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RESEARCH PAPER

Hydrogen sulphide protects against NSAID-enteropathy through modulation of bile and the microbiota

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BACKGROUND AND PURPOSE

Hydrogen sulphide is an important mediator of gastrointestinal mucosal defence. The use of non-steroidal anti-inflammatory drugs (NSAIDs) is significantly limited by their toxicity in the gastrointestinal tract. Particularly concerning is the lack of effective preventative or curative treatments for NSAID-induced intestinal damage and bleeding. We evaluated the ability of a hydrogen sulphide donor to protect against NSAID-induced enteropathy.

EXPERIMENTAL APPROACH

Intestinal ulceration and bleeding were induced in Wistar rats by oral administration of naproxen. The effects of suppression of endogenous hydrogen sulphide synthesis or administration of a hydrogen sulphide donor (diallyl disulphide) on naproxen-induced enteropathy was examined. Effects of diallyl disulphide on small intestinal inflammation and intestinal microbiota were also assessed. Bile collected after *in vivo* naproxen and diallyl disulphide administration was evaluated for cytotoxicity *in vitro* using cultured intestinal epithelial cells.

KEY RESULTS

Suppression of endogenous hydrogen sulphide synthesis by β-cyano-L-alanine exacerbated naproxen-induced enteropathy. Diallyl disulphide co-administration dose-dependently reduced the severity of naproxen-induced small intestinal damage, inflammation and bleeding. Diallyl disulphide administration attenuated naproxen-induced increases in the cytotoxicity of bile on cultured enterocytes, and prevented or reversed naproxen-induced changes in the intestinal microbiota.

CONCLUSIONS AND IMPLICATIONS

Hydrogen sulphide protects against NSAID-enteropathy in rats, in part reducing the cytotoxicity of bile and preventing NSAID-induced dysbiosis.

Abbreviations

ATB-346, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester; BCA, β-cyano-L-alanine; BHI, brain heart infusion; CBA, Columbia blood agar; CFU, colony-forming units; DADS, diallyl disulphide; DGGE, denaturing gradient gel electrophoresis; GI, gastrointestinal; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drugs; PPI, proton pump inhibitor; TLR, Toll-like receptor; UPGMA, unweighted-pair-group method with arithmetic mean

Tables of Links

TARGETS
Enzymes
Cystathionine γ-lyase
COX-1

LIGANDS
Naproxen
PGE_2

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 (Alexander *et al.*, 2013).

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs for treating the symptoms of inflammatory conditions, most notably osteoarthritis and rheumatoid arthritis. In such conditions, NSAIDs are taken chronically and have the ability to cause significant ulceration and bleeding in the gastrointestinal (GI) tract. Therapies designed to limit NSAID-induced GI injury have focused almost exclusively on gastroduodenal injury, often ignoring the serious small intestinal damage that can also occur (Wallace, 2013b). This is a concern because it is now clear that NSAID-enteropathy occurs more frequently than gastroduodenal injury (Lanas *et al.*, 2009), and it can also be more dangerous, given that there is a poor correlation of symptoms with the injury, the damage is more difficult to detect and there are no proven effective preventative or curative treatments for NSAID-enteropathy (Lanas *et al.*, 2009; Wallace, 2013a). The most common approach to reduce NSAID-induced gastroduodenal injury is via suppression of gastric acid secretion, usually through co-administration of a proton pump inhibitor (PPI) (Scheiman *et al.*, 2006). However, there is no evidence or rationale to support the notion that suppression of gastric acid secretion would have any benefit in terms of reducing damage or bleeding distal to the ligament of Treitz (i.e. beyond the proximal duodenum). On the contrary, there is emerging evidence that PPIs and histamine H_2 receptor antagonists exacerbate the small intestinal damage and bleeding caused by NSAIDs (Zhao and Encinosa, 2008; Lanas *et al.*, 2009; Wallace *et al.*, 2011; Blackler *et al.*, 2012; Satoh *et al.*, 2012). A recent cross-sectional study by Watanabe *et al.* (2013) highlighted this problem, identifying PPI usage as the greatest independent risk factor for severe ulceration and bleeding in patients with rheumatoid arthritis who were being treated with NSAIDs, with use of histamine H_2 receptor antagonists also being a significant risk factor. Studies in rodents demonstrated that the exacerbation of NSAID-enteropathy by PPIs is due to a significant shift in the intestinal microbiota, with a marked decrease in intestinal colonization by *Bifidobacteria* (Wallace *et al.*, 2011).

The damaging effects of NSAIDs in the upper GI tract are directly related to their ability to suppress mucosal synthesis of PGs (Wallace, 2008). Suppression of PG synthesis renders the mucosa susceptible to damage induced by luminal agents such as acid, digestive enzymes, bacteria, bile, and sometimes by the NSAIDs themselves (Wallace, 2013a). However, it is now clear that hydrogen sulphide (H_2S), an endogenous gaseous mediator, also plays a pivotal role in mucosal defence and in promoting repair of mucosal injury (Wallace *et al.*, 2007b; 2009; Wallace, 2010; Wallace *et al.*, 2012). Inhibition of H_2S synthesis renders the gastric mucosa more susceptible to NSAID-induced ulceration (Wallace *et al.*, 2010), whereas co-administration of H_2S donors reduces the severity of NSAID-induced damage (Fiorucci *et al.*, 2005; Wallace, 2010; Wallace *et al.*, 2014) and promotes healing (Wallace *et al.*, 2007b). The protective actions of H_2S against NSAID-induced gastric injury are at least in part due to inhibitory effects on NSAID-induced leukocyte adherence to the vascular endothelium (Zanardo *et al.*, 2006), which is an early and critical event in the pathogenesis of NSAID-induced gastropathy (Wallace *et al.*, 1990). Moreover, the vasorelaxant effects of H_2S also contribute to mucosal protection by preventing the reduction in gastric blood flow caused by NSAIDs (Fiorucci *et al.*, 2005; Mard *et al.*, 2012).

The observations of protective effects of H_2S in the GI tract have prompted the design of a new class of NSAIDs that release H_2S. These H_2S-releasing NSAIDs have been shown to produce negligible gastroduodenal damage compared with their respective parent drugs in healthy rats, in rats with impaired mucosal defence, and at doses many times greater than those required for anti-inflammatory effects (Wallace *et al.*, 2007a; 2010; Blackler *et al.*, 2012). In addition to sparing the gastric mucosa, H_2S-releasing NSAIDs also cause negligible damage in the small intestine (Wallace *et al.*, 2007a; 2010), even when co-administered with PPIs and/or aspirin, which significantly enhance the intestinal-damaging effects of conventional and COX-2-selective NSAIDs (Wallace *et al.*, 2011; Blackler *et al.*, 2012).

The pathogenesis of NSAID-enteropathy is distinct from that of NSAID-gastropathy (Wallace, 2012). Several studies have suggested critical roles for bile and for enterohepatic circulation of NSAIDs in the pathogenesis of NSAID-enteropathy (Wax *et al.*, 1970; Bjarnason *et al.*, 1993; Seitz and Boelsterli, 1998). There is also a wealth of evidence that the enteric flora contributes to the pathogenesis of NSAID-enteropathy, but it is unclear if the bacteria play a primary role in ulcer formation, or merely exacerbate injury once it has occurred (Uejima *et al.*, 1996; Hagiwara *et al.*, 2004). In the present study, we have examined the possibility that a garlic-derived H_2S donor, diallyl disulphide (DADS) (Benavides *et al.*, 2007), could prevent NSAID-induced enteropathy in a rat model. We also attempted to determine if

effects on enteric flora, bile and/or enterohepatic circulation of NSAIDs might explain any observed beneficial effects of this H_2S donor.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of the Faculty of Health Sciences at McMaster 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 148 animals were used in the experiments described here.

Effects of a H_2S donor on naproxen-induced enteropathy

Rats ($n \geq 6$ per group) were treated orally, twice daily, with naproxen (20 mg·kg^{-1}) or vehicle (DMSO and 1% carboxymethylcellulose; 5:95 ratio) for 4.5 days (nine administrations in total). Three hours after the final administration of drug or vehicle, a blood sample was drawn from the tail vein for measurement of haematocrit (Reuter *et al.*, 1997). The rats were then anaesthetized with sodium pentobarbital and blood was drawn from the aorta for measurement (by ELISA) of whole-blood thromboxane (TX) B_2, synthesis, as an index of systemic COX-1 activity (Wallace *et al.*, 1998). The small intestine was then evaluated for haemorrhagic damage, which involved measuring the area, in mm^2, of all hemorrhagic lesions. The damage areas were summed for each rat to give the 'intestinal damage score' (Wallace *et al.*, 2011). These evaluations were performed without knowledge if the treatments.

