Inhibition of APE1/Ref-1 redox signalling alleviates intestinal dysfunction and damage to myenteric neurons in a mouse model of spontaneous chronic colitis

Running Title: Targeting APE1/Ref-1 for the treatment of colitis

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Summary: This is the first study demonstrating that inhibition of APE1/Ref-1 redox signalling by APX3330 ameliorated enteric neuropathy and intestinal dysfunction via inhibition of superoxide production, DNA damage and translocation of HMGB1.

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Conflict of interest statement

The authors declare no conflict of interest.

Abstract

Background and Purpose: Inflammatory bowel disease (IBD) associates with damage to the enteric nervous system (ENS) leading to gastrointestinal (GI) dysfunction. Oxidative stress is important for the pathophysiology of inflammation-induced enteric neuropathy and GI dysfunction. Apurinic/Apyrimidinic Endonuclease 1/Redox Factor-1 (APE1/Ref-1) is a dual functioning protein that is an essential regulator of the cellular response to oxidative stress. In this study, we aimed to determine whether an APE1/Ref-1 redox domain inhibitor, APX3330 alleviates inflammation-induced oxidative stress that leads to enteric neuropathy in the *Winnie* murine model of spontaneous chronic colitis.

Experimental Approach: *Winnie* mice received APX3330 or vehicle via intraperitoneal injections over two weeks and were compared to C57BL/6 controls. *In vivo* disease activity and GI transit were evaluated. *Ex vivo* experiments were performed to assess functional parameters of colonic motility, immune cell infiltration, and changes to the ENS.

Key Results: Targeting APE1/Ref-1 redox activity with APX3330 improved disease severity, reduced immune cell infiltration, restored GI function and provided neuroprotective effects to the enteric nervous system. Inhibition of APE1/Ref-1 redox signalling leading to reduced mitochondrial superoxide production, oxidative DNA damage and translocation of high mobility group box 1 protein (HMGB1) were involved in neuroprotective effects of APX3330 in enteric neurons.

Conclusion and Implications: This study is the first to investigate inhibition of APE1/Ref-1's redox activity via APX3330 in an animal model of chronic intestinal

inflammation. Inhibition of the redox function of APE1/Ref-1 is a novel strategy that might lead to a possible application of APX3330 for the treatment of IBD.

Key words: APE1/Ref-1, APX3330, enteric nervous system, chronic intestinal inflammation, IBD, oxidative stress, DNA damage

Introduction

Inflammatory Bowel Disease (IBD) is defined by chronic, recurrent inflammation of the gastrointestinal (GI) tract, characterised by periods of remission and relapses. It is estimated that more than 5 million people globally suffer from IBD (Burisch and Munkholm 2015). IBD encompasses two major chronic idiopathic conditions, ulcerative colitis (UC) and Crohn's disease (CD). UC is confined to the rectum and colon, where it is characterised by mucosal and submucosal ulcerations. In comparison, CD is transmural and displays skip lesions throughout the GI tract resulting in strictures or fistulas (Chang et al. 2015). IBD is a complex, multifactorial disorder with the exact cause being ambiguous. Current treatments are limited by their inefficacy and toxic side-effects. Most present treatments focus on reducing clinical symptoms and do not exploit the pathogenic mechanisms of the disease, therefore, targeting pathophysiological mechanisms including enteric neuropathy may lead to more effective treatments.

The enteric nervous system (ENS) consists of networks of intrinsic neurons embedded in the wall of the GI tract and controls GI functions with little assistance from the central nervous system (de Jonge 2013). Damage to the ENS is associated with GI dysfunction (Lakhan and Kirchgessner 2010; Lomax et al. 2005). The ENS has a vital role in GI immunity as interactions between enteric neurons and immune cells are important in both normal and pathological conditions (Knowles et al. 2013). Intestinal inflammation-induced ENS damage associates with a compromised GI antioxidant capacity (Brown and Gulbransen 2017) .

Investigating oxidative stress in inflammatory conditions will provide insight into the pathogenesis of IBD and help to develop new therapies. Exposure of enteric neurons to oxidative stress results in disruption of physiological functions (Bagyánszki and Bódi

2012). Colon biopsies from IBD patients have shown increased levels of nitric oxide (NO) and oxidative stress induced DNA damage measured with a specific marker, 8-hydroxydeoxyguanosine (8-OHdG) (D'Incà et al. 2006; Keshavarzian et al. 2003).

APE1/Ref-1 is a vital dual functioning protein which regulates the cellular response to oxidative stress and acts as a DNA repair protein (Shah et al. 2017; Tell et al. 2009). The redox active domain of APE1/Ref-1 regulates cellular stress responses, angiogenesis, inflammation, and proliferation (Kelley et al. 2012; Kelley et al. 2014). In contrast, its endonuclease repair domain associates with DNA repair (Fishel et al. 2008; Luo et al. 2008; Shah et al. 2017; Vascotto et al. 2009). Multiple inflammatory conditions produce oxidative stress resulting in altered APE1/Ref-1 responses; without interception by redox activation, cell apoptosis and carcinogenesis are likely (Liu et al. 2017). Previous studies have demonstrated increased APE1/Ref-1 redox activity in tissues removed from IBD patients with active inflammation, indicating underlying oxidative stress within the GI tract (Hofseth et al. 2003; Lih-Brody et al. 1996). In addition, increased expression of APE1/Ref-1 was observed in colon sections from rats with dextran sodium sulfate (DSS)-induced colitis, which has been associated with an enhanced pro-inflammatory response (Chang et al. 2013). Thus, APE1/Ref-1 is involved in the pathophysiology of GI inflammation. Furthermore, APE1/Ref-1 endonuclease activity protects dorsal root ganglion (DRG) neurons from inflammation and chemotherapy induced oxidative stress and DNA damage (Fehrenbacher et al. 2017; Kelley and Fehrenbacher 2017).

In this study we investigated the APE1/Ref-1 redox domain inhibitor, APX3330, as a potential therapeutic agent for treatment of chronic colitis and inflammation-induced enteric neuropathy using the *Winnie* murine model of spontaneous chronic colitis. In *Winnie* mice, spontaneous colitis develops from a primary intestinal epithelial defect

conferred by a mutation in the *Muc2* mucin gene leading to intestinal inflammation at 5-6 weeks of age progressing to severe colitis by the age of 12-16 weeks. This model closely represents pathophysiological mechanisms and clinical manifestations of human IBD (Eri et al. 2010; Heazlewood et al. 2008; Rahman et al. 2015; Robinson et al. 2016).

Methods

Animals

Male and female *Winnie* mice (12 w.o; 20-30 g; n=24) were obtained from the Victoria University Werribee Animal Facility (Melbourne, Australia). Male and female C57BL/6 mice (12 w.o; 20-30 g; n=12) were obtained from the Animal Resources Centre (Perth, Australia) and were used as controls. All mice were acclimatized for 3 days prior to receiving their allocated treatments. All mice were housed at the Western Centre for Health, Research and Education (Melbourne, Victoria, Australia) in a temperature-controlled environment at 22°C with a 12-hour day/night cycle. All animals had free access to food and water. All experimental procedures in this study were conducted in agreement with the Australian National Health and Medical Research Council (NHMRC) guidelines and under animal ethics AEETH 13/001 and AEC 17/016 approved by Victorian University Animal Experimentation Ethic Committee (AEEC).

Administration of APX3330

Winnie mice were randomly divided into 2 groups: APX3330-treated and sham-treated. *Winnie* APX3330-treated mice received APX3330 (Kelley laboratory, Indianapolis, IN, USA) at a dose of 25mg/kg via *i.p* injections dissolved in cremophor (2%) (Sigma-Aldrich): ethanol (2%) in sterile water (96%) (30G needle, max volume 200µL). The dose of APX3330, route of administration and vehicle solution were based on previous studies in mice (Fishel et al. 2019; Yang et al. 2018). Mice received the treatment over two weeks, twice daily with a 12 hour interval. *Winnie* sham-treated mice received the vehicle *i.p* injections of cremophor (2%) (Sigma-Aldrich): ethanol (2%) in sterile water (96%) (30G needle, max volume 200µL) according to the same regimen as the treatment group.

Disease Activity

Disease activity in *Winnie* mice can be characterised by clinical signs including rectal prolapse, diarrhoea, changes in colon length and body weight (Eri et al. 2010; Heazlewood et al. 2008; Rahman et al. 2015; Rahman et al. 2016). Mice were monitored throughout the treatment period for clinical symptoms. Body weights were recorded daily over 14 day period and expressed as a percent of change from the body weight at the start of the treatment. Directly after culling on day 15 the whole colon was excised, and the length was measured to assess changes in colon morphology. Faecal water content was assessed by collection of fresh faecal samples at day 14 prior to culling for *ex vivo* motility experiments. Faecal samples were collected by placing each mouse in a separate sterile cage without bedding until the expulsion of the faecal mass. The samples were immediately weighed to record the wet weight then placed in an oven at +60°C for 24 hours. Dry weight was recorded and the difference between the wet and dry weight (water content) was calculated as a percent of the wet weight.

Assessment of intestinal inflammation

Measurement of faecal lipocalin (Lcn)-2 was used as a non-invasive detection of anti-inflammatory efficacy of APX3330 treatment (Robinson et al., 2017). Faecal samples collected on day 14 from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice were reconstituted in PBS-0.1 % Tween 20 (100mg/mL) to form a homogenous faecal suspension. This was centrifuged for 10 minutes at 12000 RPM at 4°C. Lcn-2 levels were estimated in the faecal pellet supernatants using Mouse NGAL ELISA Kit Lcn-2 (Abcam 199083). All samples were run in duplicate for statistical value. A microplate reader capable of measuring absorbance at 450nm, with

the correction wavelength set at 540nm, was used to detect Lcn-2 protein (pg/mL) in the faecal pellet supernatant.

