Deciphering the Pathogenesis of NSAID-Enteropathy

Using Proton Pump Inhibitors and a Hydrogen Sulfide-Releasing NSAID

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Running Title: Bile and Bacteria in NSAID-Enteropathy

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The small intestine is a significant site of ulceration and bleeding induced by nonsteroidal anti-inflammatory drugs (NSAIDs). The pathogenesis is poorly understood. The present study explored the roles of bile, bacteria and enterohepatic circulation to NSAID-enteropathy, using both a conventional NSAID (naproxen) and a gastrointestinal-safe naproxen derivative (ATB-346), as well as proton pump inhibitors. Rats were treated orally with naproxen or equimolar doses of ATB-346 over a 5-day period, with or without PPI administration, and intestinal damage was quantified. The cytotoxicity of bile from the rats was evaluated in vitro. Biliary excretion of naproxen and ATB-346 was determined. The impact of the NSAIDs and of PPIs on the composition of the intestinal microbiota was examined by deep sequencing of 16s rRNA. Naproxen caused significant intestinal damage and inflammation, while ATB-346 did not. Naproxen, but not ATB-346, dose-dependently increased the cytotoxicity of bile, and it was further increased by PPI co-administration. While biliary excretion of naproxen was significant in naproxen-treated rats, it was greatly reduced in rats treated with ATB-346. The enteric microbiota of naproxen-treated rats was distinct from that in vehicle- or ATB-346-treated rats, and PPI administration caused significant intestinal dysbiosis. The increase in cytotoxicity of bile induced by naproxen and PPIs may contribute significantly to intestinal ulceration and bleeding. Some of these effects may occur secondary to significant changes in the jejunal microbiota induced by both naproxen and PPIs.

Key Words: Gastrointestinal; Bleeding; Bile; Proton pump inhibitors; Microbiota.
Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs, because of their ability to alleviate pain and inflammation. However, the gastrointestinal (GI) toxicity of these drugs remains a major limitation to their use. The ulceration and bleeding produced by NSAIDs in the stomach and duodenum can be significantly diminished by co-administration of inhibitors of gastric acid secretion, such as proton pump inhibitors (PPIs) and histamine H$_2$ receptor antagonists (H$_2$RAs) (38). However, it has been clear for many years that these drugs offer no protection to the small intestine, where significant NSAID-induced damage and bleeding occurs (1, 23). In fact, emerging evidence indicates that inhibitors of gastric acid secretion significantly worsen the small intestinal damage caused by NSAIDs (7, 23, 37, 55, 58, 63). Although much recent research has focused on the lower intestinal damage caused by NSAIDs, there are currently no proven-effective preventative or therapeutic regiments for NSAID-enteropathy (49). Consequently, individuals with chronic conditions such as osteoarthritis and rheumatoid arthritis, which often require long-term NSAID use for symptom relief, are without any viable options to prevent NSAID-induced small intestinal injury and bleeding. This is a major clinical concern as lower GI complications are more difficult to detect, more expensive to treat, and result in longer hospital stays and higher mortality rates, compared to upper GI complications (23, 50).

The mechanisms underlying NSAID-induced damage in the distal intestine are in many ways distinct from those of NSAID-induced gastroduodenal damage (48). The damaging effects of NSAIDs in the stomach and proximal duodenum are closely related to their ability to suppress cyclooxygenase (COX) activity, and dependent upon the
presence of gastric acid. In contrast, the pathogenesis of NSAID-enteropathy is less linked to COX suppression and gastric acid, and more related to bile, enteric bacteria and enterohepatic circulation of the NSAID (3, 18, 20, 39, 46, 49, 59). The critical role of bile in the pathogenesis of NSAID-enteropathy is evident from studies showing that bile duct ligation prevented NSAID-induced intestinal damage in rats (20, 25, 42, 59), and studies that demonstrated that NSAIDs that do not undergo enterohepatic circulation do not cause small intestinal damage (3, 21, 35, 43, 59). A number of in vitro experiments have also shown that the combination of bile and an NSAID is particularly damaging to intestinal epithelial cells (33, 35, 42). The contribution of bacteria to NSAID-enteropathy was recently highlighted by a study (55) demonstrating that PPIs exacerbated NSAID-enteropathy in rats, and that this effect was a consequence of specific alterations in the intestinal microbiota by the PPI. Similar effects have now been shown for another class of inhibitors of gastric acid secretion, the H₂RAs (5, 37).

While it remains unclear how PPI-induced dysbiosis contributes to exacerbation of NSAID-induced intestinal damage and bleeding, some evidence suggests that changes in bile may be responsible (5). Several clinical reports have associated chronic use of PPIs with alterations in the GI tract, such as small intestinal bacterial overgrowth and bile acid dysmetabolism (27, 40, 61). It is possible that PPI-induced dysbiosis triggers bile acid dysmetabolism, as many intestinal microbes are capable of enzymatic modification of bile acids (4).

