NSAID-ENTEROPATHY: PATHOGENESIS AND PREVENTION

NONSTEROIDAL ANTI-INFLAMMATORY DRUG-ENTEROPATHY: THE PATHOGENIC ROLES OF BILE AND BACTERIA AND THE PROTECTIVE ROLES OF HYDROGEN SULFIDE

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TITLE: Nonsteroidal anti-inflammatory drug-enteropathy: the pathogenic roles of bile and bacteria and the protective roles of hydrogen sulfide.

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– ABSTRACT –

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a widely used class of drugs, due in part to the effective anti-inflammatory and analgesic properties they exhibit. Unfortunately, NSAIDs also exhibit substantial gastrointestinal (GI) toxicity. The mechanisms underlying the ability of NSAIDs to cause ulceration in the stomach and proximal duodenum are well understood, and this injury can largely be prevented through the suppression of gastric acid secretion by proton pump inhibitors (PPIs) or histamine H_2 receptor antagonists (H_2RAs). In contrast, the pathogenesis of small intestinal injury induced by NSAIDs (i.e., NSAID-enteropathy) is poorly understood, and there are no proven-effective therapies. This is a major clinical concern as NSAID-induced enteropathy and bleeding occur more frequently than NSAID-induced gastropathy, and is associated with significantly higher rates of morbidity and mortality. There is clear evidence that indicates important contributions to NSAID-enteropathy by bile, enteric bacteria, and the enterohepatic circulation of NSAIDs. However, it is not clear which of these mechanisms is/are the primary driver(s) of intestinal damage and injury. There is also evidence that hydrogen sulfide (H_2S) can protect the GI mucosa from ulceration and reduce the severity of NSAID-induced GI damage, although the mechanisms of H_2S-induced intestinal protection remain to be determined. Therefore, the **central aim of this thesis** was to evaluate the roles of bile, enteric bacteria, and the enterohepatic circulation of NSAIDs in the pathogenesis of NSAID-enteropathy, and to investigate the ability of H_2S to protect the small intestine from NSAID-induced damage. **Chapter 1** is an introduction to the relevant literature and **Chapter 2** is an outline of the thesis scope and

objectives. In **Chapter 3**, I demonstrated that the co-administration of an H_2S-releasing agent protected rats from NSAID-induced enteropathy, in part by preventing NSAID-induced dysbiosis and bile cytotoxicity. In **Chapter 4 and 5**, I established that the co-administration of PPIs and H_2RAs exacerbated NSAID-enteropathy in part by causing intestinal dysbiosis and enhanced bile cytotoxicity. Lastly, I demonstrated that the small intestine-sparing effects of an H_2S-releasing NSAID, ATB-346, are partly attributable to the reduced enterohepatic circulation of ATB-346 or the naproxen liberated from this drug (**Chapter 5**). In summary, the work presented in this thesis provided novel understanding of the complicated pathogenesis of NSAID-enteropathy by confirming that the nature of the bile, the enterohepatic circulation of NSAIDs, and the nature of the intestinal microbiota are of paramount importance. In addition, the results also demonstrated that hydrogen sulfide represents an effective preventative therapy for NSAID-enteropathy and that H_2S-releasing NSAIDs, such as ATB-346, have remarkable preclinical safety.

– ACKNOWLEDGMENTS –

Graduate studies were a significant transition from my undergraduate years. Initially, I felt ill suited for research because I did not see a clear return on the process. I had no predetermined course material and no tests to gauge my progression. Moreover, the answers were often unclear and only led to further questions. However, over the years I have learned that conducting good research, if nothing else, is a process. Learning to enjoy the process is important and can be highly rewarding. Experiments need to be revised, repeated, and results scrutinized, but the experiences gained from this process are lasting. While I have no doubt that my graduate work experiences will be instrumental to my professional career, I also believe that they will aid me in all of life's adventures.

To my supervisor, Dr. John L. Wallace, I thank you for giving me the opportunity to work with you over the years. During that time, I could not have asked for a better mentor. Your achievements and commitment to a variety of career endeavours, including research and a variety of academic and industry roles, are inspiring. You challenged me with thought provoking ideas and projects, while always remaining patient, encouraging, and compassionate. I cannot begin to thank you enough for the opportunities you afforded me and for your continuous support along the way. Your support has allowed me to discover my passion for research and further my career. I will always remember and appreciate the knowledge and advice you provided, both academically and in life, and for making my graduate journey so enjoyable!

To my supervisor committee, Dr. Alison Fox-Robichaud and Dr. Waliul Khan, thank you for dedicating your time, expertise, and continuous support to my graduate

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I would also like to thank my family, especially my parents, Robert and Frances, and my brother, Robin. My life will always be changing, but it is comforting knowing you are always there, providing your love and support. To my friends, thank you for making me smile and for reminding me that there is a life outside of the laboratory! And finally, thank you Sophie for your help and understanding over the years. You kept me sane during all the highs and lows, and I would not have been able to achieve this without your love and support. You inspire me to always strive for more and I cannot wait to see what lies ahead for us.

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– LIST OF ABBREVIATIONS AND SYMBOLS –

~	Approximately
©	Copyright
°C	Degree(s) Celsius
g	G-force
μM	micromolar
15-HETE	15-Hydroxyeicosatetraenoic acid
AA	Arachidonic acid
ATB-346	[2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester]
ATP	Adenosine triphosphate
ASA	Acetylsalicylic acid
BCA	β-cyano-L-alanine
BHI	Brain heart infusion
CA	Cholic acid
CBA	Columbia blood agar
CD11/18	B_2 integrins
CFU	Colony-forming units
CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CV	Cardiovascular
DADS	Diallyl disulfide
DCA	Deoxycholic acid
DGGE	Denaturing gradient gel electrophoresis
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide

EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GI	Gastrointestinal
GPCR	G-protein coupled receptor
h	Hour(s)
H_2RA	Histamine receptor antagonist
H_2S	Hydrogen sulfide
HOX	Hydroperoxidase
HT-29	Human intestinal epithelial cells
H^+/K^+ATPase	Hydrogen/potassium adenosine triphosphatase enzyme
H&E	Hematoxylin and eosin stain
ICAM-1	Intercellular adhesion molecule-1
IEC-6	Rat intestinal epithelial cells
ip	Intraperitoneal
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LPS	Lipopolysaccharide
mM	Millimolar
mmHg	Millimetre(s) of mercury
MPO	Myeloperoxidase
MRP2	Multidrug resistance-associated protein 2
ng/mL	Nanograms per millilitre
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
OA	Osteoarthritis
OTU	Operational taxonomic unit
PCA	Principle coordinates analysis
PDE	Phosphodiesterase

PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGG_2	Prostaglandin G_2
PGG/HS	Prostaglandin endoperoxide G/H synthases
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGT	Prostaglandin transporter
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity
PO	Per os
PPI	Proton pump inhibitor
ROS	Reactive oxygen species
SEM	Standard error of the mean
SI	Small intestine
SIBO	Small intestinal bacterial overgrowth
TBZ	4-hydroxythiobenzamide
tCA	taurocholic acid
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
tNSAID	Traditional nonsteroidal anti-inflammatory drug
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
UGT	UDP-glucuronosyltransferease
UPGMA	Unweighted-pair-group method with arithmetic mean
VCE	Video capsule endoscopy
VEGF	Vascular endothelial growth factor

– DECLARATION OF ACADEMIC ACHIEVEMENT –

Experiments were conceived and designed by Rory Blackler and John L. Wallace. Rory Blackler wrote this dissertation with contributions from John L. Wallace. Rory Blackler performed experiments with the assistance of others. For specific contributions to the experiments and papers in **Chapters 3-5**, please refer to the **Preface** at the beginning of each chapter.