Immediately prior to each administration of naproxen or vehicle, rats were treated with DADS (10, 30 or 60 mmol·kg^{-1} p.o.) or an equivalent volume of vehicle (1% carboxymethylcellulose). Damage was assessed and samples were taken, as described earlier. These doses of DADS were selected after completion of a preliminary dose-ranging study.

A series of experiments was performed, using the same protocol as described earlier, in which a H_2S-releasing derivative of naproxen (ATB-346) was administered at a dose of 20 mg kg^{-1} twice daily. The effects of this drug in producing intestinal damage and in altering cytotoxicity of bile (see later) were examined.

Effects of inhibition of H_2S synthesis

Rats ($n = 6$ per group) were treated orally, twice daily, with a lower dose of naproxen (10 mg·kg^{-1}) for 4.5 days. Previous studies have demonstrated that this dose of naproxen significantly reduced inflammation in a rat-adjuvant arthritis model and suppressed systemic and small intestinal COX-1 and COX-2 activity (Blackler *et al.*, 2012), but elicited a low level of damage in the small intestine. The rats were also treated twice daily with an inhibitor of cystathionine γ-lyase (β-cyano-L-alanine (BCA); 50 mg·kg^{-1} i.p.; Kawabata *et al.*, 2007), or with vehicle (PBS) immediately prior to naproxen administration. Three hours after the final dose, the small intestine was evaluated for damage and samples were collected, as described above.

PG synthesis

Three hours after the final dose of naproxen, samples of jejunum and of the corpus region of the stomach were collected for the measurement of PGE_2 synthesis, as described previously (Wallace *et al.*, 2000). Briefly, the samples were excised, weighed, and added to a tube containing 1 mL of sodium phosphate buffer (10 mmol·L^{-1}; pH 7.4). Using scissors, the tissue sample was minced for 30 s then placed in a shaking water bath (37°C) for 20 min. The samples were centrifuged (9000× *g*) for 30 s and the supernatants were collected. The concentrations of PGE_2 in the supernatants were determined by ELISA.

Intestinal inflammation

Intestinal inflammation was assessed in jejunal samples by the measurement of myeloperoxidase (MPO) activity, a quantitative index of granulocyte infiltration (Boughton-Smith *et al.*, 1988), and by histology. Samples of jejunal tissues were collected, fixed and processed by routine techniques for light microscopy (haematoxylin and eosin staining) (Wallace *et al.*, 2009).

Intestinal epithelial cell culture (IEC)

Rat IEC-6 and human intestinal epithelial (HT-29) cells were obtained from American Type Culture Centre (Manassas, VA, USA). IEC-6 cells are a non-transformed, homogenous population of epithelial-like cells that remain in an undifferentiated state, and thus, retain some features consistent with intestinal crypt cells (Quaroni *et al.*, 1979). Cultures were maintained in DMEM containing 5% (v·v^{-1}) FBS, 4 mmol·L^{-1} glutamine, 50 U·mL^{-1} penicillin and 50 μg·mL^{-1} streptomycin (complete medium) at 37°C and 5% (v·v^{-1}) CO_2. Subculture was carried out at confluence and cells between passages 17 and 20 were used for bile cytotoxicity assays. Prior to the assays, cells were seeded at 5×10^4 cells per well in 24-well plates and allowed to grow for 1–2 days post-confluence. Bile cytotoxicity assays were also conducted using HT-29 epithelial cells, which were cultured as previously described (Jobin *et al.*, 1998).

Collection of bile

One hour after the final administration of drug or vehicle, rats were anaesthetized with sodium pentobarbital. A laparotomy was performed and the bile duct was cannulated with a polyethylene cannula (PE-10; Clay Adams, Parsipany, NJ, USA). Bile was collected for 30 min. The bile was stored at –80°C until use in the cytotoxicity assay.

Bile cytotoxicity assay

Bile samples were diluted with Dulbecco's PBS (DPBS) (pH 7.4) immediately prior to incubation with IEC-6 cells. Dilutions (1:3–1:12) that fell within the physiological range of concentrations of bile acids present in the small intestine of rats (Dietschy, 1968) were assessed for their cytotoxic effects. Cells were washed with warm DPBS prior to bile application. Solutions of bile were added to IEC-6 cells for 3 h at 37°C and

5% (v·v^{-1}) CO_2. Following the incubation period, the cells were centrifuged at 250× *g* for 5 min and the supernatants collected for lactate dehydrogenase measurement, using a Cytoscan-LDH Cytotoxicity Assay Kit (G-Biosciences, St. Louis, MO, USA). Additional experiments were performed in a similar manner, but using HT-29 cells.

Biliary naproxen levels

Concentrations of naproxen in bile (using coded samples) were measured by liquid chromatography/mass spectrometry, as described previously (Blackler *et al.*, 2012). These measurements were carried out by Nucro-Technics (Scarborough, ON, Canada).

Intestinal bacterial growth

Samples of jejunum (~200 mg; with the luminal contents preserved) from rats treated with vehicle or naproxen, and co-treated with vehicle or DADS, were collected under sterile conditions and homogenized in PBS. Homogenates were kept on ice until serially diluted and plated onto Columbia blood agar (CBA, with 5% sheep blood) or brain heart infusion (BHI) agar and incubated for 48 h under either aerobic or anaerobic conditions. Plates containing between 20 and 200 colony-forming units (CFU) were analysed to determine bacterial numbers, and the results expressed as CFU per gram of tissue.

DNA extraction and polymerase chain reaction–denaturing gradient gel electrophoresis (DGGE)

Bacterial DNA was extracted from caecal content samples as previously described (Park *et al.*, 2013). DNA concentrations were determined spectrophotometrically using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The hypervariable V3 region of the bacterial 16s ribosomal RNA gene was amplified using PCR with universal bacterial primers (HDA-1 and HDA-2) (Mobixlab, McMaster University Core Facility, Hamilton, ON, Canada). DGGE was performed using a DCode universal mutation system (Bio-Rad Laboratories, Mississauga, ON, Canada). Electrophoresis was conducted at 130 V at 60°C for 4.5 h. Gels were stained with SYBR Green (Molecular Probes, Eugene, OR, USA) and viewed by UV transillumination. A scanned image of an electrophoretic gel was used to measure the staining intensity of the fragments using Quantity One software (version 4-2; Bio-Rad Laboratories). The intensity of fragments is expressed as a proportion (%) of the sum of all fragments in the same lane of the gel. Similarities among bacterial profiles were determined using the Dice coefficient, and the Ward and majority unweighted-pair-group method with arithmetic mean (UPGMA) algorithms. Construction of majority UPGMA trees was based on a resampling strategy of 200 permutations. UPGMA trees are displayed using a multidimensional scaling, which positions the entry nodes so that they occupy the best possible distance to each other to reflect the distances in the similarity/distance matrix.

Data analysis

Results are shown as means ± SEM. The data presented in this manuscript were analyzed by one-way ANOVA followed by Dunnett's or Bonferroni *post hoc* tests, with the exception of the data presented in Figure 1, which were analysed using a Student's t-test.