Gastrointestinal transit

GI transit was measured via a non-invasive radiological method in C57BL/6 control mice and APX3330-treated and sham-treated *Winnie* mice after completion of treatment on day 14 as previously described (Robinson et al., 2017). Briefly, C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice received barium sulfate as contrast (2.5mg/mL; maximum volume of 200µL; X-OPAQUE-HD), via oral gavage. Sequential x-rays were taken using HiRay Plus Porta610HF x-ray apparatus (JOC Corp, Kanagawa, Japan; 50kV, 0.3mAs, exposure time 60ms) immediately post barium sulfate administration (0min) and then every 10 minutes for the first hour, followed by every 20 minutes until expulsion of the first pellet with barium sulphate or a maximum of 4 hours. Images were processed through Fujifilm FCR Capsule XL11 and analysed on eFilm 4.2.0 software. Parameters of GI transit were measured by time (mins) to determine contrast passing through whole GI tract (whole transit time), stomach to caecum (gastro-caecal transit time; GCTT), caecum retention time and leaving caecum to anus (colonic transit time; CTT). C57BL/6 control, APX3330-treated and sham-treated *Winnie* mice were culled on the following day (day 15) and tissues were collected for *ex-vivo* studies.

Organ bath experiments for isolated colonic motility

Whole colons were removed from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice in oxygenated physiological saline and *ex vivo* organ-bath colonic motility experiments were performed as described previously (McQuade et al. 2016; Robinson et al. 2017; Swaminathan et al. 2016). Briefly, colons were positioned horizontally, cannulated at the oral and anal ends in an organ bath superfused with

Kreb's solution (composition in mM: NaCL 118, KCl 4.6, $CaCl_2$ 3.5, $MgSO_4$ 1.2, NAH_2PO_4 1, $NaHCO_3$ 25 and d-Glucose 11; bubbled with 95% O_2 and 5% CO_2) whilst maintained at a constant temperature of 37°C. The oral cannula was connected to a reservoir containing Kreb's solution that was adjusted to maintain intraluminal pressure (0 to +2cm H_2O). The anal end was coupled to an outflow tube with a maximum backpressure of 2cm H_2O. Colonic contractile activity was recorded by a video camera positioned above the organ bath. Tissues were left to equilibrate at constant intraluminal pressure for 30 minutes followed by two sequential 20 minute video recordings of colonic contractile activity. Videos were transposed into spatiotemporal maps with Scribble v2.0 software and were analysed by using MATLAB v2017a software to assess parameters of colonic motility.

Tissue collections

Distal colon tissues were harvested in oxygenated physiological saline, flushed clear of faecal content and cut along the mesenteric border. Tissues were pinned down with the mucosal side up in a Sylgard-lined Petri dish and fixed with Zamboni's fixative (2% formaldehyde containing 0.2% picric acid) overnight at 4°C. The fixative was removed by a series of washes (3 x 10 minutes) with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Sydney, Australia) followed by 3 10 minute washes with phosphate buffered saline (PBS). For cross sections, distal colon tissues were pinned and fixed as above without stretching, then covered by 50:50 optimum cutting temperature (OCT) compound (Tissue Tek, CA, USA) and frozen in liquid nitrogen-cooled isopentane and OCT and stored at -80°C until cryo-sectioned (20µm) onto glass slides. For wholemount longitudinal muscle with attached myenteric plexus (LMMP) preparations, distal colon segments were stretched to maximum capacity without tearing, fixed and washed as

above. To expose the myenteric plexus, the mucosa, submucosa and circular muscle layers were removed prior to immunohistochemical labelling.

Immunohistochemistry and Histology

Immunohistochemistry was performed as previously described (Rahman et al. 2015). Cross sections and wholemount LMMP preparations were incubated for 1 h at room temperature in 10% normalised donkey serum (Merck Millipore, Australia). Preparations were then washed (2 x 5 mins) with PBS and incubated with primary antisera **(Table 1)** overnight at room temperature in an enclosed moist environment. Cross sections and LMMP preparations were washed with 1x PBS (3 x 10 minute) and incubated with fluorophore-conjugated secondary antisera Alexa Fluor 488, 594 or 647 of the respective anti-host species (1:200; Jackson Immunoresearch, West Grove, USA) for 2 hours at room temperature. All specimens were stained with 4',6'-diamindino-2-pheylindole dihydrochloride (DAPI) to label cell nuclei. Tissues were given 3 x 10 min final washes in PBS, mounted on glass slides and cover slipped with fluorescent mounting medium (DAKO, Australia).

Tissues for histology were cryo-sectioned at 10µm, cleared and rehydrated in graded ethanol concentration. For standard hematoxylin and eosin stain (H&E) sections were immersed in histolene (3 x 4 minutes), 100% ethanol (2 minutes), 95% ethanol (2 minutes), 70% ethanol (2 minutes), rinsed in Tap water (30 seconds), then in hematoxylin (Sigma-Aldrich) (1 minute), rinsed in tap water, immersed in Scott's tap water (1 minute) and eosin (Sigma-Aldrich) (5 minutes), rinsed in tap water, immersed in 100% ethanol (2 x 1 minute) and histolene (4 minutes) and finally mounted on glass slides with DPX mounting media. A histological grading system was used to evaluate gross morphological damage from the following parameters: aberrant crypt architecture (score range: 0–3), changes to crypt length (0–3), crypt abscesses (0–3),

leukocyte infiltration (0–3), epithelial damage (0–3) and ulceration (0–3); average of three areas of 500 μm^2 per section. Slides were imaged using an Olympus BX53 microscope (Olympus Imaging, Sydney, Australia). All slides were coded, and analysis was performed blindly.

Imaging and quantitative analysis

Triple-labelled specimens were visualised and imaged via confocal microscopy (Nikon Eclipse Ti multichannel confocal laser scanning system, Nikon, Japan) using combinations of FITC, Alexa 594 and Alexa 647 filters (495nm, 559nm or 640.4nm excitation wavelength respectively). Images (1062 X 1062 pixels) were obtained with x20 (dry) or x40 and x60 (oil immersion) lenses. Immunoreactivity for CD45, β-Tubulin, and APE1/Ref-1 was analysed in cross sections and quantified as a percent of the area with specific fluorescence relative to the total 2 mm^2 area of colonic mucosa. Images of 3 colon sections from each tissue sample were captured with a x20 objective to quantify the average area of fluorescence. Immunoreactivity for GFAP, MitoSOX™ and APE1/Ref-1 in LMMP preparations was analysed in 5 randomly chosen myenteric ganglia. Fluorescence was measured as a percentage relative to the ganglion area, using ImageJ software (NIH, MD, USA). All images for CD45, β-Tubulin, APE1/Ref-1, GFAP and MitoSOX™ were acquired with equal acquisition and exposure time conditions, calibrated to standard minimum baseline fluorescence and converted to binary for analysis of the number of fluorescent pixels per image (McQuade et al. 2016; Robinson et al. 2017). Data were normalised and presented as percentage due to the variability in different ganglia and colon sizes between groups. The number of myenteric neurons were counted within 10 randomly selected ganglia

(expressed as an average number of neurons per ganglion) and within randomly chosen 4 images per preparation at x20 magnification (total area 1mm^2) (expressed as number of neurons per area). The number of neurons with translocation of HMGB1 from the nuclei to the cytoplasm was quantified with the aid of the nuclei marker DAPI. LMMP preparations were co-labelled with MAP2 and 8-OHdG to identify oxidative stress-induced DNA damage within myenteric neurons. The number of myenteric neurons co-localised with 8-OHdG was quantified as a proportion of the total number of neurons within an average of 10 myenteric ganglia. Neurons were counted using the Cell Counter plug-in in ImageJ software. All slides were coded and images were analysed blindly.

Superoxide production in the myenteric plexus

MitoSOX™ Red M36008 (Invitrogen, Australia), was used to identify mitochondrial-derived production of superoxide in the myenteric ganglia (McQuade et al. 2016). Briefly, freshly excised preparations of the distal colon from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice were dissected to expose the myenteric plexus. Samples were incubated with 5μM MitoSOX™ Red M36008 at 37ºC for 40 minutes. Tissues were washed with oxygenated Krebs solution and fixed with Zamboni's fixative for 1 hour followed by PBS washes (3 x 10 minutes). Preparations were mounted on glass slides with DAKO fluorescent mounting medium for imaging. Images were captured as described above. Images were converted into binary and fluorescent areas were measured in arbitrary units relative to ganglion area using ImageJ software (NIH, MD, USA).

Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al. 2015). Data were assessed using one-way ANOVA with Tukey's post hoc tests for multiple group comparison. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Data are presented as mean ± standard error of the mean (SEM). Value differences were considered statistically significant at $P<0.05$.