– CHAPTER 1 –

INTRODUCTION

1.1. General Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used prescription and over-the-counter medications, due in part to the potent anti-inflammatory and analgesic properties they exhibit. On a given day, it is estimated that more than 30 million people that utilize this class of drugs (Sostres *et al*., 2013). In the United States of America, NSAIDs account for 60% of the over-the-counter analgesic market and are heavily relied upon for the long-term management of inflammation and pain in chronic inflammatory conditions such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis (Sostres *et al*., 2013; Wallace, 2013b). Therefore, NSAIDs are often prescribed for chronic use, which as discussed below, can result in significant side effects in the gastrointestinal (GI) tract and cardiovascular (CV) system. Patients requiring the chronic use of NSAIDs often have co-morbidities such as diabetes, obesity, and hypertension, and are often taking a multiple pharmaceuticals, which may further increase the risk of gastrointestinal complications (Blackler *et al*., 2012; Wallace, 2013b). NSAIDs are also efficacious in the acute treatment of mild-to-moderate pain, such as menstrual cramps, gout, post-surgery pain, and headaches. Other clinical uses are also emerging, most notably cancer chemoprevention, with numerous studies indicating that the use of NSAIDs may reduce the risk of colorectal cancer and possibly other GI-related cancers (Jacobs *et al*., 2007; Elsheikh *et al*., 2014).

The wide range of therapeutic uses has subsequently rendered the world market for NSAIDs a multi-billion dollar industry. Most notably, the NSAID market in developed countries is expanding due to a growing population of elderly citizens. The

prevalence of NSAID consumption at least once weekly among the elderly (>65 years old) has been reported as high as 70%, with over half of these individuals taking NSAIDs on a daily basis (Scarpignato & Hunt, 2010). With aging populations, the associated costs and prevalence of NSAID use are likely to escalate due to concomitant increases in the prevalence of age-related diseases, such as osteoarthritis.

Despite their strong efficacy in treating pain and inflammation, NSAIDs use is accompanied by a relatively high incidence of adverse effects. The major limitation to NSAID use remains the associated GI damage and bleeding. Specifically, the ability of NSAIDs to induce damage in the stomach and duodenum has been a major focus for researchers and physicians for decades. As such, the number of NSAID-associated upper GI clinical events has substantially diminished since the introduction of gastroprotective drugs, such as proton pump inhibitors (PPIs) and histamine H_2-receptor antagonists (H_2RAs) (Scheiman *et al.*, 2006; Lanas *et al*., 2009). PPIs and H_2RAs function to suppress gastric acid secretion, as gastric acid heavily contributes to the damage caused by NSAIDs in the stomach and duodenum (Wallace, 2008). The design of 'gastric-sparing' (e.g., selective cyclooxygenase (COX)-2 inhibitors) also contributed to the decline of NSAID-associated upper GI clinical events, although the benefits of taking a selective COX-2 inhibitor over traditional (tNSAIDs) are modest (Wallace, 2013b). Selective COX-2 inhibitors, such as rofecoxib and celecoxib, cause severe GI complications less frequently than tNSAIDs (non-selective COX inhibitors), but they still result in GI-damaging effects and can adversely affect other regions of the body. For instance, patients at risk for NSAID-associated GI adverse events experience similar rates

of ulceration after taking tNSAIDs or selective COX-2 inhibitors over a 6-month period (as high as 17.1 and 16.5%, respectively) (Scheiman *et al*., 2006). In addition, selective COX-2 inhibitors and tNSAIDs have been associated with adverse renal and CV events (Cheng & Harris, 2004; Kearney *et al*., 2006). The concerns surrounding CV toxicity in patients taking selective COX-2 inhibitors resulted in the recommendation that patients also take low-dose aspirin® (i.e., acetylsalicylic acid (ASA)) for cardioprotection, which negates the small benefits gained by taking a selective COX-2 inhibitor for reduced gastroduodenal toxicity (Silverstein *et al*., 2000; Laine *et al*., 2003).

For several decades, the ability of NSAIDs to cause significant damage and bleeding in the distal regions of the small intestine was largely unappreciated and overshadowed by damage in the stomach and duodenum. It is now recognized that NSAID-induced enteropathy and bleeding occurs more frequently than NSAID-induced gastroduodenal damage (Adebayo & Bjarnason, 2006; McCarthy, 2009). NSAIDs induce clinically significant small intestinal ulceration and bleeding in upwards to 70% of patients chronically taking these drugs (Bjarnason *et al*., 1993; Graham *et al*., 2005). In a study by Lanas *et al* (2009), a disconcerting trend was observed that highlighted the distal shift of NSAID-induced GI damage occurring in patients. More specifically, there was a sharp decline in the incidence of upper GI events and a gradual increase in lower GI events between 1996-2005 (Lanas *et al.,* 2009). This is particularly troubling as the authors also reported that lower GI events resulted in significantly higher rates of reoccurrence, death, and days of hospitalization compared to upper GI events. Unfortunately, there are currently no effective preventative therapeutic agents for NSAID

enteropathy (Wallace, 2013a). Furthermore, evidence continues to emerge that antisecretory drugs, such as PPIs and H_2RAs, exacerbate the small intestinal damage and bleeding caused by NSAIDs (Zhao & Encinosa, 2008; Lanas *et al.*, 2009; Wallace *et al.*, 2011; Blackler *et al.*, 2012; Satoh *et al.*, 2012).

Although modest improvements have been made in terms of NSAID-associated GI toxicity (i.e., design of selective COX-2 inhibitors and the use of antisecretory drugs), major limitations to the use of NSAIDs remain. There are no GI- and CV-sparing NSAIDs available for patients to date. In recent years, it has become well recognized that two endogenous gaseous mediators, hydrogen sulfide (H_2S) and nitric oxide (NO), play an important role in GI mucosal defence and injury repair (Wallace, 2007). This has prompted the design of gaseous-releasing NSAIDs that demonstrate comparable efficacy to parent NSAIDs in terms of inhibiting COX activity, but are devoid of GI toxicity in animal studies (Wallace *et al*., 2010; Blackler *et al*., 2012). However, it remains to be seen whether this new class of NSAIDs are both GI- and CV-sparing in humans. In addition, there continues to be a lack of understanding of the pathogenesis of NSAID-induced enteropathy, although many studies have highlighted the role of bile, enteric bacteria, and enterohepatic circulation, as critical underlying mechanisms (Wallace, 2012). A clearer understanding of these mechanisms should provide the necessary clues to develop GI-sparing NSAIDs.

This chapter focuses on the pathogenesis of NSAID-induced enteropathy and the protective role of H_2S in the GI tract, along with brief summaries on the following subjects: the history of NSAIDs, the biosynthesis of prostaglandins and their inhibition by

NSAIDs, the contributions of COX-1 and -2 to mucosal defence, the pathogenesis of NSAID-induced gastroduodenal damage, the enterohepatic circulation of NSAIDs and interactions between bile and bacteria, and the therapeutic potential of novel H_2S-releasing NSAIDs.

1.2. History of NSAIDs

More than 5 000 years have passed since records left by Hippocrates outlined the practice of using willow bark concoctions for the treatment of rheumatic diseases, fever, and pain (Vane, 1990). However, it would not be until 1763 that the first reported "clinical trial" of willow bark administration was published. The trial verified that patients presenting with ague (fever) were effectively treated with a willow bark-containing medicament (Stone, 1763). In 1829, salicin, the active ingredient in willow bark responsible for fever suppression was isolated and crystallized by French pharmacist Leroux (Burke *et al*., 2006). By the mid 1870s, synthetic salicins (i.e., salicylic acid and sodium salicylic) that demonstrated improved efficacy and solubility properties over salicin were popular drugs for the treatment of rheumatic fever in Western and Central Europe (Vane, 1990). Although synthetic salicins were efficacious in treating pain and fever, patients complained of the strong bitter taste. In hopes of alleviating the bitter taste of salicylic acid, Felix Hoffman, an employee of Bayer Corp., first synthesized acetylsalicylic acid from salicylic acid through an acetylation reaction (Vane, 1990). With the synthesis of acetylsalicylic acid, a new class of pharmaceuticals was born, NSAIDs. Hermann Dreser, the chief pharmacologist at Bayer Corp, formally introduced

acetylsalicylic acid (ASA) in 1899 as "aspirin". ASA was marketed as an effective way of delivering salicylic acid to the body, and as a drug that demonstrated analgesic, antipyretic, and anti-inflammatory properties (Wallace, 1997; Vane, 2000).