Hydrogen sulfide (H₂S), an endogenous gaseous signaling molecule, is an important mediator of GI mucosal defence, protecting against potentially damaging luminal agents such as acid, bile, and various drugs (52), and modulating the microbiota
A new class of \( \text{H}_2\text{S}\)-releasing NSAID derivatives has demonstrated vastly improved GI safety, producing negligible damage in both the stomach and small intestine (7, 53). In particular, ATB-346, a \( \text{H}_2\text{S}\)-releasing naproxen derivative, has demonstrated superior GI safety compared to its parent NSAID (naproxen) in circumstances in which mucosal defence is significantly impaired (7, 53), even at doses many times greater than those required for anti-inflammatory effects (7). In contrast to naproxen, co-administration of ATB-346 with PPIs and/or low-dose aspirin did not elicit significant small intestinal damage or bleeding (51). The GI safety of ATB-346 is seen despite this drug causing comparable suppression of GI and systemic COX activity as produced by equimolar doses of naproxen (7, 17, 53).

In the present study, we compared the effects of naproxen and ATB-346 in the small intestine in an attempt to determine the roles of bile cytotoxicity, enterohepatic circulation and intestinal microbiota in the pathogenesis of NSAID-induced enteropathy. Specifically, we explored whether administration of naproxen vs. ATB-346 would affect the cytotoxic properties of bile, the extent to which each drug underwent enterohepatic circulation, and the changes they induced to the intestinal microbiota. In addition, we modeled the common clinical scenario of co-administration of an NSAID and a PPI to explore if PPI-associated changes in microbiota and bile might contribute to exacerbation of NSAID-enteropathy.
METHODS

Animals

Male, Wistar rats weighing 220-270 g were obtained from Charles River (Montreal, QC, Canada). All rats were housed in the Central Animal Facility at McMaster University. The rats were fed standard chow and water ad libitum, and were housed in pairs in a room with controlled temperature (22 ± 1°C), humidity (65-70%) and light cycle (12 h light/ 12 h dark). The Animal Care Committee of the Faculty of Health Sciences at McMaster University approved all experimental procedures described herein. The studies were carried out in accordance with the guidelines of the Canadian Council of Animal Care.

NSAID-Induced Enteropathy and Bile Sampling

Rats (n=5/group) were treated orally, twice daily with naproxen, ATB-346 (2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester), or vehicle (dimethylsulfoxide (DMSO)/1% carboxymethylcellulose (CMC); 5:95 ratio) for 2 days. Naproxen was administered at 3, 10, or 30 mg/kg. ATB-346 was given at doses equimolar to those of naproxen (4.4, 14.5 and 44 mg/kg). One hour after the final administration of drug or vehicle, rats were anesthetized with sodium pentobarbital. A laparotomy was performed and the bile duct was cannulated with a polyethylene cannula (PE-10; Clay Adams, Parsipany, NJ, USA) and bile collected for 30 min (35). The bile was stored at -80°C until used in the cytotoxicity assay (see below). Anaesthetized rats then had blood drawn from the aorta for measurement of whole blood thromboxane B₂ (TXB₂) synthesis, as an index of systemic COX-1 activity (51). After blood collection, the small intestine
was blindly evaluated for hemorrhagic damage. This involved measuring the area (in mm²) of all hemorrhagic lesions. The damage areas were summed for each rat to give the ‘intestinal damage score’ (55). Intestinal inflammation was quantitatively assessed in jejunal samples by the measurement of myeloperoxidase (MPO) activity, a biochemical marker of granulocyte infiltration (8). Samples of jejunum were collected for the measurement of prostaglandin (PG)E₂ synthesis, as described previously (54). The concentrations of PGE₂ in the supernatants were determined by ELISA. The PGE₂ and TXB₂ ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Biliary Excretion of Naproxen and ATB-346

Concentrations of naproxen and ATB-346 in bile collected from rats treated orally twice-daily for 2 days (4 treatments in total) with naproxen- or ATB-346 rats were measured by mass spectrometry/liquid chromatography, as described previously (7). These measurements were carried out blindly by Nucro-Technics (Scarborough, ON, Canada). The bile samples were collected as described above, at a time when plasma concentrations of naproxen for the naproxen-treated and ATB-346-treated groups were comparable (43.3 ± 3.3 and 46.0 ± 2.5 μg/mL, respectively).

Effects of NSAIDs and PPIs on Bile Cytotoxicity

Rats (n=5-6/group) were treated twice daily for a total of 9 days with omeprazole, lansoprazole (each at 10 mg/kg i.p.), or vehicle (DMSO). The doses of the PPIs were selected based on preliminary studies in which we examined their ability to markedly suppress gastric acid secretion in rats over a 12 h period, as described
previously (55). In some experiments, naproxen (10 mg/kg), ATB-346 (14.5 mg/kg), or vehicle (DMSO/1% CMC; 5:95 ratio) was co-administered orally twice daily for the final 4.5 days. One hour after the final administration of drug or vehicle, the rats were anaesthetized with sodium pentobarbital. The bile duct was cannulated and bile was collected, as described above. The small intestine was then evaluated for damage, as described above. The doses of naproxen and ATB-346 were selected because previous studies demonstrated that they produced comparable suppression of adjuvant-induced arthritis in rats, and suppressed systemic and small intestinal COX-1 and COX-2 activity to the same extent (7). Previously we demonstrated that the doses of omeprazole and lansoprazole administered in this study markedly suppressed gastric acid secretion by the 5th day of administration, when NSAID treatment was initiated (55).