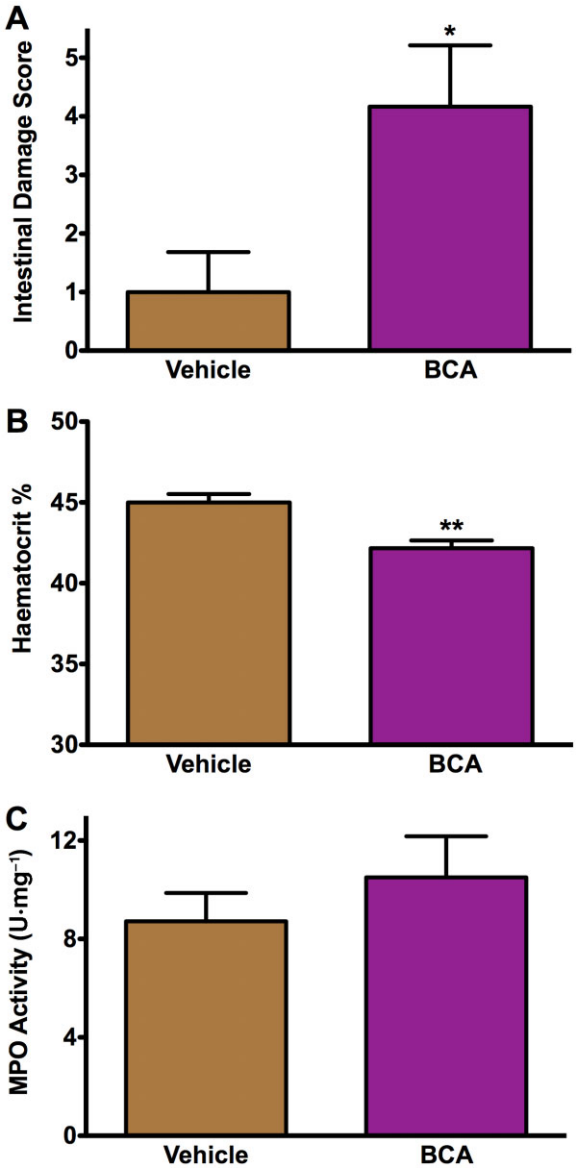

Figure 1

Inhibition of H_2S synthesis by cystathionine γ-lyase. BCA exacerbated naproxen-induced intestinal damage and bleeding. Panel A: administration of naproxen (10 mg·kg^{-1}) twice daily over 4.5 days resulted in marginal intestinal damage. Co-treatment with BCA significantly worsened naproxen-induced intestinal erosions. Panel B: rats co-treated with BCA and naproxen had significantly reduced haematocrit compared with rats treated with vehicle and naproxen. Panel C: treatment with BCA twice a day did not significantly change intestinal MPO activity. Results are shown as mean ± SEM ($n \geq 6$ per group). *$P < 0.05$, **$P < 0.01$, significantly different from vehicle; unpaired, two-tailed Student's *t*-test.

Materials

Naproxen sodium and DADS were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-cyano-L-alanine (BCA), and

the PGE_2 and TXB_2 ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI, USA). Columbia and BHI agar media plates were purchased from Becton-Dickinson (Mississauga, ON, CA). DMEM, FBS, penicillin and streptomycin were purchased from Life Technologies, Inc. (Burlington, ON, CA).

Results

Suppression of H_2S synthesis exacerbated naproxen-induced ulceration and bleeding

Administration of naproxen at 10 mg·kg^{-1} resulted in a low level of intestinal damage (Figure 1A). However, co-administration of an inhibitor of H_2S synthesis, BCA, resulted in a significant increase ($P < 0.05$) in the severity of naproxen-induced intestinal damage (Figure 1A) and a small, but significant decrease in haematocrit (Figure 1B). Jejunal granulocyte infiltration (MPO activity) in naproxen-treated rats was not affected by BCA co-treatment (Figure 1C).

DADS dose-dependently reduced enteropathy and bleeding

Administration of naproxen (20 mg·kg^{-1}) twice daily for 4.5 days resulted in severe intestinal ulceration and bleeding (Figure 2A). Rats treated with naproxen exhibited significant weight loss (~10%), and blood was evident in the intestinal lumen. Co-administration of DADS with naproxen resulted in a dose-dependent reduction in the extent of intestinal damage (Figure 2A). Naproxen treatment resulted in a 35% decrease in haematocrit ($P < 0.001$), whereas rats treated with DADS at doses of 30 or 60 mmol·kg^{-1} did not exhibit a significant change in haematocrit (Figure 2B). Co-administration of DADS (30 or 60 mmol·kg^{-1}) also significantly reduced weight loss in naproxen-treated rats ($P < 0.01$; Figure 2C).

Effects of DADS on suppression of COX activity

Naproxen administration profoundly suppressed systemic COX-1 activity (whole-blood TX synthesis; by 99%) (Figure 3A) and intestinal PGE_2 synthesis (by 64%) after twice daily dosing for 4.5 days. A similar degree of suppression of TX synthesis was observed in rats co-treated with DADS and naproxen. However, naproxen-treated rats that were co-treated with DADS at 30 or 60 mmol·kg^{-1} exhibited an increase (~1.8-fold) in intestinal PGE_2 synthesis, compared with naproxen-treated rats ($P < 0.05$) (Figure 3B). Similar effects were observed in gastric tissue (Supporting Information Fig. S1). Naproxen significantly inhibited gastric PGE_2 synthesis (by 81%), compared with vehicle-treated rats. However, co-treatment with DADS at 30 or 60 mmol·kg^{-1} resulted in significantly elevated gastric PGE_2 synthesis, compared with that in naproxen-treated rats ($P < 0.05$).

DADS dose-dependently reduced intestinal inflammation

Naproxen administration resulted in a significant (approximately fourfold) increase in jejunal granulocyte infiltration

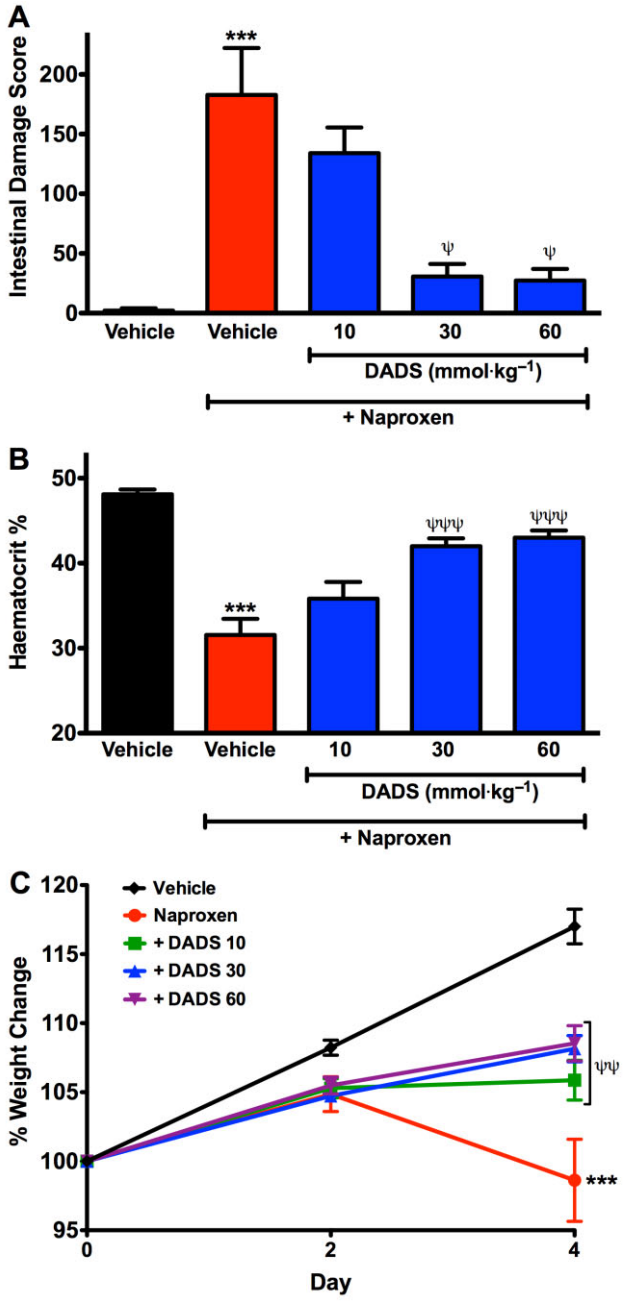

Figure 2

Dose-dependent reduction of naproxen-induced intestinal ulceration by DADS. Rats were co-treated, twice daily, with naproxen (20 mg·kg^{-1}) and vehicle or DADS (10, 30, or 60 mmol·kg^{-1}) for 4.5 days. Panel A: naproxen-induced small intestinal damage was significantly reduced by co-treatment with DADS at doses of 30 and 60 mmol·kg^{-1}kg^{-1}. Panel B: naproxen administration caused significant bleeding compared with vehicle treatment, but co-treatment with DADS at doses of 30 or 60 mmol·kg^{-1} significantly reduced the naproxen-induced decrease in haematocrit. Panel C: weight loss caused by naproxen administration was significantly reduced by co-treatment with DADS at doses of 30 or 60 mmol·kg^{-1}kg^{-1}. Results are shown as mean ± SEM ($n \geq 6$ per group). ***$P < 0.001$, significantly different from vehicle; $^{\psi}P < 0.05$, $^{\psi\psi}P < 0.01$, ($^{\psi\psi\psi}P < 0.001$, significantly different from naproxen alone; one-way ANOVA followed by Dunnett's and Bonferroni *post hoc* tests.