Despite decades of widespread ASA use and the advent of other numerous NSAIDs (e.g., indomethacin and diclofenac), it would take approximately 70 years before the mechanism of action of NSAIDs was discovered. In fact, long before the mechanism of action of NSAIDs was described two English clinicians reported evidence that NSAIDs could damage the stomach based on their gastroscopic observations of patients taking ASA (Douthwaite & Lintott, 1938). In the ensuing decades, further evidence emerged in the literature, often through case reports that noted an association between dark, rank and bloody stools and ASA use (Wallace, 2007).

Our understanding of NSAIDs was vastly expanded in the 1970s. In 1971, Sir John Vane and colleagues were credited with the discovery that NSAIDs produce their anti-inflammatory effects by inhibiting the enzymatic production of prostaglandin (PG) synthesis (Vane, 1971). These studies also demonstrated that ASA itself had pharmacological properties distinct from those of salicylic acid, and did not simply act as a pro-drug to salicylic acid (Wallace, 1997). However, it was around this time that larger clinical studies had emerged that clearly documented the increasingly apparent relationship between NSAID use and gastroduodenal ulcer formation (Levy, 1974). It is likely that the improved recognition of adverse GI effects caused by NSAIDs was driven by the enhanced potency of newly developed NSAIDs (e.g., indomethacin and fenamates) and an increased ability to visualize the upper GI tract via flexible endoscopy (Insel,

1990). Subsequently, in an effort to curtail the GI toxicity much focus turned to studying the enzymes inhibited by NSAIDs.

NSAIDs inhibit PG production by acting as reversible (excluding ASA), competitive inhibitors of COX activity (Burke *et al*., 2006). Two isoforms of COX have been identified, COX-1 and -2. COX-1 was originally identified in the mid 1970s. It was hypothesized as early as 1972 that a second COX isoform may exist (Hemler *et al*., 1976; Vane *et al*., 1998) but it was not until twenty years later that two separate groups announced the discovery of COX-2 (Kujubu *et al*., 1991; Xie *et al*., 1991). It was subsequently shown that the COX enzymes varied in expression. COX-1 was expressed constitutively in most cells, while the COX-2 isoform was rapidly expressed at markedly high levels at sites of inflammation and only minimally expressed in healthy tissues (Vane *et al*., 1994). An enticing theory quickly emerged and captured the attention of the pharmaceutical world. The theory suggested that the selective inhibition of the inducible COX-2 isoform (sparing COX-1) would reduce fever, pain, and inflammation, while sparing the GI tract of injury. In retrospect, this theory was reliant on two incorrect assumptions: 1) PGs that mediate fever, pain, and inflammation are solely generated by COX-2 and 2) the PGs produced by COX-1 are solely responsible for maintaining GI homeostasis (Wallace, 1999). Therefore, it was perceived that NSAID-induced GI toxicity was due to a lack of selectivity of tNSAIDs for COX-2 at clinically effective doses (Wallace, 1999). Numerous selective COX-2 inhibitors were designed based on the selective COX-2 theory, including celecoxib, rofecoxib, and valdecoxib. Initially, selective COX-2 inhibitors were introduced to the marketplace as alternatives to tNSAIDs

with the promise of GI safety. While some selective COX-2 inhibitors produce less gastroduodenal damage, it soon became apparent that selective COX-2 inhibitors were not devoid of GI toxicity (Wallace, 1999; Laine *et al*., 2003; Lanas *et al*., 2007; Graham *et al*., 2011). Several studies have demonstrated that the frequency and severity of small intestinal damage produced by tNSAIDs and selective COX-2 inhibitors is comparable (Maiden *et al*., 2007). For example, the VIGOR study, a large trial of rofecoxib (Vioxx), reported that 58% of patients taking rofecoxib developed small intestinal bleeds compared to 52% of patients taking naproxen, a tNSAID (Bombardier *et al*., 2000; McCarthy, 2009). The unfulfilled promise of GI safety is partially explained by the fact that, at clinically effective doses in humans, selective COX-2 inhibitors inhibit the synthesis of COX-2-derived PGs as well as suppress COX-1-derived PGs (Wallace, 1999).

The association of serious CV events with chronic NSAID use was more recently highlighted by the introduction of selective COX-2 inhibitors to the marketplace (Wallace & Vong, 2008). In fact, the heightened CV concerns associated with selective COX-2 inhibitors prompted the removal of several of these drugs from the market in recent years (i.e., rofecoxib and valdecoxib). Clinically, to reduce the risk of CV events, such as myocardial infarction and stroke, it is often recommended that patients taking selective COX-2 inhibitors also take low-dose ASA for cardioprotection (Laine *et al*., 2003; Kearney *et al*., 2006; Wallace, 2013b).

Currently, there are no 'GI-sparing' NSAIDs available for patients; however, as discussed below, promising results from NO-releasing NSAIDs and H_2S-releasing

NSAIDs in animal studies indicate that 'GI-sparing' NSAIDs may be available in the near future (Wallace, 2013a).

1.3. Prostanoids, Cyclooxygenase, and NSAIDs

1.3.1. Prostanoids. Eicosanoids are potent lipid mediators responsible for maintaining numerous homeostatic biological functions including the initiation and resolution of inflammation (Funk, 2001; Lawrence *et al*., 2002). The eicosanoid family consists of several arachidonate metabolite groups, including PGs, prostacyclin, thromboxane A_2 (TXA_2), leukotrienes, and lipoxins (Smyth *et al*., 2006). In humans, linoleic acid and lincolenic acid are two essential fatty acids that are required via ingestion for the synthesis of eicosanoids (Henzl, 2004; Murphy *et al*., 2004). In fact, linoleic acid is the precursor for arachidonic acid (AA), the most abundant eicosanoid precursor in mammalian systems (Henzl, 2004). Upon synthesis, AA is rapidly incorporated into phospholipids of the cell membrane. Eicosanoids are rapidly synthesized from AA in response to a variety of physical, chemical, and hormonal stimuli. Damaged cells release substances such as epinephrine, thrombin, and bradykinin that activate phospholipases (e.g., phospholipase A_2), which subsequently attack the membrane and liberate AA (Funk, 2001; Henzl, 2004). The availability of AA is the rate-limiting step in the rapid biosynthesis of eicosanoids (Smyth *et al*., 2006). Three major pathways may metabolize liberated AA: the COX, lipozygenase, or cytochrome P-450 pathway (Mohajer & Ma, 2000). Once synthesized, eicosanoids operate in an autocrine and paracrine manner, exerting their effects in the localized tissue (Funk, 2001). The

following paragraphs will focus on the biosynthesis of PGs and TXA_2, collectively known as prostanoids, and on the COX pathway since NSAIDs inhibit the COX isozymes responsible for prostanoid production.

Prostanoids are short-lived lipid mediators synthesized in response to mechanical stress, growth factors (e.g., epidermal growth factor), hormones, and inflammatory stimuli (Murphy *et al*., 2004). The biosynthesis of prostanoids occurs in the following general steps (Figure 1.1): 1. a stimulus activates phospholipases, liberating AA from the cellular membrane 2. free AA undergoes cyclooxygenation and hydroperoxidation by prostaglandin endoperoxide G/H synthases (PGG/HSs) (colloquially known as COXs) to produce prostaglandin H_2 (PGH_2), the precursor for all prostanoid subtypes and 3. PGH_2 is rapidly metabolized by tissue-specific isomerases to biologically active prostanoids (Funk, 2001).