Results

APX3330 treatment improved clinical symptoms in *Winnie* mice

C57BL/6 control mice did not display any symptoms of inflammation; the signs of diarrhoea, rectal prolapse and bleeding were absent; all C57BL/6 mice produced firm pellets during the 14 day period. All *Winnie* mice had chronic diarrhoea prior to start of the treatments. *Winnie* sham-treated mice had chronic diarrhoea throughout the whole treatment period; some mice had rectal prolapse evident as a rectal protrusion with oedema and bleeding **(Figure 1A)**. *Winnie* APX3330-treated mice that had rectal prolapse at the start of the treatment demonstrated reduced rectal prolapse, oedema and bleeding by day 14 of the treatment regimen **(Figure 1A)**. Colons were excised and their length was measured at day 15 **(Figure 1B)**. Colons from *Winnie* sham-treated mice had shorter lengths (75.7±3.2mm, $P<0.01$, $n=5$) than C57BL/6 control mice (90.4±3.8mm, $n=5$) and contained soft faecal masses **(Figure 1B')**. APX3330 treatment did not improve the colon lengths (70.4±1.9mm, $P<0.05$, $n=5$), however the presence of hard faecal pellets was prominent similar to C57BL/6 control mice **(Figure 1B-B')**. Faecal water content was used as a measure of diarrhoea. Fresh faecal pellets were collected on day 14 to assess water content. *Winnie* sham-treated mice had higher level of faecal water content (83.4±2.7%, $P<0.0001$, $n=7$) than C57BL/6 control

mice (57.3±0.6%, *n*=7) **(Figure 1C)**. Conversely, faeces from *Winnie* APX3330-treated mice had lower faecal water retention (73.2±2.1%, *P*<0.01, *n*=7) than *Winnie* sham-treated mice, but higher levels than C57BL/6 control mice (*P*<0.0001) **(Figure 1C)**. Haematoxylin and eosin staining was performed in colon cross sections to evaluate gross morphological alterations using parameters described in the Methods section **(Figure 1D)**. *Winnie* sham-treated mice attained a higher histological score (8.9±1.4, *P*<0.0001, *n*=5) compared to C57BL/6 control mice (0.5±0.2, *n*=5) **(Figure 1D')**. APX3330 treatment reduced histological score in *Winnie* mice (8.9±1.4, *P*<0.001, *n*=5). Body weights of C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice were measured over the 14 day period **(Figure 1E)**. *Winnie* sham-treated mice showed progressively decreasing body weight which was significantly different from day 6 until day 14 compared to C57BL/6 control mice **(Figure 1E)**. *Winnie* APX3330-treated mice grow slowly, but not equally well to control C57BL/6 mice and weighed significantly more (99.8±1.0%, *n*=8) than *Winnie* sham-treated mice at day 14 (93.3±1.8%, *P*<0.05, *n*=10) **(Figure 1E)**.

APX3330 treatment reduced inflammation in the colon

A pan-leukocyte marker anti-CD45 antibody was used in colon cross sections to investigate whether APX3330 treatment reduced immune cell infiltration in the *Winnie* mice colons **(Figure 2A)**. The density of CD45-IR cells quantified as a percent of the CD45-IR area relative to the total 2 mm² area of colonic mucosa was significantly higher in *Winnie* sham-treated mice (24.9±1.8%, *n*=5) than in C57BL/6 control (13.6±0.6%, *P*<0.01, *n*=5) and *Winnie* APX3330-treated (12.0±1.3%, *P*<0.01, *n*=5) mice **(Figure 2B)**. Severity of intestinal inflammation was assessed by the presence

of rectal prolapse and Lipocalin-2 (Lcn-2) levels in the faeces. Lcn-2, also known as neutrophil gelatinase-associated lipocalin, is a non-invasive highly sensitive and specific biomarker for intestinal inflammation (Chassaing et al. 2012). At day 14, *Winnie* sham-treated mice displayed a higher faecal Lcn-2 level (46±2.4pg/mL, *n*=5) than C57BL/6 control mice (28±2.4pg/mL, *P*<0.001, *n*=5). Treatment with APX3330 decreased faecal Lcn-2 levels (39±1.8pg/mL, *P*<0.01, *n*=5), although these were still greater than in C57BL/6 controls **(Figure 2C)**.

APX3330 treatment improved GI transit in *Winnie* mice

The efficacy of APX3330 treatment was assessed on parameters of GI transit and colonic motility. Radiographic images captured movement of the contrast agent barium sulfate from the stomach to the rectum in C57BL/6 control **(Figure 3A)**, *Winnie* sham-treated **(Figure 3A')** and *Winnie* APX3330-treated **(Figure 3A'')** mice. Total transit time in *Winnie* sham-treated (232.9±5.7mins, *n*=7) was longer than in C57BL/6 control (174.3±11.1mins, *P*<0.01, *n*=7) mice. APX3330 treatment reduced the total transit time in *Winnie* mice (163.3±18.9mins, *P*<0.05, *n*=7) to a level comparable to C57BL/6 control mice **(Figure 3B)**. No significant differences between experimental groups were observed in gastro-caecal transit time (GCTT) **(Figure 3C)**, but other parameters of GI transit were compromised in *Winnie* sham-treated mice and restored in *Winnie* APX3330-treated mice. *Winnie* sham-treated mice had a prolonged contrast retention time in the caecum (165.7±9.7mins, *n*=7) compared to C57BL/6 control mice (93.6±12.8mins, *P*<0.01, *n*=7). But the caecum retention time in *Winnie* APX3330-treated (86.4±17.8mins, *P*<0.01 compared to *Winnie* sham-treated group, *n*=7) was

similar to that of C57BL/6 control mice **(Figure 3D)**. In contrary, colonic transit time (CTT) was shorter in *Winnie* sham-treated mice (18.6±2.6mins, *n*=7) than in C57BL/6 control mice (40.8±5.2mins, *P*<0.01, *n*=7), but was restored to control levels in *Winnie* APX3330-treated (37.1±5.7mins, *P*<0.05 compared to *Winnie* sham-treated group, *n*=7) mice **(Figure 3E)**.

The effects of APX330 treatment on colonic contractile activity was assessed in *ex vivo* organ bath experiments **(Figure 4)**. Colonic migrating motor complexes (CMMCs) are identified as contractions initiated from the oral end that propagated toward the anal end for >50% of the colon length (R. R. Roberts et al. 2008; Robinson et al. 2017). Video recordings were converted into spatiotemporal maps with contractions identified as orange/red lines **(Figure 4A)**. Overall, the average length of colonic contractions in *Winnie* sham-treated mice (21.0±2.6%, *n*=6) was much shorter than in C57BL/6 control mice (79.9±4.5%, *P*<0.0001, *n*=6). APX3330 treatment increased the length of colonic contractions (74.3±1.7%, *P*<0.0001, *n*=6) **(Figure 4B)**. Lengths of CMMCs in *Winnie* sham-treated (50.2±7.2%, *n*=6) mice were significantly shorter than in C57BL/6 control mice (68.9±3.7%, *P*<0.05, *n*=6) **(Figure 4C)**. *Winnie* APX3330-treated mice had lengths of CMMCs (72.7±0.8%, *P*<0.05, *n*=6) similar to those of C57BL/6 control mice **(Figure 4C)**. CMMCs were significantly faster in *Winnie* sham-treated mice (average CMMC speed: 3.3±0.5mm/sec, *n*=6) than in C57BL/6 mice (2.0±0.1mm/sec, *P*<0.05, *n*=6). After APX3330 treatment, CMMC speed in *Winnie* mice (1.8±0.3mm/sec, *n*=6) was significantly slower than in *Winnie* sham-treated mice (*P*<0.05) and comparable to the speed of CMMCs in C57BL/6 mice **(Figure 4D)**. CMMCs in *Winnie* sham-treated mice (4.0±1.0 CMMCs/10mins, *n*=6) were significantly less frequent than in C57BL/6 mice (8.0±0.5 CMMCs/10mins, *P*<0.05,

n=6). Treatment with APX3330 tended to increase the frequency of CMMCs (6.3±1.0 CMMCs/10mins, *n*=6) compared to *Winnie* sham-treated mice, but significant difference was not achieved **(Figure 4E)**.

APX3330 treatment alleviated enteric neuropathy in *Winnie* mice

An antibody specific to neuronal microtubule protein β-tubulin (|||) was used to label nerve fibres in cross sections of the distal colon from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice **(Figure 5A)**. *Winnie* sham-treated mice had a significantly lower nerve fibre density in the distal colon (6.7±1.2%, *P*<0.001, *n*=5) than C57BL/6 control mice (14.4±1.2%, *n*=5) **(Figure 5A, 5B)**, while APX3330 treatment improved nerve fibre density in the colon (14.5±0.8, *P*<0.001, *n*=5) **(Figure 5A, 5B)**.

Myenteric neurons were identified with a pan neuronal marker anti-MAP2 antibody in LMMP preparations from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice **(Figure 5A)**. Fewer myenteric neurons per ganglion were observed in *Winnie* sham-treated mice (20±1 neurons, *n*=5) than in C57BL/6 control mice (37±4 neurons per ganglion, *P*<0.01, *n*=5) **(Figure 5A, 5C)**. APX3330 treatment alleviated the loss of myenteric neurons in *Winnie* mice (32±3 neurons per ganglion, *P*<0.05 compared to *Winnie* sham-treated group, *n*=5) **(Figure 5A, 5C)**. In *Winnie* sham-treated mice there were fewer myenteric neurons per unit area (189±17 neurons per mm^2, *n*=5) than in C57BL/6 control mice (348±36 neurons per mm^2, *P*<0.05, *n*=5) **(Figure 5A, 5D)**. APX3330 treatment alleviated loss of myenteric neurons (320±34

neurons per mm^2, $P<0.05$, $n=5$) compared to *Winnie* sham-treated mice **(Figure 5A, 5D)**.

Glial cell density was identified with an anti-GFAP antibody assessed in the myenteric plexus of the distal colon relative to the ganglion area in LMMP preparations from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice **(Figure 5A)**. *Winnie* sham-treated mice had a significant decrease in glial cell density (15.6±0.6%, $n=5$) compared to C57BL/6 control mice (26.5±0.9%, $P<0.01$, $n=5$) **(Figure 5A, 5E)**. APX3330 treatment of *Winnie* mice improved GFAP density (22.0±0.8%, $P<0.01$, $n=5$) when compared to *Winnie* sham-treated mice **(Figure 5A, 5E)**.