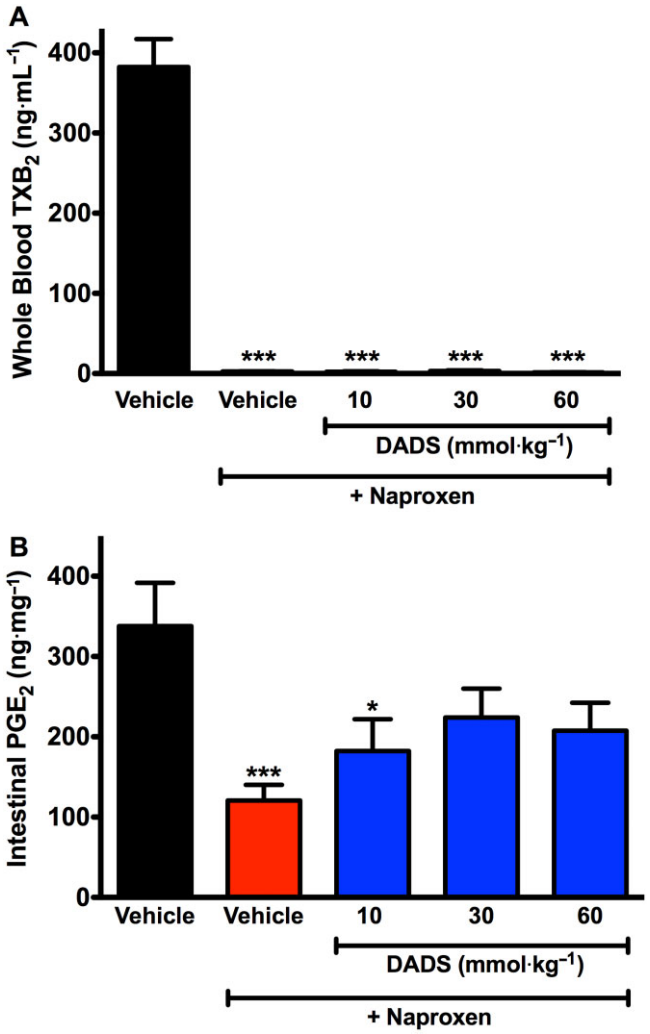

Figure 3

DADS did not prevent systemic COX inhibition by naproxen. Naproxen administration significantly suppressed (by 99%) whole-blood synthesis of TXB_2 (panel A), and this was not affected by co-administration of DADS (10, 30 or 60 mmol·kg^{-1}). Three hours after the final dose, naproxen administration also significantly inhibited (by 64%) intestinal PGE_2 synthesis compared with vehicle-treated rats (panel B). However, co-treatment with DADS at 30 and 60 mmol·kg^{-1} increased intestinal PGE_2 synthesis in naproxen-treated rats to levels comparable with vehicle-treated rats. Results are shown as mean ± SEM ($n \geq 6$ per group). *$P < 0.05$, **$P < 0.01$, significantly different from vehicle; one-way ANOVA followed by Dunnett's and Bonferroni *post hoc* tests.

(MPO activity), compared with vehicle-treated rats ($P < 0.001$). However, naproxen-induced granulocyte infiltration was prevented when rats were co-treated with DADS at doses of 30 or 60 mmol·kg^{-1} (Figure 4A). Histological examination of jejunal sections from naproxen-treated rats confirmed the extensive macroscopic erosions and granulocyte infiltration. Naproxen-treated rats exhibited a complete loss of mucosal architecture, granulocyte infiltration, and extensive subepithelial oedema, compared with vehicle-treated rats (Figure 4B and C respectively). However, mucosal structure was largely intact when naproxen-treated rats were co-treated with DADS at doses of 30 or 60 mmol·kg^{-1}, with similar appearance to vehicle-treated rats (Figure 4D).

Cytotoxic effects of bile were enhanced by naproxen

When tested at dilutions of 1:6 or 1:12, bile collected from rats given naproxen (20 mg·kg^{-1}) over 4.5 days exhibited significantly increased cytotoxic effects on IEC-6 intestinal epithelial cells, compared with bile from rats given vehicle. Results for the 1:6 dilutions are shown in Figure 5. Thus, exposure of the cells to a 1:6 dilution of bile from naproxen-treated rats for 3 h resulted in 58% cytotoxicity, compared with 26% cytotoxicity ($P < 0.001$) for bile from vehicle-treated rats (Figure 5). Similar results were also observed for the 1:12 bile dilution (data not shown).

DADS reduced naproxen-enhanced bile toxicity

The enhancement of the cytotoxicity of bile by naproxen was dose-dependently reduced in rats co-treated with DADS (Figure 5). Indeed, bile collected from rats co-treated with naproxen and DADS at 60 mmol·kg^{-1} was not significantly different, in terms of cytotoxicity, from the bile collected from rats treated only with vehicle. Similar results were also observed for the 1:12 bile dilution and in a series of experiments evaluating bile cytotoxicity on cultured HT-29 cells (Supporting Information Fig. S2).

The severity of naproxen-induced intestinal damage correlates well with the concentrations of naproxen in the bile after administration of naproxen to rats (J. L. Wallace, unpubl. data). To explore whether DADS co-administration reduced biliary concentrations of naproxen, we measured the concentrations of naproxen in bile from rats treated with naproxen (20 mg·kg^{-1}) alone or co-treated with DADS. The concentration of naproxen in the bile of naproxen-treated rats did not differ significantly when DADS was co-administered at doses of 10, 30 or 60 mmol·kg^{-1}, suggesting that DADS co-administration did not significantly alter the enterohepatic recirculation of naproxen (Supporting Information Fig. S3).

DADS administration altered the composition of the microbiota

We examined whether DADS administration would alter the composition of the intestinal microbiota. DGGE analysis of caecal contents demonstrated that treatment with DADS caused a marked shift in the composition of the microbiota. DGGE analysis was performed to compare the microbial composition in rats treated with vehicle, naproxen (20 mg·kg^{-1}) plus vehicle, or naproxen plus DADS at a protective (30 mmol·kg^{-1}) and a non-protective (10 mmol·kg^{-1}) dose. Naproxen administration caused caecal dysbiosis in rats and co-treatment with a non-protective dose of DADS (10 mmol·kg^{-1}) did not correct this shift, as analysed by the Dice coefficient and Ward algorithm to determine similarities (Figure 6A). Interestingly, the microbiota of naproxen-treated, rats co-treated with a protective dose of DADS (30 mmol·kg^{-1}), clustered with that of rats not treated with naproxen. Construction of a UPGMA tree demonstrated

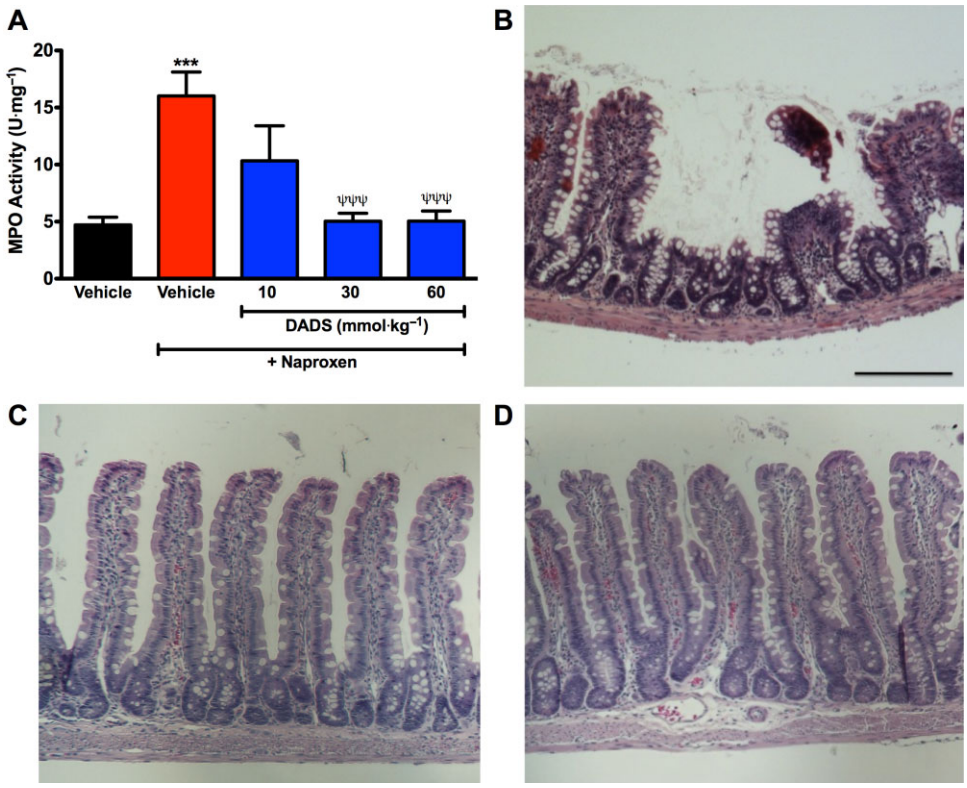

Figure 4

DADS dose-dependently prevented naproxen-induced mucosal inflammation and structural damage. Panel A: naproxen administration significantly increased intestinal MPO activity compared with vehicle-treated rats. However, co-treatment with DADS at doses of 30 or 60 mmol·kg^{-1} significantly diminished the naproxen-induced increase in MPO activity. Panel B: loss of mucosal structure in the intestine after naproxen treatment. Mucosal structure remained intact when naproxen-treated rats were co-administered DADS (30 mmol·kg^{-1}) (panel D), with a similar appearance to tissue from vehicle-treated rats (panel C). Results are shown as mean ± SEM ($n \geq 6$ per group). ***$P < 0.001$, significantly different from vehicle; ΨΨΨ$P < 0.001$, significantly different from naproxen alone; one-way ANOVA followed by Dunnett's and Bonferroni *post hoc* tests. Scale bar, 100 μm (applicable to each panel).

similar clustering and each branch had 100% re-sampling support (Figure 6B). The total number of aerobes in the jejunum of rats treated with naproxen or naproxen plus DADS (10, 30 or 60 mmol·kg^{-1}), was not significantly different from that in vehicle-treated rats (whether plated on CBA or BHI media) (Figure 6C).