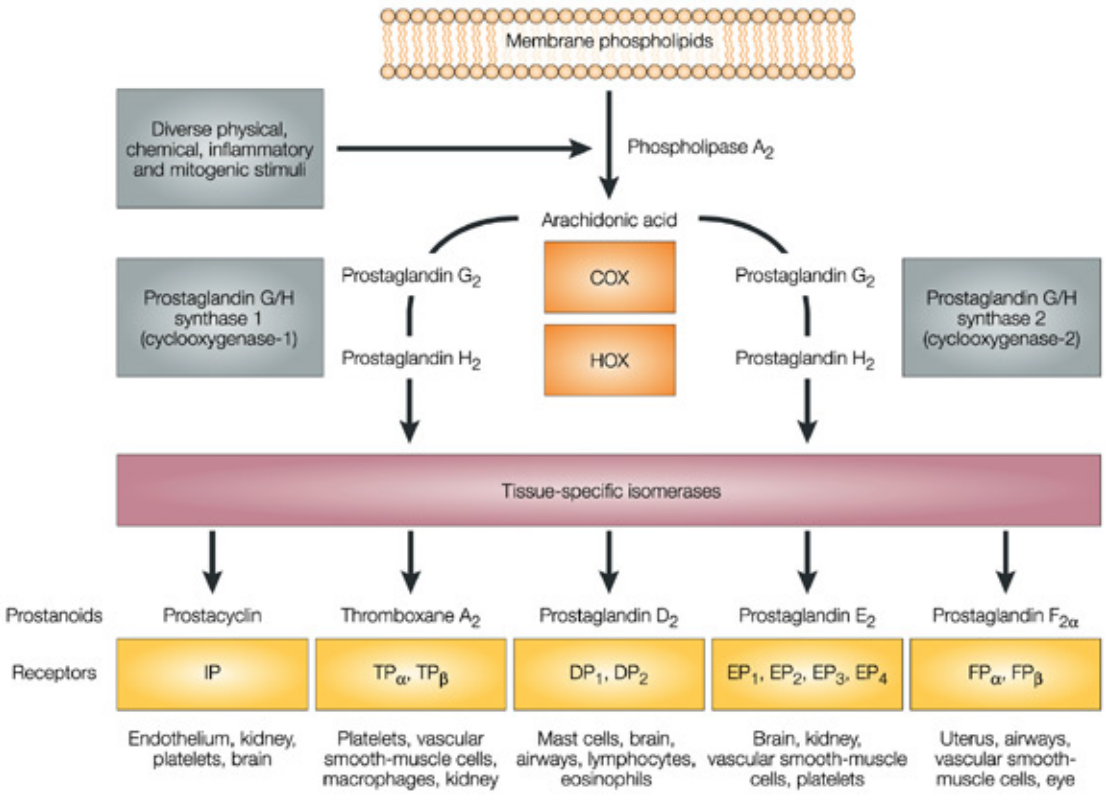

Figure 1.1. Prostaglandin Biosynthetic Pathway. A cellular stimulus triggers the activation of phospholipases in a 'generic' cell. The activated phospholipases release arachidonic acid (AA) from membrane lipids. Subsequently, COX isozymes metabolize AA to the unstable PGH_2 intermediate. Cell-specific isomerases then metabolize PGH_2 to biologically active prostanoids. The prostanoids then exert their actions locally on a family of prostaglandin receptors to mediate numerous physiological effects. [Figure from Fitzgerald, 2003]

In detail, PG synthesis is dependent on the activity of PGG/HS, which exists in two isoforms referred to as PGHS-1 (COX-1) and PGHS-2 (COX-2). These bifunctional isozymes contain both cyclooxygenase (COX) and hydroperoxidase (HOX) activities to catalyze the conversion of AA to PGG_2 and reduce PGG_2 to PGH_2, respectively

(Fitzgerald, 2003). PGH_2 is a chemically unstable precursor that acts as an immediate in the formation of all prostanoids (Funk, 2001). PGH_2 synthesis is coupled either to downstream isomerases or synthases that are expressed with some tissue specificity and generate distinct PGs (Funk, 2001; Henzl, 2004). However, the mechanism through which PGH_2 is delivered to downstream synthases remains unknown (Fitzgerald, 2003).

Generally, most cell types synthesize only one or two principal PG products. There are four major bioactive PGs and one thromboxane group generated in the mammalian system (Funk, 2001). Prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) are formed either non-enzymatically or by specific isomerases, termed PGH-PGD isomerase and PGH-PGE isomerase, respectively. Prostaglandin F_2 (PGF_2) and prostacyclin (PGI_2), along with thromboxane A_2 (TXA_2), require specific isomerases (PGF synthase, prostacyclin synthase, and thromboxane synthase, respectively) to be synthesized. Both PGI_2 and TXA_2 are unstable, active intermediates, which are rapidly and non-enzymatically inactivated to the compounds 6-keto-PGF_α and TXB_2, respectively (Murphy *et al*., 2004; Smyth *et al*., 2006). Prostanoids gain access to their G protein-coupled receptors (GPCRs) via either passive diffusion across the cell membrane or via transporters, such as prostaglandin transporter (PGT) (Schuster, 2002; Fitzgerald, 2003). There are 5 different classifications for prostanoid GPCRs, which are designated by the same letter as the natural prostanoid with the greatest affinity for the receptor (Smyth *et al*., 2009). One receptor has been identified for each of TXA_2 and PGI_2, while four distinct PGE_2 receptors ($EP_{1\text{-}4}$) and two distinct receptors for PGF_2 and have been identified (Figure 1.1) (Smyth *et al*., 2009).

PGs are not stored and must thus be continuously produced to help maintain homeostasis in the body and during an inflammatory response. In particular, PGE_2 and PGI_2 are considered pro-inflammatory and are responsible for the generation of pain and vasodilation (Wallace, 2008). Although PGs are particularly adept at initiating inflammation, they also play an important role in the resolution of inflammation (Lawerence *et al*., 2002; Ricciotti & Fitzgerald, 2011). For instance, PGD_2 has been implicated as a key mediator of the resolution of inflammation in various animal models (Serhan *et al*., 2007). Both the level and the profile of PG production change dramatically over the time course of an inflammatory response (Lawerence *et al*., 2002; Ricciotti & Fitzgerald, 2011). For instance, Gilroy and colleagues demonstrated that COX-2 expression and PGE_2 levels increased transiently in the initial stages of the inflammatory response in carrageenan-induced pleurisy in rats, however, COX-2 expression diminished after these initial stages (Gilroy *et al*., 1999). The resolution of the inflammatory response coincided again with the induction of COX-2, but high levels of PGD_2 and a metabolite of PGD_2, 15-deoxy$\Delta^{12\text{-}14}$ PGJ_2, were produced instead of PGE_2.

1.3.2. Cyclooxygenase. The COX isozymes are inserted primarily in the endoplasmic reticulum and nuclear membrane of the cell (Crofford, 1997). The binding pockets of the isozymes are exposed to bind liberated AA. Both COX-1 and -2 display comparable enzymatic function when AA is used as a substrate (Crofford, 1997). The structure of COX-1 and -2 are extraordinarily analogous, although COX-2 has a larger "side pocket" which allows it to bind a wider range of fatty acid substrates (e.g., eicosapentaenoic acid) (Vane *et al*., 1998; Smith *et al*, 2000; Fitzgerald, 2003). As

mentioned above, COX-1 is expressed constitutively in most cells, whereas COX-2 is rapidly upregulated by stimuli such as cytokines, shear stress, and growth factors. Thus, COX-1 is generally regarded as the basal enzyme responsible for homeostatic PG levels, whereas COX-2 is important in various inflammatory and "induced" settings (Funk, 2001; Fitzgerald, 2003). However, it is important to bear in mind that both COX-1 and -2 contribute to physiological and pathophysiological prostanoid production, which has been clearly demonstrated in the GI mucosa (Wallace, 2008).