**Bile Cytotoxicity Assay**

Bile samples were diluted with Dulbecco’s PBS (DPBS) (pH 7.4) immediately prior to incubation with cultured IEC-6 cells (6). A range of dilutions that encompass the physiological range of concentrations of bile acids present in the small intestinal tract of rats (14) were assessed for their cytotoxic effects (results shown are for 1:3 or 1:6). Cells were washed with warm DPBS prior incubation with diluted rat bile. Solutions of bile were incubated with IEC-6 cells for 3 h at 37°C and 5% (vol/vol) CO₂. Following the incubation period, the cells were centrifuged at 250 xg for 5 min and the supernatant collected and assayed for lactate dehydrogenase (LDH), using the Cytoscan-LDH Cytotoxicity Assay Kit (G-Biosciences, St. Louis, MO, USA), as an index of cell lysis.
Rat intestinal epithelial (IEC-6) 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 (34). Cultures were maintained in Dulbecco’s modified eagle medium (DMEM) containing 5% (vol/vol) fetal bovine serum, 4 mmol/L glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (complete medium) at 37°C and 5% (vol/vol) CO₂. Sub-culture was carried out at confluence and cells between passages 17-20 were used for bile cytotoxicity assays. Prior to the assays, cells were seeded at 5 x 10⁴ cells per well in 24-well plates and allowed to reach confluence (grown for 1-2 days post-seeding).

Effects of PPIs on Luminal pH of the jejunum

Rats (n=5 per group) were treated twice daily with omeprazole, lansoprazole (each at 10 mg/kg intraperitoneally) or vehicle (DMSO) for a total of 9 days. The contents of the intestine from a point 30 cm distal to the pyloric sphincter to the ileo-cecal valve were collected into a sterile tube. The pH of the contents was measured with a micro pH glass combination electrode (Fisher Scientific Accumet, Ottawa, ON, Canada).

DNA Extraction and Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis

Bacterial DNA was extracted from jejunal contents as previously described (32). DNA concentrations were determined spectrophotometrically using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The hypervariable V3 region of the bacterial 16s ribosomal DNA gene was amplified using polymerase chain reaction with universal
bacterial primers (HDA-1 and HDA-2) (Mobixlab, McMaster University core facility, Hamilton, ON, Canada). Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode universal mutation system (Bio-Rad, Mississauga, ON, Canada). Electrophoresis was conducted at 130 V at 60°C for 4.5 h. Gels were then stained with SYBR Green (Molecular Probes, Eugene, OR, USA) and viewed by ultraviolet 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 between bacterial profiles were determined by using the Dice coefficient (13) and Ward algorithm (56). A majority, unweighted-pair-group method with arithmetic mean (UPGMA) algorithm was used to construct a multi-dimensional scaled tree based on a resampling strategy of 200 permutations.

Deep sequencing analysis of 16S rRNA with Illumina

The V3 region of the 16S rRNA gene was amplified as previously described (2) with modifications (58). Following separation of products from primers and primer dimers by electrophoresis on a 2% agarose gel, PCR products of the correct size were recovered using a QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada). Custom, in-house Perl scripts were developed to process the sequences after Illumina sequencing (58). Cutadapt (28) was used to trim any over-read, and paired-end sequences were aligned with PANDAseq (30) with a 0.7 quality threshold. If a mismatch in the assembly of a specific set of paired-end sequences was discovered, they were
culled. Additionally, any sequences with ambiguous base calls were also discarded.

Operational taxonomic units (OTUs) were picked using AbundantOTU+, and sequences were clustered to 97% sequence identity operational taxonomic units (OTUs). Taxonomy was assigned at a 0.8 threshold using the Ribosomal Database Project (RDP) (10) classifier v.2.2 trained against the Greengenes SSU database (February, 2011 release).

For all downstream analyses we filtered the obtained OTU table excluding “Root” and excluding any sequence that was not present at least 3 times across the entire dataset.

Materials

Naproxen sodium, omeprazole, and lansoprazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). ATB-346 was provided by Antibe Therapeutics Inc. (Toronto, ON, Canada). DMEM, fetal bovine serum, penicillin, and streptomycin were purchased from Life Technologies Inc. (Burlington, ON, CA).

Statistical Analysis

Rats were randomly assigned to the treatment groups and all analyses were performed blindly. The specific statistical analyses used are described in each figure legend. An associated probability of less than 5% was considered significant.

RESULTS

Naproxen-Induced Enteropathy

Rats treated with naproxen (30 mg/kg) twice daily for 2 days resulted in severe intestinal ulceration (Fig. 1A). There was negligible intestinal damage in rats treated
with naproxen at 3 or 10 mg/kg. None of the doses of ATB-346 produced significant small intestinal damage. Consistent with the small intestinal damage, twice daily naproxen administration for 2 days resulted in a dose-dependent increase in granulocyte infiltration (MPO activity; Fig. 1B) into the jejunal tissue. In contrast, ATB-346 administration did not alter MPO activity at any of the tested doses (Fig. 1B).