To further investigate if DADS administration alone could shift the composition of the microbiota in rats, an additional experiment was conducted in which rats were treated with vehicle or DADS (10 or 30 mmol·kg^{-1}). Similar to the results mentioned earlier, the total number of aerobes and anaerobes in the jejunum of rats treated with DADS at doses of 10 or 30 mmol·kg^{-1}was not significantly different from that in vehicle-treated rats (whether plated on CBA or BHI media) (Supporting Information Fig. S3C). DGGE analysis of the caecal microbiota demonstrated that treatment of rats with DADS at a non-protective dose (10 mmol·kg^{-1}) did not cause a shift in the microbiota, compared with treatment with vehicle. However, the microbiota of rats treated with DADS at a protective dose (30 mmol·kg^{-1}) resulted in a distinct clustering of the microbiota, compared with that in rats treated with vehicle (Supporting Information Fig. S4A and B).

We also analysed the taxonomic composition of the caecal microbiota via deep sequencing of 16S rRNA (Illumina, San Diego, CA, USA). Administration of DADS at 30 mmol·kg^{-1}, but not at 10 mmol·kg^{-1}, significantly decreased multiple *Clostridiales* families, such as *Ruminococcaceae* and *Eubacteriaceae*, and decreased *Enterococcaceae* as compared with vehicle-treated rats (Supporting Information Fig. S5). Interestingly, DADS at the higher dose increased the abundance of *Mucispirillum*, a type of bacterium that colonizes the mucus layer of the mammalian tract.

Lack of intestinal damage or alteration of bile cytotoxicity with an H_2S-releasing naproxen derivative

Twice daily administration of ATB-346 for 4.5 days at a dose equimolar to naproxen at 20 mg·kg^{-1} did not cause significant damage to the small intestine of rats (scores of 0 in all six rats). When HT-29 cells were exposed to a 1:6 or 1:12 dilution of bile collected from ATB-346-treated rats, the extent of cell death observed was significantly reduced compared with that observed when the cells were exposed to the same dilution of bile from naproxen-treated rats (Supporting Information Fig. S6). ATB-346 did not significantly change the cytotoxicity of bile as compared with vehicle treatment.

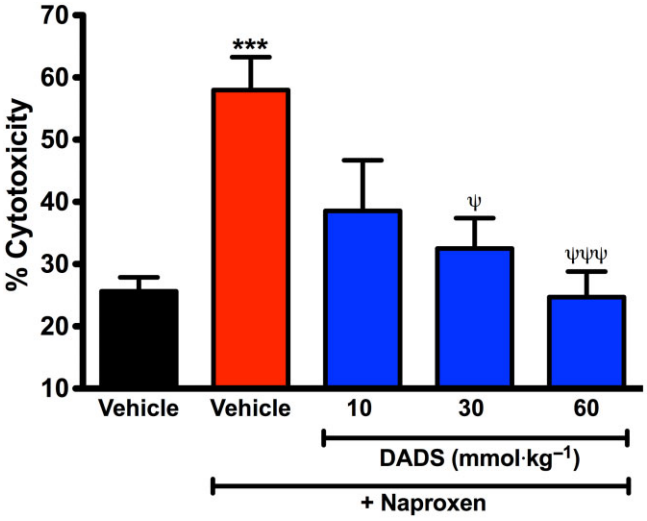

Figure 5

DADS dose-dependently reduced naproxen-induced bile cytotoxicity. Bile collected from rats treated with naproxen (20 mg·kg^{-1}) twice daily for 4.5 days was significantly more cytotoxic to cultured rat IEC-6 cells than bile collected from vehicle-treated rats. Co-treatment with DADS at 30 mmol·kg^{-1} significantly reduced the naproxen-induced increase in cytotoxicity of bile. Co-treatment with DADS at 60 mmol·kg^{-1} further reduced naproxen-induced bile cytotoxicity, to a level similar to that of bile from vehicle-treated rats. Data shown are from the 1:6 dilutions of bile samples, and are expressed as the mean ± SEM of at least six rats per group. ***$P < 0.001$, significantly different from vehicle; $^{\psi}P < 0.05$, $^{\psi\psi\psi}P < 0.001$, significantly different from naproxen alone; one-way ANOVA followed by Dunnett's and Bonferroni *post hoc* tests.

Discussion

NSAID-induced enteropathy is a significant clinical concern because of the widespread use of this class of drugs, particularly among the elderly, who have an increased propensity to develop GI ulcers (Wallace, 2013b). In recent years, considerable evidence has been provided for important roles of H_2S as a mediator of mucosal defence throughout the GI tract. Endogenous H_2S synthesis is up-regulated at sites of mucosal injury and contributes significantly to healing of the injury (Wallace *et al.*, 2007b; Wallace *et al.*, 2009; Flannigan *et al.*, 2013). Administration of H_2S donors has been shown to accelerate the healing of gastric and colonic ulcers, and to reduce mucosal inflammation (Fiorucci *et al.*, 2007; Wallace *et al.*, 2007b). In the present study, a garlic-derived H_2S donor (DADS) was shown to dose-dependently reduce the severity of ulceration and bleeding in the small intestine following administration of naproxen, one of the most prescribed NSAIDs. Furthermore, DADS administration led to significant changes in the intestinal microbiota and to the cytotoxicity of bile that could account, at least in part, for the protective effects of this H_2S donor against NSAID-enteropathy. Suppression of endogenous H_2S synthesis resulted in a significant exacerbation of naproxen-induced intestinal ulceration and bleeding. The intestinal-sparing effect of DADs and reduced cytotoxicity of bile were also observed with an H_2S-releasing derivative of naproxen (ATB-346), which suppressed PG and TX synthesis as effectively as naproxen. Indeed, with a number of H_2S-releasing derivatives of NSAIDs, we have consistently observed a greatly reduced capacity for inducing gastrointestinal damage, despite comparable suppression of COX-1 and COX-2 activity as seen with the parent NSAID (Wallace *et al.*, 2007a; 2010; Elsheikh *et al.*, 2014). These observations support the hypothesis that the beneficial effects of DADS were attributable to the H_2S it can release (Benavides *et al.*, 2007). Moreover, there does not appear to be any tachyphylaxis to the beneficial effects of H_2S in the GI tract, as GI safety was observed even after 2 weeks of daily administration of these compounds (Elsheikh *et al.*, 2014).

While the mechanism underlying the gastroduodenal damage caused by NSAIDs is clearly related to the ability of these drugs to suppress COX-1 and -2 activity, the mechanism for NSAID-enteropathy is less clear and is likely to be more complex (Wallace, 2012). COX inhibition contributes to the injury that develops in the intestine following NSAID administration, but at least three other interrelated factors appear to be more important: bile, enteric bacteria and the enterohepatic circulation of the NSAID. The latter is clear from evidence that NSAIDs that do not undergo enterohepatic recirculation do not cause significant intestinal damage (Wax *et al.*, 1970; Reuter *et al.*, 1997; Somasundaram *et al.*, 1997). After absorption, NSAIDs can be glucuronidated in the liver, and then excreted into bile. As shown in the present study, bile containing NSAIDs or NSAID-glucuronides are more damaging to intestinal epithelial cells than bile from rats that were not treated with an NSAID. This may be due, at least in part, to NSAID-induced changes in the enteric flora, leading to increased concentrations of more cytotoxic, secondary bile acids (Hofmann, 1999; Martinez-Augustin and Sanchez de Medina, 2008). Re-absorption of the NSAIDs in the ileum can only occur if the NSAID is deconjugated from the glucuronide, which requires the activity of bacterial β-glucuronidase. It has recently been demonstrated that an inhibitor of bacterial β-glucuronidase prevented NSAID-induced intestinal damage in mice (Saitta *et al.*, 2014), consistent with observations that prevention of enterohepatic circulation of NSAIDs (Wax *et al.*, 1970), or performing studies in germ-free animals (Robert and Asano, 1977; Uejima *et al.*, 1996), resulted in prevention of intestinal damage. Thus, bacteria may contribute to NSAID-enteropathy through their critical role in enterohepatic circulation, as well as in the conversion of primary to secondary bile acids. A third mechanism through which bacteria can contribute to NSAID-enteropathy is through their colonization of the initial lesions that form after NSAID administration (Elliott *et al.*, 1998), and in the case of Gram-negative bacteria, via activation of Toll-like receptor 4 (TLR-4). Watanabe *et al.* (2008) demonstrated that activation of TLR-4 contributed significantly to the intestinal injury that developed in mice and rats after administration of indomethacin. Studies involving pre-treatment with antibiotics have been less informative in establishing the contribution of enteric bacteria to NSAID-enteropathy, as it is difficult to separate a primary (preventative) effect from a secondary effect such as post-injury acceleration of healing (Kent *et al.*, 1969; Yamada *et al.*, 1993).