1.3.3. NSAID classifications. A variety of NSAIDs have been developed since the design and introduction of ASA in the marketplace. NSAIDs are a chemically heterogenous group of compounds, although most are organic acids (Burke *et al*., 2006). As organic acids, NSAIDs are generally well absorbed when administered orally, are highly bound to plasma proteins, and are excreted by the kidney via glomerular filtration or tubular secretion, or in the bile (Burke *et al*., 2006). NSAIDs are generally classified into 6 classic groups based on chemical structure (Wallace, 1992; Burke *et al*., 2006): 1. salicylates (e.g., ASA), 2. acetic acids (e.g., indomethacin and diclofenac), 3. propionic acid derivatives (e.g., naproxen and ibuprofen), 4. oxicams (e.g., piroxicam), 5. pyrazolones (e.g., phenylbutazone) and 6. fenamates (e.g., mefenamic acid). Collectively, these six groups are recognized colloquially as "traditional NSAIDs" (tNSAIDs). A new subclass of NSAID classification was added in the mid-1990s with the introduction of selective COX-2 inhibitors to the market (Wallace, 1999). In recent years the development of two novel classes of gaseous-releasing NSAIDs, nitric oxide (NO)-

releasing NSAIDs and hydrogen sulfide (H_2S)-releasing NSAIDs, has added a promising subclass to the expanding list of NSAID classifications.

1.4. Inhibition of Prostaglandin Biosynthesis by NSAIDs

The principal therapeutic effects of NSAIDs are derived from their ability to inhibit PG synthesis. Vane and colleagues first demonstrated this in 1971 when they reported that low concentrations of ASA and indomethacin inhibited the enzymatic production of PGs. NSAIDs (excluding ASA) are reversible, competitive inhibitors of COX activity. All NSAIDs suppress COX activity by interacting with the bis-oxygenase subunit. This action prevents the introduction of molecular oxygen and binding of AA in the COX site. As previously mentioned, the COX isozymes also have peroxidase activity, however, NSAIDs have little effect on inhibiting this activity (Smith *et al*., 2000).

The ability of NSAIDs to inhibit either COX isozyme can vary. All tNSAIDs can inhibit COX-1 and -2 but they generally bind more tightly to COX-1 (Smith *et al*., 2000). On the other hand, selective COX-2 inhibitors exhibit selectivity for COX-2, but often also inhibit COX-1 at clinically effective doses. All NSAIDs compete with AA for the COX active site, but can exhibit varying modes of inhibition. The first mode is a rapid, simple, reversible competitive inhibition (e.g., ibuprofen and naproxen) of the COX isozymes. The second is a rapid, lower affinity, reversible binding mode that is followed by a time-dependent, higher affinity, slowly reversible binding (e.g., indomethacin). Finally, the rather unique third mode of inhibition is a rapid, reversible binding followed by irreversible, covalent modification (acetylation) (e.g., ASA) (Smith *et al*., 2000).

NSAIDs that exhibit the first mode of inhibition do not modify the conformation of COX, whereas the second mode of inhibition results in an enzyme-inhibitor complex and an ensuing conformational change (Burke *et al*., 2006). This conformational change is not permanent, which allows a slow regression to the initial COX enzyme conformation, thus restoring the ability for PG synthesis (Smith *et al*., 1996). The third mode of inhibition, exclusive to ASA, involves the covalent modification (an irreversible conformation change) of COX-1 and -2 by an acetylation reaction (Smith *et al*., 2000; Burke *et al*., 2006). The resulting acetyl group prevents AA from entering the active site by partially blocking the binding pocket. This modification permanently inactivates the COX enzyme and prevents the synthesis of prostanoids even after the NSAID detaches. Indeed, the ability of ASA to irreversibly inhibit (i.e., acetylate) COX-1 is the basis for its unique, long-lived cardioprotective effects. Unlike most cells, platelets cannot synthesize new COX-1 enzymes to replace the enzymes that are deactivated (via acetylation) by ASA. Therefore, these platelets lose the ability to synthesis and release TXA_2, an important prostanoid that promotes platelet aggregation (Smith *et al*., 1996). Interestingly, unlike acetylated COX-1, which cannot oxidize AA at all, acetylated COX-2 can still oxidize AA. However, instead of synthesizing PGH_2, acetylated COX-2 produces 15-hydroxyeicosatetraenoic acid (15-HETE) from AA (Lecomte *et al*., 1994). 5-lipoxygenase can then metabolize 15-HETE to yield 15-epilipoxin A4, which along with many other lipoxins, are potent anti-inflammatory mediators (Serhan & Oliw, 2001; Lawrence *et al*., 2002).

The selectivity of selective COX-2 inhibitors is derived from exhibit a mixed mode of COX inhibition. These drugs inhibit COX-2 via the second mode of inhibition discussed above (time-dependent, reversible conformational change), whereas they inhibit COX-1 via a rapid, competitive, reversible mode of inhibition (Smith *et al*., 2000). This results in extended periods of COX-2 activity inhibition compared to COX-1.

1.5. Contributions of COX-1 and -2 to Mucosal Defence

The inhibition of COX is central to the anti-inflammatory and analgesic actions of NSAIDs, as it prevents the synthesis of pro-inflammatory PGs. Alas, the synthesis of PGs by COX-1 and -2 also plays a crucial role in many physiological processes (Wallace, 2008). PGs are important mediators of GI mucosal defence and repair. Indeed, PGs help modulate virtually all aspects of mucosal defense, such as the secretion of luminal factors, the maintenance of mucosal blood flow, and the acceleration of ulcer healing (Wallace, 2008). Table 1.1 illustrates the many levels of gastric mucosal defence that PGs help mediate. The inhibition of PG synthesis renders the mucosa much more susceptible to damage induced by countless luminal irritants, such a gastric acid, bacteria, bile, and drugs (Wallace, 2008). For instance, the main PGs synthesized by the gastroduodenal mucosa (PGE_2 and PGI_2) are potent vasodilators that ensure mucosal blood flow is maintained to help neutralize back-diffusing acid when the epithelial barrier has been compromised (Wallace, 2012). The inhibition of PG synthesis compromises the ability of the GI mucosa to restore its structure and function following injury. For instance, Wallace and McKnight demonstrated that the administration of a topical irritant (hypertonic

saline) to fasted rats resulted in extensive gastric epithelial damage, but that the damage quickly resolved without the development of hemorrhage or ulceration. However, pre-treatment with an NSAID to inhibit PG synthesis rapidly worsened the epithelial damage caused by the irritant and prevented tissue repair, and resulted in severe hemorrhagic and erosive damage (Wallace & Mcknight, 1990).