Suppression of Cyclooxygenase Activity

Naproxen (10 mg/kg) administration suppressed systemic COX-1 activity (whole blood thromboxane synthesis) by 98% and intestinal PGE₂ synthesis by 96% after twice daily dosing for 2 days (Fig. 2). An equimolar dose of ATB-346 produced a comparable level of suppression of whole blood thromboxane synthesis and intestinal PGE₂ synthesis. With 4 days of administration of the drugs the level of suppression of whole blood thromboxane synthesis and intestinal PGE₂ synthesis rose to >99% and >97%, respectively, for both naproxen and ATB-346.

Naproxen, but not ATB-346, Increased the Cytotoxicity of Bile

Bile collected from rats that had been treated with naproxen twice daily for 2 days exhibited a clear dose-dependent increase in cytotoxicity in the IEC-6 intestinal epithelial cell assay, as compared to bile from vehicle-treated rats (Figure 3A). However, bile collected from rats treated with ATB-346 at equimolar doses to naproxen, did not exhibit any enhancement of cytotoxicity as compared to that from vehicle-treated rats. The cytotoxicity of bile from naproxen-treated rats increased further when the bile was
from rats treated for 4 days rather than for 2 days, while bile from rats treated with ATB-346 for 4 days continued to show no increase in cytotoxicity (data not shown).

The effects of direct addition of naproxen into samples of bile on cytotoxicity were also assessed. Bile spiked with naproxen at 1 or 5 μg/mL exhibited no significant increase in cytotoxicity versus that seen with bile alone (data not shown). These concentrations bracket those that were detected in bile from rats treated with naproxen (see below).

Biliary Levels of Naproxen and ATB-346

There were dramatic differences in the concentrations of naproxen in bile collected from rats 3 h after the final administration of naproxen or ATB-346 (Fig. 3B). In naproxen-treated rats the concentrations of naproxen in bile increased in a dose-dependent manner (to ~5 μg/mL at the highest dose), and were significantly greater than the concentrations of naproxen in bile from rats treated with equimolar doses of ATB-346 (Fig. 3B). In contrast, treatment of rats with equimolar doses of ATB-346 resulted in markedly lower levels of naproxen in bile. ATB-346 was not detected in any samples.

Effects of Naproxen and ATB-346 on Intestinal Microbiota

Administration of naproxen (10 mg/kg), ATB-346 (14.5 mg/kg) or vehicle twice daily for 4 days altered the taxonomic composition of the jejunal microbiota in rats, as analyzed by partial 16S rRNA gene profiling using Illumina sequencing. In particular, naproxen induced a decrease in unclassified genera within the *Lachnospiraceae* family
(from the gram-positive *Clostridia* class) and increased abundance of the *Bacteroides* genus (from the gram-negative *Bacteroidia* class). On the other hand, ATB-346 administration resulted in a significant (p<0.05) increase in the abundance of *Lactobacillaceae* family (Figure 4).

In a separate experiment, rats were treated with a higher dose of naproxen (20 mg/kg) twice daily for 2 days to explore whether changes in particular bacteria correlated with the development of intestinal injury. Naproxen administration caused significant intestinal injury, resulting in a mean damage score of 90 ± 22. Interestingly, increased intestinal injury from naproxen treatment was positively correlated with higher abundances of several gram-negative microbial families (see Table 1). Naproxen administration also caused a significant decrease in the relative abundance of unclassified bacteria within the *Lachnospiraceae* family (p<0.05).

**PPIs Exacerbated Intestinal Injury and Inflammation**

Treatment of rats with naproxen at a dose below the threshold for inducing macroscopic intestinal ulcers did not cause significant inflammation, as measured by jejunal MPO activity. Treatment with a PPI also did not cause significant intestinal inflammation. However, co-treatment with naproxen and PPIs exhibited significant intestinal inflammation (p<0.05 Fig. 5A). This was observed with both omeprazole and lansoprazole. In contrast, administration of ATB-346 (14.5 mg/kg) for 4 days did not significantly affect jejunal levels of granulocyte infiltration when given alone, or when co-administered with a PPI (omeprazole) (Figure 5B).
**PPIs Increased Bile Cytotoxicity**

As described above, bile collected from rats treated twice daily for 4 days with naproxen (10 mg/kg) had enhanced cytotoxic effects compared to bile collected from rats treated with vehicle (Figure 5C). In rats co-treated with naproxen and a PPI, a significant increase in the cytotoxic effects of bile was observed as compared to bile from rats treated with naproxen alone (p<0.01). Bile collected from rats treated with an equimolar dose of ATB-346 exhibited similar cytotoxicity to bile collected from rats treated with vehicle, and this was not significantly affected by co-administration of omeprazole (Fig. 5D).