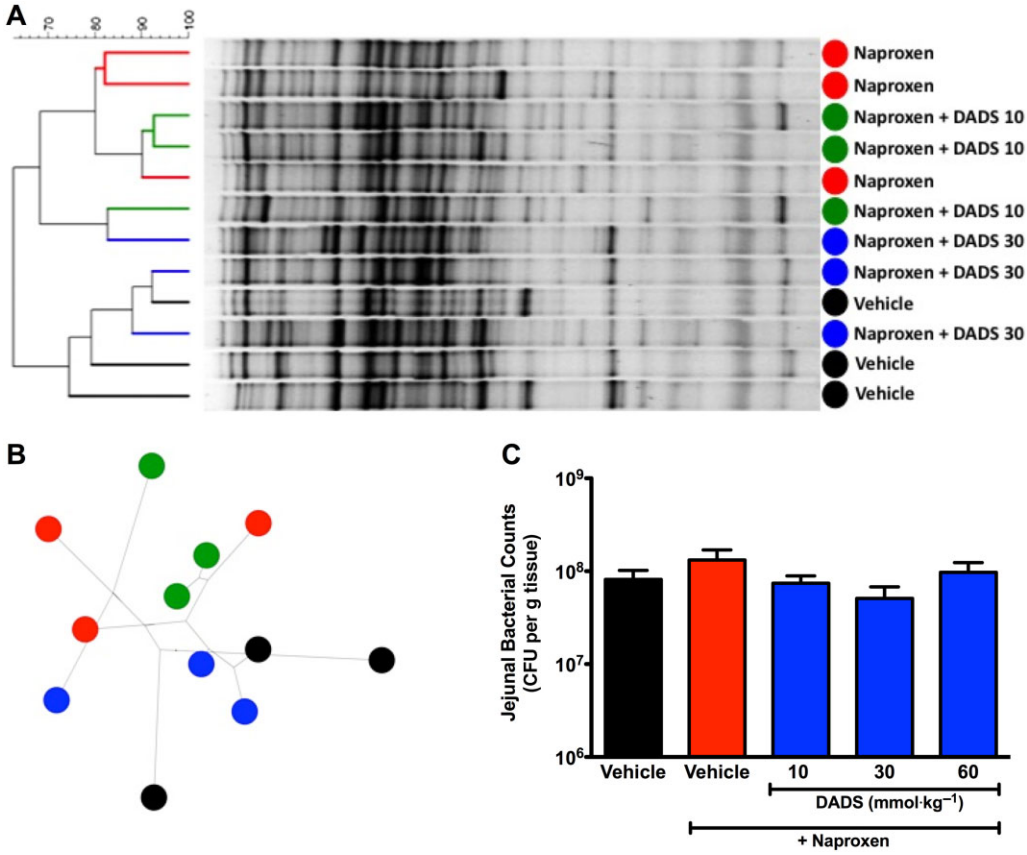

Figure 6

Co-treatment with DADS prevented naproxen-induced dysbiosis. Panel A: DGGE analysis revealed that naproxen (20 mg·kg^{-1}) administration to rats caused dysbiosis of the caecal microbiota, with distinct clustering from vehicle-treated rats. Co-treatment with DADS at 30 mmol·kg^{-1} shifted the microbiota of naproxen-treated rats back to being similar to that of vehicle-treated rats. Using a resampling technique (majority UPGMA algorithm), the dendrogram clustering observed in Panel A was confirmed, indicating a robust difference in microbiota composition between groups (panel B). Panel C: The total number of aerobes in the jejunum did not significantly differ in rats treated with vehicle, naproxen, or naproxen plus DADS (10, 30 or 60 mmol·kg^{-1}) twice daily for 4.5 days. Results in panel C are from samples plated on CBA and shown as mean ± SEM ($n \leq 5$ per group). The data were analysed by a one-way ANOVA followed by Dunnett's multiple comparison test.

In the present study, treatment with DADS resulted in a reduction of the *in vitro* cytotoxicity of bile to the same level as in rats not treated with an NSAID, triggered marked changes in the intestinal microbiota, but did not alter enterohepatic recirculation of naproxen (i.e. the levels of naproxen in bile were unaltered by DADS treatment). This suggests that DADS did not significantly change bacterial deconjugation of naproxen-glucuronide, a necessary step for the NSAID to be re-absorbed in the ileum. We cannot rule out the possibility that treatment with DADS may have altered the levels of naproxen-glucuronide in bile. In a previous study, we observed that the ratio of biliary naproxen-glucuronide : naproxen after administration of an H_2S-releasing naproxen derivative decreased over time with repeated administration of this compound (Blackler *et al.*, 2012). Concentrations of naproxen in the bile of rats treated with naproxen (20 μg·kg^{-1}) were ~3 μg·mL^{-1}. This concentration of naproxen (~1.2 μM) is well below the concentration required to significantly increase the cytotoxicity of the bile in our *in vitro* assay (only at concentrations of naproxen of >10 μM was an increase in the cytotoxicity of bile observed).

Another mechanism through which NSAIDs have been suggested to cause small intestinal ulceration is through their ability to uncouple oxidative phosphorylation, leading to death of epithelial cells (Somasundaram *et al.*, 1997). If this is the case, it raises the intriguing possibility that the protective effects of H_2S could be related to the ability of this gaseous mediator to act as an electron donor in mitochondrial respiration (Goubern *et al.*, 2007). H_2S has been shown capable of rescuing mitochondrial function during hypoxia and anoxia, by virtue of this action, contributing to its cytoprotective effects in the GI tract and elsewhere (Elrod *et al.*, 2007; Kimura *et al.*, 2010; Campolo *et al.*, 2013). Epithelial cells in the GI tract have been reported to be the most efficient cells at using H_2S as a mitochondrial energy source (Mimoun *et al.*, 2012).

Treatment with DADS also resulted in a statistically significant increase in intestinal PG synthesis. As expected, naproxen markedly inhibited intestinal PGE_2 synthesis and whole-blood TX synthesis (the latter is almost entirely derived from platelets) (Wallace *et al.*, 1998). In rats pretreated with DADS at the two higher doses, which were

protective against intestinal injury, the levels of PGE_2 synthesis were significantly greater than in rats pretreated with vehicle. However, the differences in intestinal PGE_2 synthesis between the groups treated with 'protective' doses of DADS and the group treated with a non-protective dose of DADS were negligible, suggesting that altered PGE_2 synthesis was unlikely to have contributed significantly to the protective effects of DADS.

As mentioned earlier, the pathogenesis of NSAID-enteropathy is more complicated than that of NSAID-gastropathy. The multifactorial aspect of NSAID-enteropathy may explain why doses of DADS in the $mmol \cdot kg^{-1}$ range were required to observe a protective effect against small intestinal damage, whereas doses of DADS in the $\mu mol \cdot kg^{-1}$ range were effective in preventing naproxen-induced gastric damage (Wallace *et al.*, 2010). However, the ability of an H_2S donor to protect against NSAID-induced intestinal damage may also depend on the nature of the donor, and the manner in which H_2S is delivered by the donor relative to the delivery and absorption of the NSAID. We observed that ATB-346, an H_2S-releasing derivative of naproxen, at a dose equivalent to a $20\ mg \cdot kg^{-1}$ dose of naproxen, did not produce significant small intestinal damage, consistent with previous observations (Wallace *et al.*, 2010; Blackler *et al.*, 2012). This effect was seen even though, at this dose, ATB-346 would deliver less than 1% of the H_2S that is released by the protective doses of DADS. Interestingly, as was the case with DADS co-administration, the cytotoxicity of bile from rats treated with ATB-346 was significantly reduced compared with bile from rats treated with naproxen.