APX3330 treatment reduced oxidative stress and associated DNA damage

The fluorescent mitochondrial superoxide marker MitoSOX™ Red was used to evaluate superoxide production in distal colon myenteric ganglia from C57BL/6 control, *Winnie* sham-treated, *Winnie* APX3330-treated mice **(Figure 6A)**. High levels of MitoSOX™ fluorescence were evident in the myenteric plexus of *Winnie* sham-treated mice (30.9±2.7%, $P<0.05$, $n=5$) when compared to C57BL/6 control mice (16.4±0.4%, $n=5$) **(Figure 6A, 6B)**. Increased superoxide production in the myenteric plexus was alleviated in the colons from *Winnie* APX3330-treated mice (13.8±2.3%, $P<0.01$, $n=5$) (**Figure 6A, 6B)**.

Translocation of high mobility group box 1 (HMGB1) protein from nuclei to cytoplasm identifies its conversion to a damage associated molecular pattern (DAMP) that is released by dying cells to stimulate an immune response (Paudel et al., 2018).

HMGB1 translocation from neuronal nuclei to cytoplasm was analysed within LMMP preparations from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice **(Figure 6A')**. In *Winnie* sham-treated mice many more cells exhibited HMGB1 translocation into the cytosol (17±3 neurons per ganglion, $P<0.01$, $n=5$) than in C57BL/6 control mice (0±0 neuron per ganglion, $n=5$) **(Figure 6A', 6C)**. APX3330 treatment attenuated cytoplasmic translocation of HMGB1 in the myenteric ganglia (1±0 neuron per ganglion, $P<0.05$, $n=5$) compared to *Winnie* sham-treated mice **(Figure 6A', 6C).**

APE1/Ref-1 expression was determined by immunofluorescence within the myenteric plexus in the colon cross sections and LMMP preparations from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice **(Figure 7A, 7A')**. A significant increase in APE1/Ref-1 intensity was observed in the colon cross sections from *Winnie* sham-treated mice (32.2±3.3%, $P<0.0001$, $n=5$) compared to C57BL/6 control mice (11.7±0.9%, n=5) **(Figure 7A, 7B)**. APX3330 treatment reduced this overexpression of APE1/Ref-1 in colon cross sections (13.8±1.3%, $P<0.001$, $n=5$) compared to *Winnie* sham-treated mice **(Figure 7A, 7B)**. The level of APE1/Ref-1 immunoreactivity was increased within the myenteric ganglia of *Winnie* sham-treated mice (17.4±1.1%, $P<0.05$, $n=5$) compared to C57BL/6 control mice (11.8±1.5%, $n=5$) **(Figure 7A', 7C)**. In contrast, immunoreactivity in the myenteric ganglia from *Winnie* APX3330-treated mice (6.2±1.1%, $P<0.001$ compared to *Winnie* sham-treated group, $n=5$) was similar to C57BL/6 controls **(Figure 7A', 7C)**.

Myenteric neurons were co-immunolabelled with a panneuronal marker, MAP2, and oxidative DNA damage marker, 8-OHdG **(Figure 8A)**. Overall, 8-OHdG immunofluorescence density was significantly greater in *Winnie* sham-treated mice

(17.6±1.8%, *P*<0.01, *n*=5) than in C57BL/6 control mice (4.6±1.1%, *n*=5) **(Figure 8A, 8B)**. This immunoreactivity subsided in *Winnie* APX3330-treated mice (3.8±1.7%, *P*<0.01, *n*=5) to levels comparable to C57BL/6 control mice **(Figure 8A, 8B)**. These results were consistent with the number of myenteric neurons colocalised with 8-OHdG which was significantly larger in *Winnie* sham-treated mice (26±8.0%, *P*<0.01, *n*=5) than in C57BL/6 control mice (1.2±1.0%, *n*=5) **(Figure 8A, 8C)** or in *Winnie* APX3330-treated mice (1.9±0.8%, *P*<0.01 compared to *Winnie* sham-treated group, *n*=5) **(Figure 8A, 8C)**.

Discussion

This is the first study to investigate the effects of APX3330, a small molecule inhibitor of APE1/Ref-1 redox signalling, in a clinically relevant animal model of IBD. Our study provides novel findings on the presence of APE1/Ref-1 in enteric neurons and increased level of APE1/Ref-1 in both the mucosa and myenteric ganglia, significant increase in mitochondrial ROS production, leading to oxidative DNA damage and translocation of HMGB1 in the colons from *Winnie* mice. Targeting this pathway with APX3330 ameliorated enteric neuropathy, colonic dysmotility and altered GI transit while providing anti-inflammatory and antioxidant effects in the *Winnie* murine model of spontaneous chronic colitis. These results demonstrate that APX3330 had prominent therapeutic effects in a preclinical animal model of IBD.

The current treatments for IBD are limited due to inefficacy, intolerance, resistance, side-effects and inability to maintain remission (Ghadir et al. 2016; Nitzan et al. 2016). Frontline treatments tend to involve immunosuppression which has long-term detrimental effects leading to diminished quality of life in IBD patients (Abegunde et

al. 2016; Gionchetti et al. 2016; Hanauer 2016; Harris 2006). Several new biological treatments have been introduced into clinic that have helped to reduce use of corticosteroids, but many patients stop responding to the clinically approved drugs, so there is still a large unmet need for IBD treatment (Neurath 2017).

Oxidative stress is a major contributing factor to tissue injury in IBD patients. Reduced mucosal antioxidant capacity and plasma antioxidants have been shown in patients with active CD and UC (Achitei et al. 2013; Ishihara et al. 2009; Lih-Brody et al. 1996). However, free radical antioxidants failed in clinical trials, as treatment with general antioxidants does not affect specific signalling and localized oxidative pathways or reversal of DNA damage within cells and subsequently within subcellular elements of cells (Aviello and Knaus, 2017). Therefore, new therapeutic strategies to block the major sources of oxidative stress are essential. Our study targeted a key molecule involved in oxidative DNA damage/repair mechanisms, APE1/Ref-1, which underpins cellular homeostasis and regulates multiple downstream inflammatory mediators that fuel uncontrolled homeostatic disbalance causing detrimental effects observed in IBD. APX3330 has the unique ability to suppress inflammation and chemotherapy-induced enhanced redox signalling activity of APE1/Ref-1 while providing neuroprotection in dorsal root ganglion neurons through an increase in oxidative DNA damage repair (Fehrenbacher et al. 2017; Kelley et al. 2016; Kim and Chang 2014; Kim et al. 2015). A recent phase I clinical trial of APX3330 in cancer patients demonstrated no significant toxicities/adverse effects, disease stabilization in six patients with four on drug for an extended time (252, 337, 357, and 421 days), predicted pharmacokinetics and target engagement (NCT03375086) (Chu et al. 2019; Shahda et al. 2019). Here we tested whether APX3330 alleviates intestinal inflammation, GI dysfunction and damage to enteric neurons in a pre-clinical model of spontaneous chronic colitis.

Rectal bleeding, prolapse, chronic diarrhoea, lack of body weight gain are clinical symptoms of chronic colitis in the *Winnie* mouse model (Eri et al. 2010; Heazlewood et al. 2008; Rahman et al. 2015; Rahman et al. 2016). Treatment with APX3330 improved clinical signs associated with colonic inflammation in this model of chronic colitis. APX3330 attenuated rectal bleeding, improved stool consistency and prevented loss of body weight in *Winnie* mice. Gross morphological damage of the colon demonstrated aberrant crypt architecture, crypt elongation, abscesses, epithelial damage and ulceration were prominent in *Winnie* sham-treated mice. Increased colonic crypt length due to epithelial hyperplasia is consistently observed in *Winnie* mice with chronic colitis (Heaselwood et al., 2008; Eri et al. 2011; Rahman et al., 2015) as well in other models of chronic colitis (Erben et al 2014; Prattis and Jurjus, 2015), although in chemically-induced models of colitis (TNBS, DSS) both crypt elongation as well as crypt destruction with mucosal erosion have been reported (Fuss et al 2002; Jiminez et al 2015). These parameters of gross morphological damage were alleviated by APX3330 treatment. High levels of colonic leukocyte infiltration and faecal Lcn-2 are reliable markers of inflammation which are prominent in multiple animal models of experimental colitis including *Winnie* mice (Rahman et al. 2015; Robinson et al. 2017). APX3330 alleviated the immune response in the *Winnie* mice after a two-week treatment regimen. This was shown by reduced CD45+ leukocyte infiltration throughout the distal colon and reduced levels of faecal Lcn-2 in *Winnie* APX3330-treated mice. Further studies need to be performed to define APX3330 effects on specific types of immune cells infiltrating the colon as well as specific inflammatory mediators.

Winnie mice show an increase in total GI transit time due to a substantial delay in the caecum, although accelerated colonic transit is evident, consistent with previous

reports in this model (Robinson et al. 2017). Similarly, constipation in the proximal colon (James et al. 2018) and accelerated distal colonic transit (Haase et al. 2016; Rao et al. 1987) are common in patients with severe UC. Persistent alterations of GI transit in IBD patients and animal models beyond periods of acute inflammation indicate that damage to the intestinal innervation controlling GI functions is involved (Moynes et al. 2013). Intrinsic and extrinsic innervations are integral to the functions of the GI tract (Uesaka et al. 2016). Significant changes to intestinal transit result from modulation of the sympathetic and parasympathetic innervation of the GI tract observed in both *Winnie* and chemically-induced models of colitis (Lomax et al. 2005; Moynes et al. 2014; Rahman et al. 2015; Robinson et al. 2017). In addition to changes in GI transit time, there was a notable decrease in the average length of colonic contractions with a reduction in length, increased speed and lower frequency of CMMCs demonstrating distorted colonic motor activity in *Winnie* mice compared to controls. APX3330 treatment successfully restored total GI and colonic transit time *in vivo* as well as colonic CMMC lengths to control levels *ex vivo*. These changes correlate with increased colonic transit time observed in *in vivo* experiments. Although frequency of CMMCs has not returned to control levels, overall propulsive activities of the colon become more prominent. This positive modulation of the GI motility suggests that APX3330 treatment has direct or indirect effects on intestinal innervation. Whilst GI transit is influenced by both extrinsic and intrinsic neurons, the *ex vivo* contractile activity of the isolated colon is driven mainly by enteric neurons within the myenteric ganglia (Roberts et al. 2007). Accordingly, we focused on the mechanisms underlying these positive effects of APX3330 treatment in the myenteric plexus.