**PPIs Caused Intestinal Dysbiosis and Altered Luminal pH**

Previous studies suggested that treatment of rats with omeprazole resulted in significant changes in the intestinal microbiota, particularly a marked decrease in Actinobacteria (*Bifidobacter spp.*) (55). This was further investigated in the present study, using omeprazole and lansoprazole. Treatment of rats with omeprazole or lansoprazole for 9 days resulted in a marked shift in the composition of the intestinal bacterial community. As shown in Figure 6A, DGGE analysis of jejunal contents demonstrated distinct clustering of microbiota profiles of vehicle-treated rats versus overlapping clustering of microbiota from omeprazole- and lansoprazole-treated rats (as analyzed by the Dice coefficient and Ward algorithm to determine similarities) (13,56). Construction of a majority UPGMA tree demonstrated similar clustering with nearly 100% resampling support for each branch (Figure 6B).
The ability of treatment with either of the PPIs to markedly increase the cytotoxicity of bile was recapitulated in this experiment (Fig. 6C). Moreover, there were significant changes in the pH of the intestinal luminal contents in rats treated with omeprazole or lansoprazole as compared to that of vehicle-treated rats (Fig. 6D). Surprisingly, treatment with either of the PPIs resulted in a significant decrease in jejunal luminal pH.

To further investigate how PPI administration altered the intestinal microbiota in rats, we analyzed the taxonomic composition of the jejunal microbiota in rats by partial 16S rRNA gene profiling using Illumina sequencing. The *Firmicutes* phylum, specifically the *Lactobacillaceae* family, represented the vast majority (>95%) of bacterial abundance in jejunal microbiota samples in both PPI- and vehicle-treated rats. Administration of omeprazole or lansoprazole (each at 10 mg/kg) twice daily for 9 days resulted in a significant (p<0.05) increases in abundance of *γ-Proteobacteria* (Fig. 7), including the families *Pseudomonadaceae* and *Enterobacteriaceae*, compared to vehicle-treated rats. Omeprazole-treated rats exhibited a significant (p<0.05) decrease in the abundance of *Actinobacteria* compared to vehicle-treated rats, while lansoprazole treatment increased the relative abundance of Bacteroidetes (Fig. 7).

**DISCUSSION**

The GI toxicity of NSAIDs remains the major limitation to the use of this class of drugs. The mechanisms of NSAID-induced damage in the stomach and proximal duodenum are well understood (47) and that damage can largely be prevented by the
co-administration of inhibitors of gastric acid secretion, such as PPIs or H2RAs (23, 38).

Conversely, the complex pathogenesis of NSAID-induced damage in the more distal intestine remains incompletely understood and there are no proven-effective preventative or curative treatments (49). Moreover, it is becoming apparent that inhibitors of gastric acid secretion can significantly worsen NSAID-enteropathy (37, 55, 58). Considerable evidence indicates that interactions among bile, intestinal microbes and the enterohepatic circulation of NSAIDs are important contributors to the development of NSAID-induced intestinal damage (49). In recent years, it has been demonstrated that H2S is a particularly potent cytoprotective agent in the GI tract (52).

Indeed, an H2S-releasing derivative of naproxen (ATB-346) was previously shown to spare the rat gastrointestinal tract of damage, while the parent drug caused extensive mucosal injury and bleeding (7, 53).

In the present study, administration of a commonly prescribed NSAID (naproxen) dose-dependently caused intestinal ulceration and inflammation in rats. Naproxen is excreted in bile and undergoes extensive enterohepatic circulation. Treatment with naproxen significantly increased the cytotoxic effects of bile on cultured intestinal epithelial cells, consistent with effects seen with other NSAIDs in similar models (12, 64).

In contrast to the effects of naproxen, treatment of rats with ATB-346, an H2S-releasing derivative of naproxen, did not elicit intestinal damage or inflammation. Treatment with ATB-346 also did not increase the cytotoxicity of bile on cultured intestinal epithelial cells. Interestingly, although ATB-346 is metabolized to naproxen, the levels of naproxen in bile from ATB-346-treated rats were substantially lower than those observed in rats.
treated with an equimolar dose of naproxen, despite plasma levels of naproxen being comparable in the two groups.

The enterohepatic recirculation of NSAIDs is a major factor contributing to the intestinal damage caused by these drugs (3, 9, 35, 42, 59). When NSAIDs undergo enterohepatic recirculation, the intestinal epithelial cells are repeatedly exposed to the topical damaging effects of the NSAID and its metabolites (45). The substantially reduced enterohepatic circulation of ATB-346, or of naproxen liberated from this drug, may contribute significantly to its reduced intestinal-damaging effects. The in vitro bile cytotoxicity studies demonstrated that bile containing increased levels of naproxen (as well as metabolites such as naproxen-glucuronides) is significantly more damaging to intestinal epithelial cells. The fact that spiking bile with naproxen in vitro did not increase its cytotoxicity is consistent with previous studies suggesting that it is the naproxen-glucuronides secreted in bile that account for the increased damage it causes to intestinal epithelial cells (39). However, the enhancement of bile cytotoxicity following naproxen administration may also be related to changes in the composition of bile itself. Enteric bacteria can contribute to the pathogenesis of NSAID-enteropathy in numerous ways, including modification of the composition of the bile (4, 14). NSAID administration to rats has been shown to increase the concentration of secondary bile acids in bile (62). Secondary bile acids are more toxic to intestinal epithelial cells than primary bile acids (19, 64). Conversion of primary to secondary bile acids is dependent upon enteric bacterial enzymes, so NSAID-induced shifts in the microbiota could lead to altered cytotoxicity of bile. Moreover, an association between bile dysmetabolism and intestinal dysbiosis has been reported in patients chronically taking inhibitors of gastric
Acid secretion (40, 41). Enteric bacteria also play a major role in the enterohepatic circulation of NSAIDs. The re-absorption of NSAIDs in the small intestine requires hydrolysis of NSAID-glucuronides by bacterial β-glucuronidase (26). Pharmacological inhibition of bacterial β-glucuronidase has been shown to prevent NSAID-induced intestinal damage in mice (36).