Table 1.1 Gastric mucosal defence mechanisms

1. **Pre-epithelial mechanisms**
   - Mucus
   - Bicarbonate secretion
   - Surface active phospholipids
2. **Epithelial mechanisms**
   - Tight junction complex
   - Restitution
   - Growth factor secretion
   - Cell proliferation
3. **Sub-epithelial mechanisms**
   - Microcirculation
   - Leukocytes

[Table adapted from Matsui et al., 2011]

COX-1 and -2 have overlapping roles in the maintenance of GI mucosal defence (Wallace *et al*., 2000). The failure of selective COX-2 inhibitors to spare the GI tract from damage and bleeding is a strong example of this overlap. While COX-1 expression contributes the majority of PGs produced in the GI mucosa of a healthy individual, numerous studies have demonstrated that when the GI mucosa is inflamed. COX-2

expression is markedly upregulated and the contribution of COX-2-derived eicosnaoids to mucosal defence is much greater (Souza *et al*., 2003). For example, substantially increased COX-2 expression is observed following acid-induced mucosa damage, periods of ischemia, or when COX-1 is inhibited (Davies *et al*., 1997; Maricic *et al*., 1999; Gretzer *et al*., 2001). It appears that the upregulation of COX-2-derived PGs following mucosal damage is a means of fortifying mucosal defence mechanisms (e.g., increasing blood flow and bicarbonate secretion) and enhancing injury repair (e.g., ulcer healing) (Reuter *et al*., 1996; Smith & Langenbach, 2001; Ma *et al*., 2002). The ability of COX-1 and -2 to compensate for one another to prevent significant gastrointestinal damage from occurring has been demonstrated in several studies using a rat model of NSAID-induced gastroenteropathy. Wallace and colleagues demonstrated that NSAID-induced gastroduodenal damage requires the inhibition of both COX-1 and -2 by administering various combinations of COX-1 or -2 specific inhibitors (Wallace *et al*., 2000). The authors provided further support for this hypothesis by varying the dose of ketorolac, an NSAID highly selective for COX-1, administered to rats (Figure 1.2).

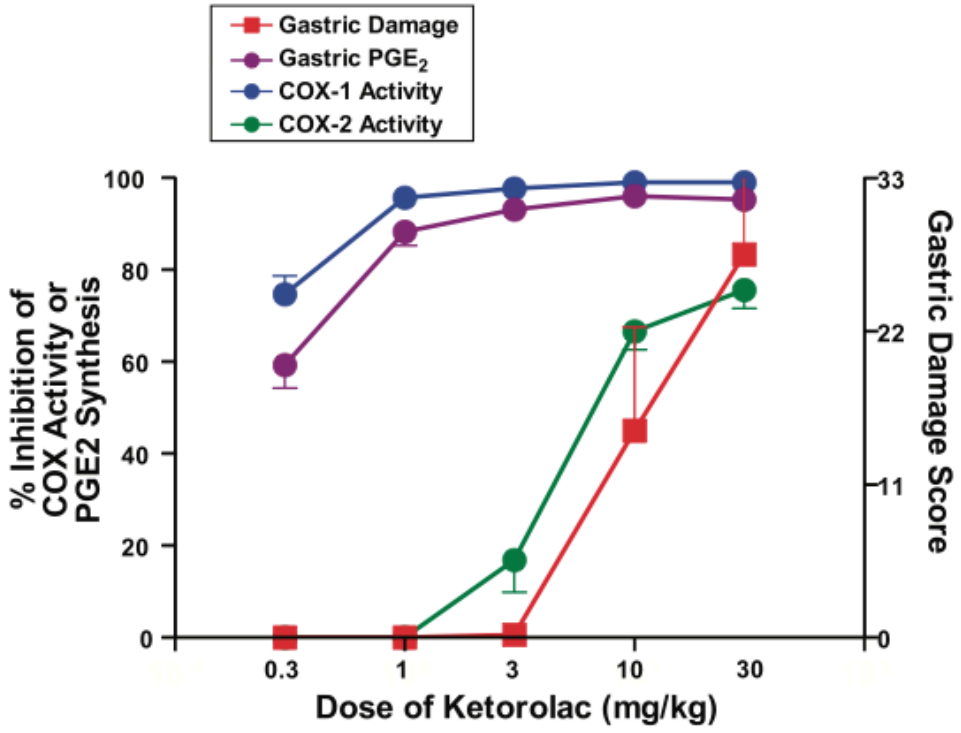

Figure 1.2. Dose-related inhibitory effects of ketorolac on COX-1 and -2 activity and gastric PGE_2 synthesis administration, and the relation to gastric damage. Low doses of ketorolac caused COX-1 activity inhibition only and resulted in no gastric damage whereas administration of higher doses that produced significant concomitant inhibition of COX-1 and -2, caused extensive gastric damage. [Figure from Wallace *et al*., 2000]

It has also been noted that the administration of low-dose ASA, a more potent inhibitor of COX-1 than of COX-2, to rats causes no gastric or small intestinal damage, but results in a rapid increase in COX-2 expression (Fiorucci *et al*., 2002; Blackler *et al*., 2012). However, when the rats are co-administered a selective COX-2 inhibitor, they develop extensive gastric and small intestinal damage. Similarly, the co-administration of low-dose ASA and a selective COX-2 inhibitor in humans increased the incidence and severity of gastrointestinal damage and bleeding (Laine *et al*., 2003; Wallace, 2013b). It

is possible that this phenomenon can be explained in terms of COX inhibition. The compensatory increase in COX-2 following low-dose ASA administration likely increased the resistance of the GI mucosa to injury by causing elevated PG synthesis. However, co-treatment with a selective COX-2 inhibitor prevented the ASA-induced COX-2-derived PGs from being produced, leading to a compromised mucosal state and subsequent damage.

The notion that mucosal defense is dually mediated by both COX-1 and -2 is further exemplified by studies where mice have been genetically modified to be deficient of one of the COX isozymes. For example, COX-1-deficient mice had low endogenous levels of gastric mucosal PG synthesis, but did not spontaneously develop gastric ulcers (Langenbach *et al*., 1995). Also, COX-2-deficient mice demonstrated an impaired ability to resolve inflammation, suggesting that COX-2 is an important contributor to the production of anti-inflammatory mediators (Wallace *et al*., 1998).

Although both COX-1 and -2 contribute to the physiological maintenance of the gastrointestinal tract, NSAID-induced COX inhibition does not fully explain the pathogenesis of NSAID-induced gastroenteropathy. This is especially true in the unique mechanisms of NSAID-enteropathy.

1.6. Pathogenesis of NSAID-induced Gastropathy

As reported in their 1938 gastroscopy study, Douthwaite and Lintott provided the first clear demonstration that NSAIDs could elicit bleeding patients' stomachs. Since that time, the mechanisms underlying the ability of NSAIDs to cause ulceration in the

stomach and proximal duodenum (i.e., gastropathy) have been well documented and understood. Despite major advances in our understanding of NSAID-induced gastropathy, GI toxicity remains the major limitation to NSAID use (Scarpignato & Hunt, 2010; Wallace, 2012). There are currently no 'GI-sparing NSAIDs' available, although it is now understood that damage in the stomach and duodenum can largely be prevented by the suppression of gastric acid secretion. The following paragraphs will address the mechanisms of NSAID-induced gastropathy and the prevention of gastropathy by antisecretory drugs (i.e., PPIs and H_2RAs) and muco-protective drugs.

Approximately 30 to 50% of NSAID users have endoscopic lesions, primarily in the antral and corpal regions of the stomach, and often without clinical manifestations (Sostres *et al*., 2013). However, erosions in the antral region of the stomach can often progress to subepithelial hemorrhages and ulcerations (i.e., penetration through the muscularis mucosa), resulting in life threatening perforations and bleeds (McCarthy, 1990; Sostres *et al*., 2010). Upper GI bleeds are particular dangerous given that platelets are unable to aggregate in acidic (i.e., pH<4) environments (Green *et al*., 1978). The main risk factors for NSAID-induced gastroduodenal injury include older age (≥60 years), prior history of peptic ulceration, concomitant use of anticoagulants (including low-dose ASA) and/or corticosteroids, and the use of high-dose or multiple NSAIDs (Laine, 2006). Patients at high risk for upper GI clinical events are often co-prescribed an inhibitor of gastric acid secretion or placed on a selective COX-2 inhibitor in addition to low-dose ASA, although both of these treatment regiments have their own inherent risks (Scheiman & Hindley, 2010; Wallace, 2013b).

