 55: 10700–10715.

Yu H, Buff SM, Baatz JE, Virella-Lowell I (2008). Oral instillation with surfactant phospholipid: a reliable alternative to intratracheal injection in mouse studies. Lab Anim 42: 294–304.