Intestinal inflammation leads to enteric neuropathy resulting in compromised GI function (Mawe 2015; Moynes et al. 2013). The severity of inflammation in the colon

has an impact on myenteric neuronal loss in *Winnie* mice (Rahman et al. 2015). Loss of myenteric neurons and neuronal fibres occurs in multiple animal models of intestinal inflammation and in IBD patients (Bernardini et al. 2012; Boyer et al. 2005; Linden et al. 2005; Nurgali et al. 2007; Nurgali et al. 2011; Rahman et al. 2016). In addition, reduced expression of GFAP-positive glial cells within myenteric ganglia is seen in *Winnie* mice in this study. In contrast, studies of colonic biopsies from IBD patients, animal models of colitis and *in vitro* cultures yielded divergent results on the expression of GFAP in inflammatory conditions (Pochard et al. 2018). The reduced immunofluorescence density of GFAP-positive glial cells in our study may be due to either loss of GFAP-positive glial cells, phenotypic changes or decreased expression of the protein corresponding to a loss of glial reactivity. Other types of glial cells should be considered in future studies of this model. Enteric glial cells regulate intestinal homeostasis and protect enteric neurons from oxidative stress-induced death (Abdo et al. 2009; Roberts et al. 2013). Loss of GFAP-positive glial cells causes degeneration of enteric neurons and intestinal inflammation (Bush et al., 1998; Cornet et al., 2001). Altered signalling between enteric glia and neurons contributes to colonic dysmotility (McClain et al., 2014). However, in inflammatory conditions, activated enteric glial cells mediate neuronal death via activation of purinergic signalling (Brown et al. 2016). Purinergic transmission plays an important role in both excitatory and inhibitory in neuronal pathways as well as sensory transduction in the ENS (Bornstein 2008). Purinergic signalling via P2 receptors plays an important role in controlling nuclear maintenance of APE1/Ref-1 (Pines et al. 2005). Activation of P2 receptors and intracellular ROS production cause APE1/Ref-1 translocation from the nucleus to cytoplasm (Pines et al. 2005) which was also observed in our study. The role of

purinergic signalling in regulation of APE1/Ref-1 function in various functional types of enteric neurons needs to be further elucidated.

APX3330 treatment alleviated loss of myenteric neurons, improved nerve fibre and GFAP immunoreactivity in the myenteric ganglia when compared to *Winnie* sham-treated mice. APX3330 has been proven to be neuroprotective in sensory neurons after insults caused by ionizing radiation, cheotherapeutic agents and inflammatory stimuli all producing oxidative DNA damage acted upon by APE1/Ref-1 (Fehrenbacher et al. 2017; Jiang et al. 2009; Kelley et al. 2014; Kim et al. 2015; Vasko et al. 2011). These studies showed that enhancement of the DNA repair function of APE1/Ref-1, but not its redox signalling, underlies neuroprotective effects of APX3330 in sensory neurons. This was demonstrated using both a genetic and small molecule approach with APX3330 (Jiang et al. 2009; Kelley et al. 2014; Kelley et al. 2016; Kim et al. 2015). Facilitation of the endonuclease activity of APE1/Ref-1 stimulates base excision repair (BER) of oxidative DNA damage in neurons (Kelley et al. 2014). Our study provides novel findings on the presence of APE1/Ref-1 in enteric neurons and increased level of APE1/Ref-1 in both the mucosa and myenteric ganglia in the colons from *Winnie* mice. These results are consistent with findings in the colon tissues removed from IBD patients with active inflammation (Hofseth et al. 2003) and in the colonic epithelium of an animal model of DSS-induced colitis (Chang et al. 2013). Inflammation caused by pathogenic bacteria, ionising radiation, ROS and toxic agents transiently increase intracellular APE1/Ref-1 in gastric epithelial cells and sensory neurons (O'Hara et al. 2006; Vasko et al. 2011). Our results indicate that APX3330 treatment alleviates the APE1/Ref-1 redox activity in *Winnie* mice after the two-week treatment regimen. As APX3330 regulates the inflammatory response, loss of enteric neurons and

immunoreactivity of GFAP in our model of IBD, we tested if these effects result from dysregulation of ROS production in the myenteric plexus.

In the GI tract, the main sources of ROS are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes (NOX and dual oxidase (DUOX)), NOS and the mitochondrial electron transport chain (mETC). NOX and DUOX produce ROS not as a by-product, but as a primary function. GI tissues express multiple NOX isoforms, including NOX1, NOX2, NOX4 and DUOX2 within different cell types including macrophages, lymphocytes and neutrophils, epithelial cells, fibroblasts and glandular cells throughout the GI tract (Lam et al., 2015). Recent study demonstrated that enteric glial cells increase expression of NOX-2 in response to inflammatory stimuli (Kimono et al. 2019). NOX expression in enteric neurons of the GI tract has not been investigated. It is known that mitochondria can regulate, activate, differentiate and aid survival of immune cells (Angajala et al. 2018). Deregulation of the mitochondrial electron transport chain (mETC), with increased mitochondrial ROS (mtROS) levels, was observed in IBD patients (Dashdorj et al. 2013). Oxidants produced by GI cells provide an antibacterial defence (Wink et al. 2011). Uncontrolled and persistent overproduction of ROS due to up-regulation of oxidases or altered mitochondrial function and/or inadequate removal of ROS by antioxidant systems causes tissue injury and exacerbates inflammation eventually leading to other chronic complications, such as fibrosis, neoplasia and extra-intestinal symptoms (Rezaie et al.2007; Aviello and Knaus, 2017). In addition, it has been shown that the production of ROS induces DNA damage leading to neuronal loss (Harrison et al. 2005). In our study, oxidative stress in myenteric neurons and surrounding cells was shown by the increased mitochondrial superoxide production in *Winnie* mice. Myenteric neurons in *Winnie* mice exhibit increased mitochondrial superoxide and oxidative stress-induced

DNA damage, as seen in multiple studies that describe increase in oxidative DNA damage in the colonic mucosa of IBD patients (D'Incà et al. 2006; Dincer et al. 2007; Lih-Brody et al. 1996) and chemically-induced colitis (Chang et al. 2013). Our results demonstrate that APX3330 reduced oxidative stress via its redox inhibition of APE1/Ref-1 and oxidative DNA damage in chronic colitis. On the other hand, suppressing APE1/Ref-1's redox activity with APX3330 enhances APE1/Ref-1's endonuclease repair activity via the BER pathway which takes place in both the nuclei and mitochondria. APE1/Ref-1's endonuclease activity is responsible for repairing DNA damage induced by oxidative stress, alkylating agents, and ionising radiation (Krokan and Bjørås 2013; Shah et al. 2017). Further studies required to elucidate APE1/Ref-1's endonuclease repair activity in enteric neurons after APX3330 treatment.

DNA damage can induce translocation of HMGB1 from the nucleus to the cytoplasm (Park et al. 2004; Tang et al. 2016; Tsung et al. 2007). Significantly higher numbers of cells in the myenteric plexus of *Winnie* mice displayed translocation of HMGB1 from the nucleus to the cytoplasm indicating cellular stress. HMGB1 has various functions which depend on its location outside or inside the cell. Acetylated cytosolic HMGB1 released from cells into extracellular space acts as a proinflammatory mediator interacting with immune cells and stimulating production of cytokines and chemokines (Janko et al. 2014; Paudel et al. 2018) leading to neuroinflammation, hyperexcitability and neuronal death (Kaneko et al. 2017; Okuma et al. 2012). APE1/Ref-1 regulates the release of cytosolic HMGB1 and the inflammatory signalling to extracellular HMGB1 (Yuk et al. 2009). After APX3330 treatment HMGB1 was retained in the nuclei of myenteric neurons, thereby reducing release of HMGB1 and presumably increasing survival of colonic myenteric neurons in *Winnie* mice. This is consistent with findings

that nuclear HMGB1 directly interacts with and enhances the endonuclease activity of APE1/Ref-1 (Balliano et al. 2017) acting as a cofactor modulating BER capacity in cells (Prasad et al. 2007).

Conclusion

This study is the first to provide evidence for therapeutic effects of APX3330 in alleviating inflammatory responses, disease severity, GI dysfunction and enteric neuropathy. Inhibition of mitochondrial superoxide production, APE1/Ref-1 redox signalling, oxidative DNA damage and translocation of HMGB1 are some of the mechanisms involved in neuroprotective effects of APX3330 in enteric neurons (**Figure 9**). Further studies are needed to elucidate more detailed molecular mechanisms underlying the role of APE1/Ref-1, its redox signalling and DNA damage/repair functions in enteric neurons as well as long term effects of APX3330 treatment. However, results of the recent clinical trial in oncology patients with advanced solid tumors demonstrated that long term treatment with APX3330 did not show any safety concerns (Chu et al. 2019; Shahda et al. 2019). Thus, specific inhibition of the redox signalling function of the APE1/Ref-1 molecule is a novel strategy that might lead to a possible application of APX3330 for the treatment of IBD with good tolerability.

References

Abdo, Hind, et al. (2009), 'Enteric glial cells protect neurons from oxidative stress in part via reduced glutathione', *The FASEB Journal,* 24 (4), 1082-94.

Abegunde, Ayokunle T., Muhammad, Bashir H., and Ali, Tauseef (2016), 'Preventive health measures in inflammatory bowel disease', *World Journal of Gastroenterology,* 22 (34), 7625-44.