There are two major components to NSAID-induced gastroduodenal ulceration and bleeding: (1) the local, topical damaging actions on the epithelium and (2) the systemic actions (i.e., COX inhibition) (Figure 1.3).

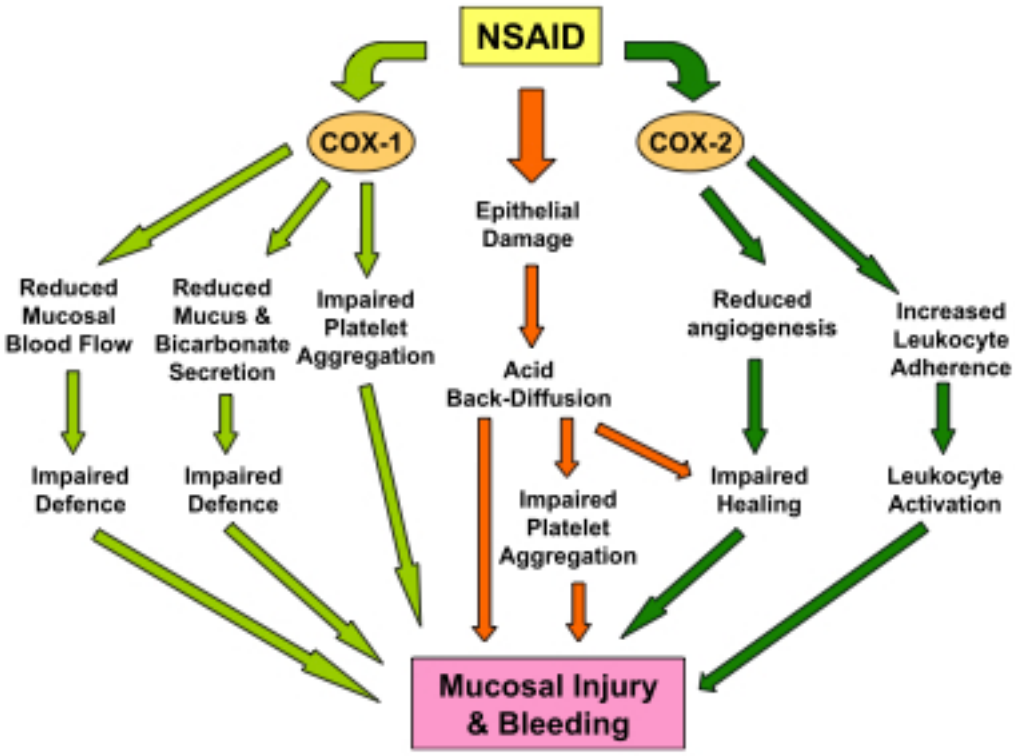

Figure 1.3. Pathogenesis of NSAID-induced gastropathy. The exposure of local epithelium to the direct, cytotoxic effects (red arrows) of NSAIDs can cause mucosal injury/bleeding. Similarly, the inhibition of cyclooxygenase (COX)-1 and -2 activity (green arrows) contributes to mucosal injury/bleeding by compromising mucosal defence and impairing healing. However, the effects elicited by selective inhibition of COX-1 or -2 only, or topical damage alone, is unlikely to produce clinically significant gastroduodenal damage. [Figure from Wallace, 2008]

The topical effects of NSAIDs on the epithelium result in several damaging outcomes. Many NSAIDs are weak, organic acids (e.g. naproxen) and are capable of

damaging the cellular membrane of epithelial cells when in direct contact (Tarnawski *et al*., 1988). In the presence of stomach acid, these NSAIDs are deprotonated, charged compounds that can become trapped within epithelial cells and induce osmotic lysis (Somasundaram *et al*., 1995). Furthermore, NSAIDs can cause the uncoupling of oxidative phosphorylation in epithelial cells that leads to cell death (Somasundaram *et al*., 1995). NSAIDs can also directly disrupt the layer of surface-active phospholipids on the mucosal surface and thus render it susceptible to damage by various luminal agents, most notably gastric acid (Giraud *et al*., 1999; Lichtenberger *et al*., 2006). It has also been reported that NSAIDs directly inhibit epithelial repair by interfering with epithelial growth factor (EGF) signaling pathways, which are important in promoting epithelial cell proliferation following injury (Pai *et al*., 2001; Kajanne *et al*., 2007). However, the topical damaging effects of NSAIDs are not critical to the development of the clinically significant gastroduodenal damage caused by NSAIDs. Numerous studies have demonstrated that the topical exposure of the gastroduodenal mucosa to NSAIDs via oral administration is not required for ulcer formation. Indeed, NSAID-induced gastroduodenal ulcers still occur by means of parenteral (e.g., topical or intravenous) administration (Whittle *et al*., 1985; Estes *et al*., 1993; Wallace & McKnight, 1993). Furthermore, NSAIDs designed to limit topical gastric damage (i.e., enteric-coated and/or prodrug NSAID formulations) exhibit comparable incidences of gastric ulceration and bleeding to that of the parent NSAID (Graham *et al*., 1985; Carson *et al*., 1987; Wallace, 2008).

Conversely, the primary mechanism of action through which NSAIDs damage the gastroduodenal mucosa is the suppression of mucosal PG synthesis. For example, the extent to which an NSAID inhibits mucosal PG synthesis correlates very well with its ability to induce gastroduodenal damage (Whittle, 1981; Rainsford & Willis, 1982). A strong temporal correlation also exists between the inhibition of mucosal PG synthesis and the onset of gastroduodenal damage (Whittle, 1981; Wallace, 2008). However, it is important to note that marked suppression of gastric PG synthesis does not always result in gastric damage (Figure 1.2) (Ligumsky *et al*., 1983; Wallace *et al*, 2000). The suppression of gastric PG synthesis renders the mucosa vulnerable to the damaging effects of luminal agents, such as gastric acid, pepsin, bacteria (e.g. *Helicobacter pylori*), and to the topical damaging effects of NSAIDs themselves (Wallace, 2008; Matsui *et al*., 2011). The reason for this being, several aspects of gastric mucosal defense are mediated by PGs, such as mucus and bicarbonate secretions, mucosal blood flow, epithelial cell turnover and repair, and even mucosal immunocyte function (Wallace & Tigley, 1995; Wallace, 2008).

An early and critical event involved in NSAID-induced gastropathy is the damage to the vascular endothelium caused by an increase in leukocyte adherence (primarily neutrophils) shortly after NSAID administration (Wallace, 2008). Several studies have demonstrated that the administration of NSAIDs to rats resulted in an increase in the number of neutrophils adhering to the vascular endothelium in the gastric microcirculation (Wallace *et al*., 1990; Wallace *et al*., 1993). This results from an NSAID-induced alteration in inflammatory mediator production (e.g., decreased PG

synthesis and increased tumor necrosis factor-alpha (TNF-α) release), which subsequently increases leukocyte-endothelial adhesion by upregulating the expression of both endothelial (e.g., intercellular adhesion molecule-1 (ICAM-1)) and leukocyte (CD11/18) adhesion molecules (Wallace *et al*., 1993; Andrews *et al*., 1994). An increase in the release of TNF-α and leukotriene B_4 can be observed following NSAID administration in rats, and likely potentiates leukocyte adherence in part by stimulating the expression of adhesion molecules (Asako *et al*., 1992; Santucci *et al*., 1994; Appleyard *et al*., 1996). It has been reported that an increase in leukocyte adherence to the gastric endothelium can contribute to mucosal injury via two main mechanisms (Wallace, 2008): 1. Neutrophil adherence in the microcirculation could further the obstruction of mucosal blood flow caused by the inhibition of PGI_2 and PGE_2 synthesis (Wallace & Granger, 1999), 2. Adhered, and thus likely activated, neutrophils release reactive oxygen metabolites and proteases that are capable of mediating the mucosal injury caused by NSAIDs (Vaananen *et al*., 1991; Wallace, 1997). The role of NSAID-induced neutrophil adherence is further supported by studies where pharmacological interventions were used to prevent NSAID gastropathy. Rats made neutropenic through treatment with an anti-neutrophil antibody did not develop hemorrhagic lesions upon NSAID administration (Wallace *et al*., 1990). In addition, the pre-treatment of rats with monoclonal antibodies for ICAM-1 or CD11/18 prevented NSAID-induced leukocyte adherence and protects the stomach from NSAID-induced damage (Wallace *et al*., 1993). It has also been reported that the suppression of PGI_2, an important inhibitor of neutrophil activation and adherence, may contribute to the increased neutrophil adherence observed after NSAID administration (Wallace, 1992).