Enteric microbiota can exacerbate intestinal injury and impede the healing of ulcers (16, 57). Several studies have demonstrated that NSAID administration caused significant changes in the intestinal microbiota, often increasing the abundance of gram-negative bacteria (11, 18, 21, 43, 46). The results of the present study are consistent with the previous studies with other NSAIDs: treatment with naproxen resulted in increased abundance of gram-negative bacterial families and a decreased abundance of several Firmicutes families, such as Lachnospiraceae. Interestingly, several members of the Lachnospiraceae family produce butyric acid, a short-chain fatty acid that has been shown to have an important role in maintaining the integrity of the intestinal mucosa (24). The enlarged presence of gram-negative bacteria could contribute to NSAID-induced intestinal injury by exacerbating the inflammatory response through activation of toll-like receptor 4 (TLR-4) (57). Indeed, we observed a significant correlation between the abundance of gram-negative bacteria and intestinal damage in naproxen-treated rats in the present study.

The present study also explored the mechanisms underlying the exacerbation of NSAID-enteropathy by PPIs. As mentioned above, inhibition of gastric acid secretion can lead to significant dysbiosis (40, 41, 55), and this was also shown to underlie a significant increase in susceptibility to intestinal damage in rats treated with PPIs (55). We
observed that treatment with a PPI resulted in an increased abundance of jejunal gram-negative $\gamma$-Proteobacteria and decreased abundance of jejunal gram-positive bacteria such as Actinobacteria and Firmicutes. The $\gamma$-Proteobacteria phylum includes many familiar enteric pathogens, such as Salmonella and Escherichia coli. It has been previously demonstrated that germ-free rodents monocolonized with E. coli are susceptible to NSAID-enteropathy, whereas germ-free rodents monocolonized with a gram-positive commensal bacteria did not develop intestinal ulcers (46). In our previous study on PPI-induced exacerbation of NSAID-enteropathy in rats, we observed a similar loss of Actinobacteria following PPI administration, and demonstrated that restoration of intestinal Bifidobacteria (a genus in the Actinobacteria phylum) prevented naproxen-induced intestinal ulceration and bleeding (55). A surprising finding in our study was the significant decrease in pH in the jejunum of rats treated with a PPI versus rats treated with vehicle. Such an effect could have played a part in the changes in the jejunal microbiota that were observed, but we cannot rule out the possibility that the altered pH was a consequence of the changes in the microbiota.

PPI treatment also resulted in a marked enhancement of bile cytotoxicity, which may account in part for the exacerbation of intestinal damage in rats co-treated with naproxen and a PPI. We previously reported that the cytotoxicity of bile is increased by H$_2$RAs (5), and exacerbation of intestinal damage is observed with the combination of H$_2$RAs and an NSAID (5, 37). The changes in the intestinal microbiota observed in the present study may contribute to the increase in bile cytotoxicity, by promoting the production of more cytotoxic, secondary bile acids in lumen of the small intestine (4, 29).
Although suppression of gastric acid secretion was associated with marked enhancement of bile cytotoxicity, this alone was not sufficient to produce overt intestinal damage. It is likely that suppression of intestinal PG synthesis (such as with naproxen administration), along with the enhanced cytotoxicity of bile, is necessary for ulcer induction. The lack of intestinal damage with ATB-346, despite inhibition of PG synthesis to a comparable extent as with naproxen, may be attributed to the lack of effect of this drug on the cytotoxicity of bile, and possibly to other actions of the H₂S that is liberated from this compound. H₂S has been shown to exert a range of potential cytoprotective effects in the GI tract (52), including stimulation of mucus secretion and favourable modulation of the intestinal microbiota (31). Moreover, H₂S can substitute for oxygen in driving mitochondrial production of adenosine triphosphate (22), and this may be particularly important in the context of NSAID-enteropathy, given the observations that NSAIDs can inhibit oxidative phosphorylation (42).

In summary, the present study provides evidence consistent with the hypothesis that biliary excretion of NSAIDs, topical irritant effects of bile and the intestinal microbiota contribute significantly to the intestinal ulceration caused by these drugs. Drug-induced changes in the microbiota could contribute significantly to the observed changes in cytotoxicity of bile, and to exacerbation of mucosal injury once it occurs. Exacerbation of NSAID-induced intestinal injury by inhibitors of gastric acid secretion is likely also a consequence of the effects of these drugs on the intestinal microbiota. ATB-346 spares the small intestine of injury despite profoundly suppressing intestinal PG synthesis, possibly because of substantially reduced biliary secretion of this drug and its
metabolites, but likely also due to H$_2$S-mediated cytoprotective and microbiota-modifying effects.

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**Disclosures**

Dr. Wallace is a founder and director of Antibe Therapeutics Inc., which is developing hydrogen sulfide-releasing anti-inflammatory drugs, including ATB-346.