Achitei, D., et al. (2013), 'Different Profile of Peripheral Antioxidant Enzymes and Lipid Peroxidation in Active and Non-active Inflammatory Bowel Disease Patients', *Digestive Diseases and Sciences,* 58 (5), 1244-49.

Angajala, Anusha, et al. (2018), 'Diverse Roles of Mitochondria in Immune Responses: Novel Insights Into Immuno-Metabolism', *Frontiers in immunology,* 9, 1605-05.

Aviello G, Knaus UG (2017), 'ROS in gastrointestinal inflammation: Rescue Or Sabotage?', *Br J Pharmacol*. 174(12), 1704-1718.

Bagyánszki, Mária and Bódi, Nikolett (2012), 'Diabetes-related alterations in the enteric nervous system and its microenvironment', *World Journal of Diabetes,* 3 (5), 80-93.

Balliano, Angela, et al. (2017), 'HMGB1 Stimulates Activity of Polymerase β on Nucleosome Substrates', *Biochemistry,* 56 (4), 647-56.

Bernardini, Nunzia, et al. (2012), 'Immunohistochemical analysis of myenteric ganglia and interstitial cells of Cajal in ulcerative colitis', *Journal of cellular and molecular medicine,* 16 (2), 318-27.

Bornstein, J. C. (2008), 'Purinergic mechanisms in the control of gastrointestinal motility', *Purinergic Signalling,* 4 (3), 197-212.

Boyer, L., et al. (2005), 'Myenteric plexus injury and apoptosis in experimental colitis', *Auton Neurosci,* 117 (1), 41-53.

Brown, Isola A. M. and Gulbransen, Brian D. (2017), 'The antioxidant glutathione protects against enteric neuron death in situ, but its depletion is protective during colitis', *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 314 (1), G39-G52.

Brown, Isola A. M., et al. (2016), 'Enteric glia mediate neuron death in colitis through purinergic pathways that require connexin-43 and nitric oxide', *Cellular and molecular gastroenterology and hepatology,* 2 (1), 77-91.

Burisch, Johan and Munkholm, Pia (2015), 'The epidemiology of inflammatory bowel disease', *Scandinavian Journal of Gastroenterology,* 50 (8), 942-51.

Chang, Chen-Wang, et al. (2015), 'Intestinal Stricture in Crohn's Disease', *Intestinal Research,* 13 (1), 19-26.

Chang, In-Youb, et al. (2013), 'Apurinic/apyrimidinic endonuclease 1, the sensitive marker for DNA deterioration in dextran sulfate sodium-induced acute colitis', *Redox Report,* 18 (5), 165-73.

Chassaing, Benoit, et al. (2012), 'Fecal Lipocalin 2, a Sensitive and Broadly Dynamic Non-Invasive Biomarker for Intestinal Inflammation', *PLOS ONE,* 7 (9), e44328.

Chu, Lincy, et al. (2019), 'CTC enumeration and characterization as a pharmacodynamic marker in the phase I clinical study of APX3330, an APE1/Ref-1 inhibitor, in patients with advanced solid tumors', *Journal of Clinical Oncology,* 37 (15_suppl), e14531-e31.

Curtis, Michael J., et al. (2015), 'Experimental design and analysis and their reporting: new guidance for publication in BJP', *British Journal of Pharmacology,* 172 (14), 3461-71.

Dashdorj A, Jyothi KR, Lim S, Jo A, Nguyen MN, Ha J, *et al.* (2013), 'Mitochondria-targeted antioxidant MitoQ ameliorates experimental mouse colitis by suppressing NLRP3 inflammasome-mediated inflammatory cytokines', *BMC Med.* 11: 178.

D'Incà, Renata, et al. (2006), 'Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia', *Inflammatory Bowel Diseases,* 10 (1), 23-27.

de Jonge, Wouter J. (2013), 'The Gut's Little Brain in Control of Intestinal Immunity', *ISRN Gastroenterology,* 2013, 630159.

Dincer, Yıldız, et al. (2007), 'Oxidative DNA Damage and Antioxidant Activity in Patients with Inflammatory Bowel Disease', *Digestive Diseases and Sciences,* 52 (7), 1636-41.

Erben U, Loddenkemper C, Doerfel K, et al. (2014), 'A guide to histomorphological evaluation of intestinal inflammation in mouse models', *Int J Clin Exp Pathol*. 7(8), 4557-4576.
Eri, R. D., et al. (2011), 'An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity', *Mucosal Immunology,* 4, 354-364.
Fehrenbacher, Jill C., et al. (2017), 'DNA damage mediates changes in neuronal sensitivity induced by the inflammatory mediators, MCP-1 and LPS, and can be reversed by enhancing the DNA repair function of APE1', *Neuroscience,* 366, 23-35.
Fishel, Melissa L., et al. (2008), 'Knockdown of the DNA repair and redox signaling protein Ape1/Ref-1 blocks ovarian cancer cell and tumor growth', *DNA repair,* 7 (2), 177-86.
Fishel, Melissa L., et al. (2019), 'Anti-tumor activity and mechanistic characterization of APE1/Ref-1 inhibitors in bladder cancer', *Molecular Cancer Therapeutics*, molcanther.1166.2018.
Fuss IJ, Boirivant M, Lacy B, Strober W. (2002), 'The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis', *J Immunol*. 168(2), 900-908.
Ghadir, Mohammad Reza, et al. (2016), 'Nonadherence to Medication in Inflammatory Bowel Disease: Rate and Reasons', *Middle East Journal of Digestive Diseases,* 8 (2), 116-21.
Gionchetti, Paolo, et al. (2016), 'Use of corticosteroids and immunosuppressive drugs in inflammatory bowel disease: Clinical practice guidelines of the Italian Group for the Study of Inflammatory Bowel Disease', *Digestive and Liver Disease,* 49 (6), 604-17.
Haase, A. M., et al. (2016), 'Regional gastrointestinal transit times in severe ulcerative colitis', *Neurogastroenterology & Motility,* 28 (2), 217-24.
Hanauer, Stephen B. (2016), 'Oral or Topical 5-ASA in Ulcerative Colitis', *Digestive Diseases (Basel, Switzerland),* 34 (1-2), 122-24.
Harris, Raymond C. (2006), 'COX-2 and the Kidney', *Journal of Cardiovascular Pharmacology,* 47.
Harrison, Jason F., et al. (2005), 'Oxidative stress-induced apoptosis in neurons correlates with mitochondrial DNA base excision repair pathway imbalance', *Nucleic acids research,* 33 (14), 4660-71.
Heazlewood, Chad K., et al. (2008), 'Aberrant Mucin Assembly in Mice Causes Endoplasmic Reticulum Stress and Spontaneous Inflammation Resembling Ulcerative Colitis', *PLOS Medicine,* 5 (3), e54.
Hofseth, Lorne J., et al. (2003), 'The adaptive imbalance in base excision–repair enzymes generates microsatellite instability in chronic inflammation', *Journal of Clinical Investigation,* 112 (12), 1887-94.
Ishihara, Tomoaki, et al. (2009), 'Therapeutic Effect of Lecithinized Superoxide Dismutase against Colitis', *Journal of Pharmacology and Experimental Therapeutics,* 328 (1), 152.
James, Sally L., et al. (2018), 'Characterization of ulcerative colitis-associated constipation syndrome (proximal constipation)', *JGH open : an open access journal of gastroenterology and hepatology,* 2 (5), 217-22.
Janko, Christina, et al. (2014), 'Redox modulation of HMGB1-related signaling', *Antioxidants & redox signaling,* 20 (7), 1075-85.
Jiang, Yanlin, et al. (2009), 'Role of APE1 in differentiated neuroblastoma SH-SY5Y cells in response to oxidative stress: use of APE1 small molecule inhibitors to delineate APE1 functions', *DNA repair,* 8 (11), 1273-82.
Jiminez JA, Uwiera TC, Douglas Inglis G, Uwiera RR. (2015), 'Animal models to study acute and chronic intestinal inflammation in mammals'. *Gut Pathog*. 7, 29.
Kaneko, Y., et al. (2017), 'Extracellular HMGB1 Modulates Glutamate Metabolism Associated with Kainic Acid-Induced Epilepsy-Like Hyperactivity in Primary Rat Neural Cells', *Cellular Physiology and Biochemistry,* 41 (3), 947-59.
Kelley, Mark R. and Fehrenbacher, Jill C. (2017), 'Challenges and opportunities identifying therapeutic targets for chemotherapy-induced peripheral neuropathy resulting from oxidative DNA damage', *Neural regeneration research,* 12 (1), 72-74.