Changes in the microbiota induced by administration of DADS may have contributed significantly to the ability of this H_2S donor to reduce the severity of naproxen-induced intestinal damage. As outlined earlier, enteric bacteria can contribute to the pathogenesis of NSAID-enteropathy in several ways, affecting both the cytotoxicity of bile, the enterohepatic circulation of the NSAID and the ability of ulcers to heal. In this study, we have demonstrated that naproxen administration also caused significant changes in the enteric microflora, as has been reported previously (Uejima *et al.*, 1996; Reuter *et al.*, 1997; Hagiwara *et al.*, 2004; Watanabe *et al.*, 2008). Co-administration of 'protective' doses of DADS with naproxen resulted in a normalization of the microbiota (i.e. to be similar to that in rats not receiving naproxen). Of course, this latter effect could simply be a consequence of the prevention of naproxen-induced intestinal damage. A causal relationship between DADS-induced changes in the microbiota and reduced intestinal damage has not been established. However, it is noteworthy that administration of DADS alone resulted in significant changes in the enteric microflora, including causing a marked decrease in multiple *Clostridiales* families, and a substantial increase in abundance of *Mucispirillum*. These effects were observed with a dose of DADS that was effective in reducing naproxen-induced enteropathy ($30\ mmol \cdot kg^{-1}$), but not at a dose that was ineffective in reducing naproxen-induced enteropathy ($10\ mmol \cdot kg^{-1}$).

In summary, the present study extends previous observations that H_2S has protective effects in the GI tract, with the demonstration that DADS could substantially reduce the severity of intestinal ulceration and bleeding induced by repeated administration of an NSAID. The pathogenesis of NSAID-enteropathy is complicated, involving cytotoxic effects of bile, changes in intestinal microbiota and enterohepatic circulation of the NSAID. The present study provides evidence that administration of an H_2S donor can significantly affect two of these factors: reducing the cytotoxicity of bile and significantly altering the enteric microbiota. These two effects may be related, as changes in enteric bacteria can lead to altered bile metabolism, and in turn to an alteration of cytotoxicity.

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Author contributions

R. W. B., J. P. M., A. M. and M. W. performed the experiments; R. W. B., J. P. M., P. B. and J. L. W. designed the experiments; R. W. B., J. P. M., A. M., M. W., P. B., M. G. S. and J. L. W. analysed the data; and R. W. B., M. W. and J. L. W. wrote the paper.

Conflict of interest

Dr Wallace is Founder and one of the Directors of Antibe Therapeutics, Inc., a company developing novel anti-inflammatory drugs.

References

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.

Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell S, Patel RP *et al.* (2007). Hydrogen sulfide mediates the vasoactivity of garlic. Proc Natl Acad Sci U S A 104: 17977–17982.

Bjarnason I, Hayllar J, MacPherson AJ, Russell AS (1993). Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 104: 1832–1847.

Blackler R, Syer S, Bolla M, Ongini E, Wallace JL (2012). Gastrointestinal-sparing effects of novel NSAIDs in rats with compromised mucosal defence. PLoS ONE 7: e35196.

Boughton-Smith NK, Wallace JL, Whittle BJ (1988). Relationship between arachidonic acid metabolism, myeloperoxidase activity and leukocyte infiltration in a rat model of inflammatory bowel disease. Agents Actions 25: 115–123.

Campolo M, Esposito E, Ahmad A, Di Paola R, Wallace JL, Cuzzocrea S (2013). A hydrogen sulfide-releasing cyclooxygenase inhibitor markedly accelerates recovery from experimental spinal cord injury. FASEB J 27: 4489–4499.

Dietschy JM (1968). Mechanisms for the intestinal absorption of bile acids. J Lipid Res 9: 297–309.

Elliott SN, Buret A, McKnight W, Miller MJ, Wallace JL (1998). Bacteria rapidly colonize and modulate healing of gastric ulcers in rats. Am J Physiol 275: G425–G432.

Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L *et al.* (2007). Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA 104: 15560–15565.

Elsheikh W, Blackler RW, Flannigan KL, Wallace JL (2014). Enhanced chemopreventative effects of a hydrogen sulfide-releasing NSAID (ATB-346) in experimental colorectal cancer. Nitric Oxide 41: 131–137.

Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Medcarelli A, Orlandi S *et al.* (2005). Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. Gastroenterology 129: 1210–1224.

Fiorucci S, Orlandi S, Mencarelli A, Caliendo G, Santagada V, Distrutti E *et al.* (2007). Enhanced activity of a hydrogen sulphide-releasing derivative of mesalamine (ATB-429) in a mouse model of colitis. Br J Pharmacol 150: 996–1002.

Flannigan KL, Ferraz JG, Wang R, Wallace JL (2013). Enhanced synthesis and diminished degradation of hydrogen sulfide in experimental colitis: a site-specific, pro-resolution mechanism. PLoS ONE 8: e71962.

Goubern M, Andriamihaja M, Nubel T, Blachier F, Bouillaud F (2007). Sulfide, the first inorganic substrate for human cells. FASEB J 21: 1699–1706.

Hagiwara M, Kataoka K, Arimochi H, Kuwahara T, Ohnishi Y (2004). Role of unbalanced growth of gram-negative bacteria in ileal ulcer formation in rats treated with a nonsteroidal anti-inflammatory drug. J Med Invest 51: 43–51.

Hofmann AF (1999). The continuing importance of bile acids in liver and intestinal disease. Arch Intern Med 159: 2647–2658.

Jobin C, Morteau O, Hans DS, Sartor RB (1998). Specific NF-kappaB blockade selectively inhibits tumour necrosis factor-alpha-induced COX-2 but not constitutive COX-1 gene expression in HT-29 cells. Immunology 95: 537–543.

Kawabata A, Ishiki T, Nagasawa K, Yoshida S, Maeda Y, Takahashi T *et al.* (2007). Hydrogen sulfide as a novel nociceptive messenger. Pain 132: 74–81.

Kent TH, Cardelli RM, Stamler FW (1969). Small intestinal ulcersand intestinal flora in rats given indomethacin. Am J Pathol 54: 237–249.

Kimura Y, Goto Y, Kimura H (2010). Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. Antioxid Redox Signal 12: 1–13.

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.

Lanas A, Garcia-Rodriguez LA, Polo-Tomas M, Ponce M, Alonso-Abreu I, Perez-Aisa MA *et al.* (2009). Time trends and impact of upper and lower gastrointestinal bleeding and perforation in clinical practice. Am J Gastroenterol 104: 1633–1641.

Mard SA, Neisi N, Solgi G, Hassanpour M, Darbor M, Maleki M (2012). Gastroprotective effect of NaHS against mucosal lesions induced by ischemia-reperfusion injury in rat. Dig Dis Sci 57: 1496–1503.

Martinez-Augustin O, Sanchez de Medina F (2008). Intestinal bile acid physiology and pathophysiology. World J Gastroenterol 14: 5630–5640.

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.

Mimoun S, Andriamihaja M, Chaumontet C, Atanasiu C, Benamouzig R, Blouin JM *et al.* (2012). Detoxification of H_2S by differentiated colonic epithelial cells: implication of the sulfide oxidizing unit and of the cell respiratory capacity. Antioxid Redox Signal 17: 1–10.

Park AJ, Collins J, Blennerhassett PA, Ghia JE, Verdu EF, Bercik P *et al.* (2013). Altered colonic function and microbiota profile in a mouse model of chronic depression. Neurogastroenterol Motil 25: 733–e575.

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): D1098–1106.

Quaroni A, Wands J, Trelstad RL, Isselbacher KJ (1979). Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. J Cell Biol 80: 248–265.

Reuter BK, Davies NM, Wallace JL (1997). Nonsteroidal anti-inflammatory drug enteropathy in rats: role of permeability, bacteria, and enterohepatic circulation. Gastroenterology 112: 109–117.

Robert A, Asano T (1977). Resistance of germfree rats to indomethacin-induced intestinal lesions. Prostaglandins 14: 333–341.

Saitta KS, Zhang C, Lee KK, Fujimoto K, Redinbo MR, Boelsterli UA (2014). Bacterial β-glucuronidase inhibition protects mice against enteropathy induced by indomethacin, ketoprofen or diclofenac: mode of action and pharmacokinetics. Xenobiotica 44: 28–35.

Satoh H, Amagase K, Takeuchi K (2012). Exacerbation of nonsteroidal anti-inflammatory drug-induced small intestinal lesions by antisecretory drugs in rats: the role of intestinal motility. J Pharmacol Exp Ther 343: 270–277.