**Author’s contributions**

RWB, JLW conception and design of research; RWB, GDP, AM, GJDS, and KLF performed experiments; RWB, GDP, AM, GJDS, AGB, JLW analyzed data; RWB, GDP, AM, GJDS, MGS, AGB interpreted results of experiments; RWB, GDP, AM, JLW prepared figures; RWB, GDP, GJDS, AGB, JLW edited and revised the manuscript; RWB, GDP, AM, GJDS, KLF, PB, MGS, AGB and JLW approved the final version of manuscript.
REFERENCES


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


55. Wallace JL, Syer S, Denou E, de Palma G, Vong L, McKnight W, Jury J, Bolla M, Bercik P, Collins SM, Verdu E, Ongini E. Proton pump inhibitors exacerbate NSAID-


**Figure Legends**

**Figure 1:** Effects of naproxen and ATB-346 (a hydrogen sulfide-releasing naproxen derivative) on small intestinal damage and inflammation. **Panel A:** Oral administration of naproxen twice daily for two days caused small intestinal damage that increased in severity in a dose-dependent manner. In contrast, ATB-346 administration caused less intestinal damage at all doses tested. At the dose of 30 mg/kg, there was significantly more damage with naproxen than with an equimolar dose of ATB-346 (**p<0.01; n=5 per group; Bonferroni’s Multiple Comparison test). **Panel B:** Naproxen increased intestinal inflammation (myeloperoxidase (MPO) activity) in a dose-dependent manner (**p<0.001 versus vehicle-treated rats; Dunnett’s Multiple Comparison test). At all doses tested, ATB-346 did not affect intestinal MPO activity. At the dose of 10 mg/kg and 30 mg/kg, intestinal MPO levels were significantly greater in rats treated with naproxen versus those treated with ATB-346 (\(\psi p<0.05\) and \(\psi\psi p<0.01\), respectively; Student’s t-test). Each group consisted of five rats. Results are shown as mean ± SEM.

**Figure 2:** ATB-346 and naproxen administration resulted in comparable suppression of cyclooxygenase activity. Naproxen (10 mg/kg) or ATB-346 (equimolar) administration twice-daily for 2 days significantly suppressed (by ≥95%) whole blood synthesis of thromboxane (TXB\(_2\)) versus vehicle administration (**p<0.001; panel A). 90 minutes after the final dose of naproxen or ATB-346 to rats, intestinal prostaglandin (PG) E\(_2\) synthesis was significantly inhibited (by ≥96%) compared to vehicle-treated rats (**p<0.01; panel B). Each group consisted of 5 rats. Results are shown as mean ± SEM.
and the data were analyzed by a one-way analysis of variance followed by Dunnett’s post-hoc test.

**Figure 3:** Naproxen administration dose-dependently increased cytotoxicity of bile and biliary concentrations of naproxen. **Panel A:** Bile collected from rats treated with naproxen (10 or 30 mg/kg) twice-daily for 2 days was significantly more cytotoxic to cultured rat intestinal epithelial cells (IEC-6) than bile collected from vehicle-treated rats (**p<0.001; Dunnett’s Multiple Comparison test). However, bile collected from rats treated with ATB-346 had significantly reduced cytotoxicity of bile as compared to rats treated with naproxen at equimolar doses (\( \psi p<0.05 \) for 10 mg/kg; \( \psi\psi\psi p<0.001 \) for 30 mg/kg; Student’s t-test). Data are shown as the mean ± SEM of at least 5 rats per group.

**Panel B:** Concentrations of naproxen in bile collected from rats treated with naproxen increased in a dose-dependent manner. At all doses tested, ATB-346 administration resulted in markedly reduced concentrations of naproxen in bile as compared to those in rats treated with equimolar doses of naproxen (\( *p<0.05, **p<0.01, ***p<0.001 \) versus the corresponding naproxen-treated group). ATB-346 was not detected in any samples of bile. The bile was collected from rats 3 hours after the final administration of the test drug or vehicle. Results are shown as mean ± SEM (\( n=5 \) per group). The data were analyzed by an unpaired, two-tailed Student’s t-test.

**Figure 4:** Treatment with naproxen or ATB-346 altered the taxonomic composition of the intestinal microbiota. Deep sequencing analysis of 16S rRNA via Illumina revealed changes in the taxonomic composition at the Family level of the jejunal microbiota.
following twice-daily administration to rats of naproxen (10 mg/kg), ATB-346 (14.5 mg/kg) or vehicle for 4 days (n=5 per group). Each coloured section represents the relative abundance of bacteria present within each group. Treatment with naproxen or ATB-346 significantly diminished the relative abundance of *Lachnospiraceae* compared to vehicle-treated rats. Treatment with naproxen significantly increased the abundance (relative to vehicle-treated rats) of *Bacteroidaceae*, whereas treatment with ATB-346 significantly increased the abundance of *Lactobacillaceae*. (*p<0.05 versus vehicle-treated; n≥5 per group; Mann-Whitney test).