Kelley, Mark R., Georgiadis, Millie M., and Fishel, Melissa L. (2012), 'APE1/Ref-1 Role in Redox Signaling: Translational Applications of Targeting the Redox Function of the DNA Repair/Redox Protein APE1/Ref-1', *Current Molecular Pharmacology,* 5 (1), 36-53.
Kelley, Mark R., et al. (2014), 'Role of the DNA Base Excision Repair Protein, APE1 in Cisplatin, Oxaliplatin, or Carboplatin Induced Sensory Neuropathy', *PLoS ONE,* 9 (9), e106485.
Kelley, Mark R., et al. (2016), 'Identification and Characterization of New Chemical Entities Targeting Apurinic/Apyrimidinic Endonuclease 1 for the Prevention of Chemotherapy-Induced Peripheral Neuropathy', *The Journal of pharmacology and experimental therapeutics,* 359 (2), 300-09.
Keshavarzian, A., et al. (2003), 'Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease', *Gut,* 52 (5), 720-28.
Kim, Eun Ran and Chang, Dong Kyung (2014), 'Colorectal cancer in inflammatory bowel disease: The risk, pathogenesis, prevention and diagnosis', *World Journal of Gastroenterology : WJG,* 20 (29), 9872-81.
Kim, Hyun-Suk, et al. (2015), 'APE1, the DNA base excision repair protein, regulates the removal of platinum adducts in sensory neuronal cultures by NER', *Mutation research,* 779, 96-104.
Kimono D, Sarkar S, Albadrani M, et al. (2019), 'Dysbiosis-associated enteric glial cell immune-activation and redox imbalance modulate tight junction protein expression in Gulf War illness pathology', *Front Physiol*. 10, 1229.
Knowles, Charles H., et al. (2013), 'New perspectives in the diagnosis and management of enteric neuropathies', *Nature Reviews Gastroenterology &Amp; Hepatology,* 10, 206.
Krokan, Hans E. and Bjørås, Magnar (2013), 'Base Excision Repair', *Cold Spring Harbor Perspectives in Biology,* 5 (4), a012583.
Lakhan, Shaheen E. and Kirchgessner, Annette (2010), 'Neuroinflammation in inflammatory bowel disease', *Journal of Neuroinflammation,* 7 (1), 37.
Lam G, Apostolopoulos V, Zulli A, and Nurgali K (2015), 'NADPH oxidases and inflammatory bowel disease', *Curr Med Chem*. 22(17), 2100-2109.
Lih-Brody, Lisa, et al. (1996), 'Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease', *Digestive Diseases and Sciences,* 41 (10), 2078-86.
Linden, D. R., et al. (2005), 'Indiscriminate loss of myenteric neurones in the TNBS-inflamed guinea-pig distal colon', *Neurogastroenterol Motil,* 17 (5), 751-60.
Liu, Hao, et al. (2017), 'Redox Imbalance in the Development of Colorectal Cancer', *Journal of Cancer,* 8 (9), 1586-97.
Lomax, A. E., Fernández, E., and Sharkey, K. A. (2005), 'Plasticity of the enteric nervous system during intestinal inflammation', *Neurogastroenterology & Motility,* 17 (1), 4-15.
Luo, Meihua, et al. (2008), 'Role of the Multifunctional DNA Repair and Redox Signaling Protein Ape1/Ref-1 in Cancer and Endothelial Cells: Small-Molecule Inhibition of the Redox Function of Ape1', *Antioxidants & Redox Signaling,* 10 (11), 1853-67.
Mawe, Gary M. (2015), 'Colitis-induced neuroplasticity disrupts motility in the inflamed and post-inflamed colon', *The Journal of Clinical Investigation,* 125 (3), 949-55.
McQuade, Rachel M., et al. (2016), 'Role of oxidative stress in oxaliplatin-induced enteric neuropathy and colonic dysmotility in mice', *British Journal of Pharmacology,* 173 (24), 3502-21.
Moynes, Derek M., et al. (2013), 'Effects of Inflammation on the Innervation of the Colon', *Toxicologic Pathology,* 42 (1), 111-17.
Moynes, Derek M.; Lucas, Graydon H.; Beyak, Michael J.; and Lomax, Alan E. (2014), 'Effects of Inflammation on the Innervation of the Colon'.
Neurath, M. F. (2017), 'Current and emerging therapeutic targets for IBD', *Nat Rev Gastroenterol Hepatol,* 14 (5), 269-78.
Nitzan, Orna, et al. (2016), 'Role of antibiotics for treatment of inflammatory bowel disease', *World Journal of Gastroenterology,* 22 (3), 1078-87.

Nurgali, Kulmira, et al. (2007), 'Phenotypic changes of morphologically identified guinea-pig myenteric neurons following intestinal inflammation', *The Journal of Physiology,* 583 (Pt 2), 593-609.
Nurgali, Kulmira, et al. (2011), 'Morphological and functional changes in guinea-pig neurons projecting to the ileal mucosa at early stages after inflammatory damage', *The Journal of Physiology,* 589 (Pt 2), 325-39.
O'Hara, Ann M., et al. (2006), 'Interleukin-8 Induction by Helicobacter pylori; in Gastric Epithelial Cells is Dependent on Apurinic/Apyrimidinic Endonuclease-1/Redox Factor-1', *The Journal of Immunology,* 177 (11), 7990.
Okuma, Yu, et al. (2012), 'Anti–high mobility group box-1 antibody therapy for traumatic brain injury', *Annals of Neurology,* 72 (3), 373-84.
Park, J. S., et al. (2004), 'Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein', *J Biol Chem,* 279 (9), 7370-7.
Paudel, Yam Nath, et al. (2018), 'HMGB1: A Common Biomarker and Potential Target for TBI, Neuroinflammation, Epilepsy, and Cognitive Dysfunction', *Frontiers in neuroscience,* 12, 628-28.
Pines, Alex, et al. (2005), 'Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP', *Nucleic acids research,* 33 (14), 4379-94.
Pochard, Camille, et al. (2018), 'The multiple faces of inflammatory enteric glial cells: is Crohn's disease a gliopathy?', *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 315 (1), G1-G11.
Prasad, Rajendra, et al. (2007), 'HMGB1 is a cofactor in mammalian base excision repair', *Molecular cell,* 27 (5), 829-41.
Prattis S, Jurjus A (2015), 'Spontaneous and transgenic rodent models of inflammatory bowel disease'. *Lab Anim Res,* 31(2), 47-68.Rahman, Ahmed A., et al. (2015), 'Alterations in the distal colon innervation in Winnie mouse model of spontaneous chronic colitis', *Cell and Tissue Research,* 362 (3), 497-512.
Rahman, Ahmed A., et al. (2016), 'Rectal prolapse in Winnie mice with spontaneous chronic colitis: changes in intrinsic and extrinsic innervation of the rectum', *Cell And Tissue Research,* 366 (2), 285-99.
Rao, S. S., et al. (1987), 'Studies on the mechanism of bowel disturbance in ulcerative colitis', *Gastroenterology,* 93 (5), 934-40.
Rezaie A, Parker RD, Abdollahi M (2007), 'Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause?' *Dig Dis Sci* 52, 2015–2021.
Roberts, Jane A., et al. (2013), 'Oxidative stress disrupts purinergic neuromuscular transmission in the inflamed colon', *The Journal of physiology,* 591 (15), 3725-37.
Roberts, Rachael R., et al. (2007), 'Development of colonic motility in the neonatal mouse-studies using spatiotemporal maps', *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 292 (3), G930-G38.
Roberts, Rachael R., et al. (2008), 'Disturbances of colonic motility in mouse models of Hirschsprung's disease', *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 294 (4), G996-G1008.
Robinson, Ainsley M., et al. (2016), 'Fecal Microbiota and Metabolome in a Mouse Model of Spontaneous Chronic Colitis: Relevance to Human Inflammatory Bowel Disease', *Inflammatory Bowel Diseases,* 22 (12), 2767-87.
Robinson, Ainsley M., et al. (2017), 'Alterations of colonic function in the Winnie mouse model of spontaneous chronic colitis', *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 312 (1), G85-G102.
Shah, Fenil, et al. (2017), 'Exploiting the Ref-1-APE1 node in cancer signaling and other diseases: from bench to clinic', *npj Precision Oncology,* 1 (1), 19.

Shahda, Safi, et al. (2019), 'A phase I study of the APE1 protein inhibitor APX3330 in patients with advanced solid tumors', *Journal of Clinical Oncology,* 37 (15_suppl), 3097-97.
Swaminathan, Mathusi, et al. (2016), 'Video Imaging and Spatiotemporal Maps to Analyze Gastrointestinal Motility in Mice', *JoVE,* (108), e53828.
Tang, Yiting, et al. (2016), 'Regulation of Posttranslational Modifications of HMGB1 During Immune Responses', *Antioxidants & redox signaling,* 24 (12), 620-34.
Tell, Gianluca, et al. (2009), 'The Many Functions of APE1/Ref-1: Not Only a DNA Repair Enzyme', *Antioxidants & Redox Signaling,* 11 (3), 601-19.
Tsung, Allan, et al. (2007), 'HMGB1 release induced by liver ischemia involves Toll-like receptor 4–dependent reactive oxygen species production and calcium-mediated signaling', *The Journal of Experimental Medicine,* 204 (12), 2913.
Uesaka, Toshihiro, et al. (2016), 'Development of the intrinsic and extrinsic innervation of the gut', *Developmental Biology,* 417 (2), 158-67.
Vascotto, Carlo, et al. (2009), 'APE1/Ref-1 Interacts with NPM1 within Nucleoli and Plays a Role in the rRNA Quality Control Process', *Molecular and Cellular Biology,* 29 (7), 1834-54.
Vasko, Michael R., et al. (2011), 'The repair function of the multifunctional DNA repair/redox protein APE1 is neuroprotective after ionizing radiation', *DNA repair,* 10 (9), 942-52.
Wink, David A., et al. (2011), 'Nitric oxide and redox mechanisms in the immune response', *Journal of leukocyte biology,* 89 (6), 873-91.
Yang, Xiao, et al. (2018), 'The regulatory role of APE1 in epithelial-to-mesenchymal transition and in determining EGFR-TKI responsiveness in non-small-cell lung cancer', *Cancer Medicine,* 7 (9), 4406-19.
Yuk, J. M., et al. (2009), 'A dual regulatory role of apurinic/apyrimidinic endonuclease 1/redox factor-1 in HMGB1-induced inflammatory responses', *Antioxid Redox Signal,* 11 (3), 575-88.