Scheiman JM, Yeomans ND, Talley NJ, Vakil N, Chan FK, Tulassay Z (2006). Prevention of ulcers by esomeprazole in at-risk patients using non-selective NSAIDs and COX-2 inhibitors. Am J Gastroenterol 101: 701–710.

Seitz S, Boelsterli UA (1998). Diclofenac acyl glucuronide, a major biliary metabolite, is directly involved in small intestinal injury in rats. Gastroenterology 115: 1476–1482.

Somasundaram S, Rafi S, Hayllar J, Sigthorsson G, Jacob M, Price AB *et al.* (1997). Mitochondrial damage: a possible mechanism of the 'topical' phase of NSAID induced injury to the rat intestine. Gut 41: 344–353.

Uejima M, Kinouchi T, Kataoka K, Hiraoka I, Ohnishi Y (1996). Role of intestinal bacteria in ileal ulcer formation in rats treated with a nonsteroidal antiinflammatory drug. Microbiol Immunol 40: 553–560.

Wallace JL (2008). Prostaglandins, NSAIDs, and gastric mucosal protection; why doesn't the stomach digest itself? Physiol Rev 88: 1547–1565.

Wallace JL (2010). Physiological and pathophysiological roles of hydrogen sulfide in the gastrointestinal tract. Antioxid Redox Signal 12: 1125–1133.

Wallace JL (2012). NSAID gastropathy and enteropathy: distinct pathogenesis likely necessitates distinct prevention strategies. Br J Pharmacol 165: 67–74.

Wallace JL (2013a). Mechanisms, prevention and clinical implications of nonsteroidal anti-inflammatoty drug-enteropathy. World J Gastroenterol 19: 1861–1876.

Wallace JL (2013b). Polypharmacy of osteoarthritis: the perfect intestinal storm. Dig Dis Sci 58: 3088–3093.

Wallace JL, Keenan CM, Granger DN (1990). Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. Am J Physiol 259: 462–467.

Wallace JL, Bak A, McKnight W, Asfaha S, Sharkey KA, MacNaughton WK (1998). Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. Gastroenterology 115: 101–109.

Wallace JL, McKnight W, Reuter BK, Vergnolle N (2000). NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. Gastroenterology 119: 706–714.

Wallace JL, Caliendo G, Santagada V, Cirino G, Fiorucci S (2007a). Gastrointestinal safety and anti-inflammatory effects of a hydrogen sulfide-releasing diclofenac derivative in the rat. Gastroenterology 132: 261–271.

Wallace JL, Dicay M, McKnight W, Martin GR (2007b). Hydrogen sulfide enhances ulcer healing. FASEB J 21: 4070–4076.

Wallace JL, Vong L, McKnight W, Dicay M, Martin GR (2009). Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology 137: 569–578.

Wallace JL, Caliendo G, Santagada V, Cirino G (2010). Markedly reduced toxicity of a hydrogen sulphide-releasing derivative of naproxen (ATB-346). Br J Pharmacol 159: 1236–1246.

Wallace JL, Syer S, Denou E, de Palma G, Vong L, McKnight W *et al*. (2011). Proton pump inhibitors exacerbate NSAID-induced small intestinal injury by inducing dysbiosis. Gastroenterology 141: 1314–1322.

Wallace JL, Ferraz JG, Muscara MN (2012). Hydrogen sulfide: an endogenous mediator of resolution of inflammation and injury. Antioxid Redox Signal 17: 58–67.

Wallace JL, Blackler RW, Chan MV, Da Silva GJ, Elsheikh W, Flannigan KL *et al*. (2014). Anti-inflammatory and cytoprotective actions of hydrogen sulfide: translation to theapeutics. Antioxid Redox Signal. Epub ahead of print.

Watanabe T, Higuchi K, Kobata A, Nishio H, Tanigawa T, Shiba M *et al*. (2008). Non-steroidal anti-inflammatory drug-induced small intestinal damage is Toll-like receptor 4 dependent. Gut 57: 181–187.

Watanabe T, Tanigawa T, Nadatani Y, Nagami Y, Sugimori S, Okazaki H *et al*. (2013). Risk factors for severe nonsteroidal anti-inflammatory drug-induced small intestinal damage. Dig Liver Dis 45: 390–395.

Wax J, Clinger WA, Varner P, Bass P, Winder CV (1970). Relationship of the enterohepatic cycle to ulcerogenesis in the rat small bowel with flufenamic acid. Gastroenterology 58: 772–780.

Yamada T, Deitch E, Specian RD, Perry MA, Sartor RB, Grisham MB (1993). Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. Inflammation 17: 641–662.

Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 20: 2118–2120.

Zhao Y, Encinosa W (2008). Hospitalizations for gastrointestinal bleeding in 1998 and 2006: Statistical brief #65. *Health Care Cost and Utilization Project (HCUP) Statistical Briefs*. Agency for Health Care Policy and Research.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effects of DADS on inhibition of gastric PGE_2 synthesis by naproxen. Three hours after the final dose, naproxen significantly suppressed gastric PGE_2 synthesis (***$P < 0.001$). However, co-treatment with DADS at 30 or 60 mmol·kg^{-1} significantly increased gastric PGE_2 synthesis in naproxen-treated rats ($^{\psi}P < 0.05$). Results are shown as mean ± SEM ($n \geq 6$ per group). The data were analysed by a one-way ANOVA followed by Dunnett's and Bonferroni *post hoc* tests.

Figure S2 DADS dose-dependently reduced naproxen-induced bile cytotoxicity on HT-29 cultured cells. Similar to the bile cytotoxicity results from IEC-6 cell cultures, bile collected from rats treated with naproxen (20 mg·kg^{-1}) twice daily for 4.5 days was significantly more cytotoxic than bile collected from vehicle-treated rats (***$P < 0.001$). Bile collected from rats co-treated with DADS at 60 mmol·kg^{-1} was significantly less cytotoxic than bile from naproxen-treated rats (*$P < 0.05$). The bile samples were diluted 1:6 in Dulbecco's PBS (DPBS) prior to being added to the cultured cells. Results are shown as mean ± SEM ($n = 6$ per group). The data were analysed by a one-way ANOVA followed by Dunnett's multiple comparison test.

Figure S3 Biliary concentrations of naproxen were unaltered by co-administration of DADS. Naproxen (20 mg·kg^{-1}) was co-administered with vehicle or with DADS (10, 30 or 60 mmol·kg^{-1}) twice daily for 4.5 days. Bile was collected 1 h after the final drug administration. Biliary naproxen concentrations were measured by liquid chromatography/mass spectrometry. Results are shown as mean ± SEM ($n = 6$ per group). The data were analysed by a one-way ANOVA followed by Dunnett's and Bonferroni *post hoc* tests.

Figure S4 Treatment with DADS caused a shift in the microbiota composition. Panel A: DGGE analysis revealed that there were significant differences in the composition of caecal microbiota in vehicle-treated and DADS-treated (30 mmol·kg^{-1}) rats. The clustering observed in the dendrogram constructed using the Dice coefficient and Ward algorithm in panel A was confirmed using majority UPGMA algorithm (panel B). Panel C: the total number of aerobes and anaerobes in the jejunum did not significantly differ in rats treated with vehicle or with DADS (10 or 30 mmol·kg^{-1}) twice daily for 4.5 days. Results in panel C are from samples plated on CBA and shown as mean ± SEM ($n \leq 5$ per group). The data were analysed by a one-way ANOVA followed by Dunnett's multiple comparison test.

Figure S5 Effects of DADS at a protective dose (30 mmol·kg^{-1}) and a non-protective dose (10 mmol·kg^{-1}) on the rat microbiota were examined. The taxonomic composition of the caecal microbiota from rats treated with DADS or vehicle was evaluated via deep sequencing of 16S rRNA with Illumina. Data are shown as the mean ± SEM of five rats per group. *$P < 0.05$ versus the vehicle-treated group (one-way ANOVA followed by Bonferroni test).

Figure S6 ATB-346 administration did not enhance the cytotoxic effects of bile on human intestinal epithelial (HT-29)

cells (n = 12 per group). Bile collected from rats treated with naproxen (10 mg kg^{-1}) twice daily for 4 days resulted in a significant increase in cytotoxicity as compared with the effects of bile from vehicle-treated rats (***$P < 0.001$). In contrast, bile from rats treated with an equimolar dose of ATB-346 exhibited significantly less cytotoxicity than bile from naproxen-treated rats ($^{\Psi\Psi}P < 0.01$), and not significantly different from bile from vehicle-treated rats. Results are from the 1:6 and 1:12 dilutions and shown as mean ± SEM. The data were analysed by one-way ANOVA and Bonferroni test. ATB-346 is 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester, a H_2S-releasing derivative of naproxen.