**Figure 5:** Treatment with a proton pump inhibitor (PPI) increased bile cytotoxicity and intestinal inflammation. **Panel A:** There was no significant change in jejunal myeloperoxidase (MPO) activity in vehicle-treated rats co-administered naproxen (10 mg/kg; twice daily for 4 days), but co-administration of naproxen to omeprazole- or lansoprazole-treated rats resulted in a significant increase in MPO activity (*p<0.05, **p<0.01 versus vehicle-treated group). Rats co-treated with naproxen and omeprazole exhibited increased MPO activity as compared to vehicle- + naproxen-treated rats (*Δp<0.05). **Panel B:** Co-administration of ATB-346 to vehicle- or omeprazole-treated rats did not increase intestinal MPO activity. Bile was collected 3 hours after the final administration of the test drugs. **Panel C:** Bile collected from vehicle-treated rats co-treated with naproxen (10 mg/kg, twice-daily for 4 days) and a PPI was significantly more cytotoxic to cultured rat intestinal epithelial cells (IEC-6) than bile collected from rats treated with vehicle (*p<0.05). Naproxen administration to rats also receiving omeprazole or lansoprazole (twice daily for 9 days) resulted in significantly enhanced...
bile cytotoxicity as compared to bile collected from vehicle-treated rats co-treated with naproxen ($^{5}p<0.05$, $^{55}p<0.01$). Likewise, bile from PPI + naproxen co-treated rats was significantly more cytotoxic than bile from rats treated with vehicle alone (***$p<0.001$).

Panel D: In contrast to naproxen, the cytotoxic effects of bile collected from rats treated with ATB-346 (14.5 mg/kg) and a PPI were similar to that of bile collected from vehicle-treated rats. Co-administration of ATB-346 to omeprazole-treated rats did not alter bile cytotoxicity as compared to vehicle-treated rats. The data are expressed as the mean ± SEM of 6 rats per group and were analyzed by an unpaired, two-tailed Student’s t-test and a one-way analysis of variance followed by Dunnett’s and Bonferroni’s post-hoc tests.

Figure 6: Proton pump inhibitors (PPIs) cause intestinal dysbiosis, significantly enhance the cytotoxic properties of bile, and alter intestinal luminal pH. The dysbiosis was demonstrated using two methods (panels A and B). Panel A: Denaturing gradient gel electrophoresis (DGGE) analysis revealed that administration of omeprazole or lansoprazole to rats caused dysbiosis of the jejunal microbiota, with clustering distinct from that of vehicle-treated rats. Note that the DGGE results from all of the vehicle-treated rats are clustered together, separate from those from the PPI-treated rats. Similarly, in Panel B, where a resampling technique was employed (majority unweighted-pair-group method with arithmetic mean algorithm), the Ward dendrogram shows clustering of the microbiota profiles from vehicle-treated rats distinct from those of PPI-treated rats. Panel C: Bile collected from rats treated with omeprazole or lansoprazole, each at 10 mg/kg twice daily for 9 days, exhibited significantly enhanced
cytotoxicity when incubated for 3 h on rat intestinal epithelial (IEC-6) cells (**p<0.001 versus vehicle-treated group). Panel D: Administration of omeprazole or lansoprazole significantly lowered the pH of the jejunum lumen as compared to vehicle-treated rats (**p<0.01, ***p<0.001). Results are shown as mean ± SEM and the data were analyzed by a one-way analysis of variance followed by Dunnett’s post-hoc tests.

Figure 7: Proton pump inhibitors alter the small intestinal microbiota in rats. Partial 16S rRNA gene profiling using Illumina sequencing revealed marked changes in the taxonomic composition at the phylum level of jejunal microbiota of rats that received omeprazole or lansoprazole (each at 10 mg/kg) or vehicle twice-daily for 9 days (n=4-5 rats/group). The jejunal microbiota was dominated (>95%) by bacteria belonging to the phylum Firmicutes (lower panels). The upper panels depict the composition of the remaining ≤5% of the jejunal microbiota. Both omeprazole and lansoprazole significantly increased the relative abundance of γ-Proteobacteria as compared to vehicle-treated rats. Treatment with omeprazole significantly reduced the relative abundance of Actinobacteria in comparison with the group treated with vehicle, while treatment with lansoprazole significantly increased the relative abundance of Bacteroidetes. (*p<0.05 versus vehicle-treated; n≥5 per group; Mann-Whitney test).
TABLE 1
Associations between abundance of bacterial family colonization and severity of naproxen-induced intestinal injury

<table>
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<tr>
<th>Bacterial Family</th>
<th>$r^2$</th>
<th>p-value</th>
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<tr>
<td>Bacteroidaceae</td>
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<td>Porphyromonadaceae</td>
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<td>Enterococcaceae</td>
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<tr>
<td>Lachnospiraceae</td>
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<td>0.019</td>
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A

Bar graph showing the % Cytotoxicity of Naproxen and ATB-346 at different doses (mg/kg naproxen equivalent). The Y-axis represents % Cytotoxicity ranging from 0 to 50. The X-axis represents the dose (mg/kg naproxen equivalent) ranging from Vehicle to 30.

B

Bar graph showing the Biliary [Naproxen] (ng/mL) at different doses (mg/kg naproxen equivalent). The Y-axis represents Biliary [Naproxen] ranging from 0 to 3000. The X-axis represents the dose (mg/kg naproxen equivalent) ranging from 3 to 30.