Table 1. Primary antisera utilised for Immunohistochemistry

Primary Antisera	Target	Host Species	Titration	Specimen	Supplier
Anti-CD45 (ab10558)	Leukocyte Infiltration	Mouse	1:500	Cross-sections	Abcam
Anti-β-tubulin III (ab18207)	Nerve fibers	Rabbit	1:1000	Cross-sections	Abcam
Anti-MAP2 (ab5392)	Neurons	Chicken	1:5000	LMMP	Abcam
Anti-GFAP (ab53554)	Glial Cells	Goat	1:500	LMMP	Abcam
Anti-APE1/Ref-1	APE1 protein	Mouse	1:1000	Cross Sections LMMP	Produced by Mark Kelley's lab
Anti-HMGB1 (ab227168)	Damage-associated molecular protein	Mouse	1:500	LMMP	Abcam
Anti-8-OHdG (ab48508)	DNA Damage	Mouse	1:200	LMMP	Abcam

Figure Legends

Figure 1. The effects of APX3330 treatment on clinical symptoms in *Winnie* mice

A. Images of a C57BL/6 control mouse and a *Winnie* sham-treated mouse with severe intestinal inflammation and rectal prolapse with blood vessel proliferation and oedema before and after 14 days of APX3330 treatment. **B, B'.** Colons from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice were excised and their length (mm) measured at day 15 (*n*=5/group). **C.** Faecal water content (the difference between the wet and dry weight) was calculated as a percent of the wet weight of fresh faecal pellets measured at day 14 of treatment (n=7/group). **D, D'.** Gross morphology of the distal colon was assessed by H&E staining and histological scoring was quantified in distal colon cross sections from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). **E.** Body weight loss or gain in C57BL/6 control (*n*=10), *Winnie* sham-treated (*n*=10) and *Winnie* APX3330-treated (*n*=8) mice over the 14 day period. Data expressed as mean ± SEM, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 compared to C57BL/6 control mice; ^*P*<0.05, ^^*P*<0.01 compared to *Winnie* sham-treated mice.

Figure 2. The anti-inflammatory effects of APX3330 treatment in the colon of *Winnie* mice

A, A'. CD45+ leukocytes were labelled using a leukocyte marker anti-CD45+ (green) antibody in the colon cross sections. Mucosal epithelial cells are labelled with nuclei marker DAPI (blue) (**A**: scale bar = 50µm, x40 magnification; **A'**: scale bar = 30µm, x60 magnification). **B.** Density of CD45+-IR cells normalised to the width of the colon

sections in C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). **C.** Lipocalin-2 (Lcn-2) levels in faecal pellets were quantified from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). Data expressed as mean ± SEM, ***P*<0.01, ****P*<0.001 compared to C57BL/6 control mice; ^^*P*<0.01 compared to *Winnie* sham-treated mice.

Figure 3. The effects of APX3330 treatment on gastrointestinal transit

A-A''. Radiographic images captured movement of the contrast agent, barium sulfate, from the stomach to the expulsion of the first pellet (shown in red rectangles) in C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=7/group). **B.** Overall gastrointestinal transit time extended in *Winnie* sham-treated mice and subsided in *Winnie* APX3330-treated mice. **C.** Gastro-caecal transit time (GCTT) demonstrated no significant changes between C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice. **D.** A prolonged caecum retention time was observed in *Winnie* sham-treated mice and was attenuated in *Winnie* APX3330-treated mice. **E.** Colonic transit time (CTT) was accelerated in *Winnie* sham-treated mice and was comparable to C57BL/6 control mice improved in *Winnie* APX3330-treated animals. Data expressed as mean ± SEM, ***P*<0.01 compared to C57BL/6 control mice; ^*P*<0.05, ^^*P*<0.01 compared to *Winnie* sham-treated mice.

Figure 4. The effects of APX3330 treatment on parameters of colonic motility

A. Video recordings from *ex vivo* whole colon samples were transposed into spatiotemporal maps. Contractions were distinguished as red and relaxation as blue from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice

(*n*=6/group). **B.** Average length of contractions in proportion to the whole colon length **C.** Average length of colonic migrating motor complexes (CMMCs, white arrows in **A**) defined as contractions initiated from the oral end that propagated toward the anal end for >50% of the colon length. **D.** Speed of propagation of CMMCs. **E.** Frequency of CMMCs quantified as the number of contractions per 10 minutes. Data expressed as mean ± SEM, **P*<0.05, *****P*<0.0001 compared to C57BL/6 control mice; ^*P*<0.05, ^^^^*P*<0.0001 compared to *Winnie* sham-treated mice.

Figure 5. APX3330 treatment promoted myenteric neuronal survival and ameliorated nerve fiber and glial cell density in the myenteric plexus in *Winnie* mice

A. Neuronal microtubule proteins were labelled by immunofluorescence using β-tubulin (III) (purple) antibody to identify nerve fibers innervating the colon in cross sections (scale bar = 100µm, x20 magnification). Anti-MAP2-IR antibody staining (blue) for myenteric neurons in LMMP preparations (scale bar = 50µm, x40 magnification). Glial cells were labelled with an anti-GFAP antibody (orange) in the myenteric plexus of the colon from C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (scale bar = 50µm, x40 magnification). **B.** Density of β-tubulin (III)-IR nerve fibers normalised to colon thickness in C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). **C.** Quantitative analysis of myenteric neurons per ganglion C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). **D.** Myenteric neurons were quantified per area in LMMP preparations of C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). **E.** Density of GFAP-IR glial cells per ganglia in C57BL/6

control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). Data expressed as mean ± SEM, **P*<0.05, ***P*<0.01, ****P*<0.001, compared to C57BL/6 control mice; ^*P*<0.05, ^^*P*<0.01, ^^^*P*<0.001, compared to *Winnie* sham-treated mice.

Figure 6. Effects of APX3330 treatment on superoxide production and HMGB1 translocation in the myenteric plexus of *Winnie* mice

A. LMMP preparations of the distal colon were labelled with Mitochondrial superoxide marker MitoSOX™ (red) indicative of oxidative stress. Increased MitoSOX™ immunofluorescence was evident in the myenteric ganglia of *Winnie* sham-treated (*n*=5) compared to C57BL/6 control (*n*=5) mice (scale bar=100µm, x40 magnification). This was alleviated in *Winnie* APX3330-treated (*n*=5) mice (scale bar=100µm) **A'.** HMGB1 immunoreactivity (green) colocalised with nuclei marker DAPI (blue) in the myenteric plexus. Translocation of nuclear HMGB1 to cytosol (marked with white arrows) was observed in *Winnie* sham-treated compared to C57BL/6 control (mice and was averted in *Winnie* APX3330-treated mice (*n*=5/group) (scale bar=50µm, x60 magnification). **B.** MitoSOX fluorescence intensity was assessed relative to ganglion area. **C.** The number of cells with translocation of HMBG1 from nucleus to cytoplasm was quantified in the myenteric ganglia. Data expressed as mean ± SEM, **P*<0.05, ***P*<0.01 compared to C57BL/6 control mice; ^*P*<0.05, ^^*P*<0.01 compared to *Winnie* sham-treated mice.

Figure 7. The effects of APX3330 treatment on APE1/Ref-1 expression in myenteric neurons of *Winnie* mice

A. APE1/Ref-1 labelled by immunofluorescence (red) in the colon cross sections. **A'.** APE1/Ref-1 immunofluorescence (green) in the myenteric plexus in wholemount LMMP preparations. **B.** Density of APE1/Ref-1 fluorescence in the colon cross sections relative to the total area of colonic mucosa in C57BL/6, *Winnie* sham-treated, *Winnie* APX3330-treated mice (*n*=5/group) (scale bar=100µm, x20 magnification). **C.** Density of APE1/Ref-1 fluorescence quantified as a percentage relative to the ganglion area in the myenteric plexus in C57BL/6 control, *Winnie* sham-treated, *Winnie* APX3330-treated mice (*n*=5/group) (scale bar=50µm, x40 magnification). Data expressed as mean ± SEM, **P*<0.05, *****P*<0.0001 compared to C57BL/6 control mice; ^^^*P*<0.001 compared to *Winnie* sham-treated mice.

Figure 8. The effects of APX3330 treatment on DNA damage in myenteric neurons of *Winnie* mice

A. DNA damage was observed with an anti-8-OHdG (green) antibody in myenteric neurons immunoreactive for MAP2 (purple) (scale bar=50µm, x40 magnification). **B.** The density of 8-OHdG-IR cells was quantified as a percentage relative to the ganglion area within myenteric plexus in C57BL/6 control, *Winnie* sham-treated and *Winnie* APX3330-treated mice (*n*=5/group). **C.** The number of neurons with colocalisation of 8-OHdG-IR and MAP2-IR relative to the total number of neurons per ganglion area. Data expressed as mean ± SEM, ***P*<0.01 compared to C57BL/6 control; ^^*P*<0.01 compared to *Winnie* sham-treated mice.

Figure 9. Effects of APX3330 treatment on enteric neurons and GI functions.

In the inflamed colon, high levels of colonic leukocyte infiltration and faecal Lcn-2 were alleviated by APX3330 treatment. Chronic intestinal inflammation associates with the

damage to the enteric nervous system embedded within the intestinal wall and controlling gastrointestinal (GI) functions. In enteric neurons, APX3330 treatment APX3330 reduces oxidative stress via inhibition of APE1/Ref-1's redox signalling, mitochondrial superoxide production and oxidative DNA damage. On the other hand, suppressing APE1/Ref-1 redox activity enhances APE1/Ref-1's endonuclease repair activity. DNA damage induces translocation of HMGB1 from the nucleus to the cytoplasm. Acetylated cytosolic HMGB1 released from cells into extracellular space interacts with immune cells and stimulates production of cytokines and chemokines leading to neuroinflammation, hyperexcitability and neuronal death (*dashed line:* not studied in our model). After APX3330 treatment HMGB1 was retained in the nuclei of myenteric neurons. Targeting this pathway with APX3330 ameliorated enteric neuropathy, colonic dysmotility and altered GI transit leading to improved clinical signs associated with chronic colitis (created with BioRender.com).