The impairment of mucosal healing that is observed following NSAID administration is likely due in part to the effect of NSAIDs on platelets, which are important contributors to ulcer healing (Wallace, 2008). The acceleration of ulcer healing by platelets is related to the release of vascular endothelial growth factor (VEGF), a proangiogenic protein that stimulates blood vessel growth (Wallace, 2008). Reduced healing is observed in rats that are immunodepleted of platelets, and the transfusion or oral administration of platelets can restore normal healing in thrombocytopenic rats (Ma *et al*., 2001). NSAID administration in rats results in a significant decrease in serum levels of VEGF and increased levels of endostatin, an antiangiogenic factor (Ma *et al*., 2001).

While the suppression of PG synthesis is paramount to the pathogenesis of NSAID gastroduodenal injury, such injury can be prevented through the delivery of exogenous mediators of mucosal defence. For instance, oral administration of exogenous PGs can prevent gastric damage in rats (Robert *et al*., 1979). Likewise, co-prescribing a PGE_1 analogue, misoprostol, to NSAID users has been shown to effectively reduce the incidence of gastroduodenal ulcers (Graham *et al*., 1993). However, there is a high incidence of adverse effects (e.g., diarrhea) in patients taking misoprostol, which limits the use of this drug. The administration of two gaseous mediators (i.e., nitric oxide (NO) and hydrogen sulfide (H_2S)) has also been shown to reduce NSAID-induced gastroduodenal damage in animal models (Wallace, 2008; Wallace, 2010). The ability to reduce leukocyte-endothelial cell adhesion may partially explain why both NO and H_2S were able to prevent or reduce NSAID-induced gastroduodenal injury (Wallace *et al*.,

1997b; Zanardo *et al*., 2006). NO and H_2S are also potent vasodilators and can thus counteract the reduced mucosal blood flow caused by NSAID administration (Wallace, 2007). Furthermore, the administration of NO and H_2S has been shown to promote ulcer healing and resolve mucosal inflammation (Wallace, 2013a).

Although the administration of exogenous mediators of mucosal defence can limit or prevent NSAID-gastropathy, the most commonly used agents for preventing and treating NSAID-induced gastroduodenal damage are inhibitors of gastric acid secretion (i.e., antisecretory drugs). Extensive clinical evidence demonstrates that H_2RAs and PPIs, two classes of antisecretory drugs, reduce upper GI tract bleeding and ulcerations caused by NSAIDs, as well as accelerate the healing of pre-existing injury (Taha *et al*., 1996; Scheiman *et al*., 2006; Scarpignato & Hunt, 2010). H_2RAs are competitive antagonists of histamine at the parietal cell H_2 receptor and thus, when bound, inhibit gastric acid secretion by parietal cells in the stomach (Marks, 1992). The development of H_2RAs (e.g., cimetidine and famotidine) in the 1970s was hailed as a major breakthrough in the treatment of peptic ulcers (Marks, 1992). However, H_2RAs cannot completely prevent the secretion of gastric acid as parietal cells can also secrete acid in response to acetylcholine and gastrin. In the late 1980s, a new class of antisecretory drugs (i.e., PPIs) emerged with a mode of action quite different from H_2RAs. The first and second PPI to enter the market were omeprazole and lansoprazole, respectively (Satoh, 2013). PPIs act by irreversibly blocking the hydrogen/potassium adenosine triphosphatase (H^+/K^+ ATPase) enzyme of the parietal cell (Satoh, 2013). The H^+/K^+ ATPase enzyme, commonly referred to as the gastric proton pump, is directly responsible for secreting H^+ ions into the gastric lumen.

By irreversibly binding to the gastric proton pump, PPIs permanently deactivate it and thus prevent it from secreting H^+ ions (Satoh, 2013). Therefore, PPI administration causes a pronounced and long-lasting reduction in gastric acid production. The use of PPIs has largely superseded H_2RAs as they demonstrate superior efficacy and ulcer healing rates in patients (Eriksson *et al*., 1995). The success of antisecretory drugs in preventing NSAID gastroduodenal injury is highlighted by the numerous combination tablets of an NSAID and a PPI (or H_2RA) becoming available in the market (Wallace, 2012). However, as discussed in section 1.8, it is becoming increasing clear that the co-administration of antisecretory drugs and NSAIDs contributes to a significant aggravation of NSAID-enteropathy (Wallace, 2013b).

1.7. The Enterohepatic Circulation of NSAIDs and Interactions Between Bile and Bacteria

1.7.1. The enterohepatic circulation of NSAIDs

Hepatocytes produce bile by secreting bile acids, cholesterol, phospholipids, and other solutes into the canaliculi, between adjacent hepatocytes (Floch, 2002). The bile flows through the biliary tree, and eventually into the common bile duct for delivery to the small intestine (Floch, 2002). Once the bile enters the small intestine, some biliary constituents (e.g., bile acids) are readily absorbed via enterocytes, then transferred to the portal blood to be extracted by hepatocytes, and subsequently re-secreted into the bile. This process, known as the enterohepatic circulation, enables the cycling of many xenobiotic compounds in addition to the redelivery of endogenous constituents (e.g., bile

acids). Specific transporters are required for the canalicular and intestinal transport of xenobiotic compounds, and several studies have demonstrated a minimal difference between humans and rodents in the expression and regulation of these transporters and thus, rodent are often effective translational models for studying the enterohepatic circulation of xenobiotics (Treinen-Molsen & Kanz, 2006).

It has been recognized that bile plays a critical role in NSAID-enteropathy due to the damaging effects of bile acids on the intestinal epithelium, and due to the high concentration of NSAID, or the respective drug metabolites, in the bile (Wallace, 2013a). Indeed, the vast majority of NSAIDs undergo enterohepatic circulation. In addition, biliary drug delivery is an important factor in NSAID-induced intestinal injury. Initially, NSAIDs are absorbed and delivered via the blood to the liver where they are metabolized via phase I and phase II (i.e., conjugated) biotransformations (Burke *et al*., 2006). Phase II conjugation reactions (i.e., glucuronidation, acetylation, and sulfation reactions) increase the polarity of NSAIDs and respective metabolites before the secretion of these into the bile or urine (Gonzalez & Tukey, 2006). NSAIDs with a carboxylic acid functional group undergo glucuronidation (attachment of glucuronic acid), which is catalyzed by several types of UDP-glucuronosyltransfereases (UGTs) (Gonzalez & Tukey, 2006). This renders the multi-drug resistance-associated protein 2 (MRP2) as the dominant exporter responsible for the secretion of conjugated NSAIDs into the bile (Gerk & Vore, 2002). Once delivered via the bile back into the small intestine, conjugated NSAIDS are deconjugated by bacterial β-glucuronidase. Deconjugation is necessary for the passive reabsorption of NSAIDs across enterocytes and typically occurs in the distal

regions of the small intestine as these regions harbour larger bacterial communities that express β-glucuronidase (Floch, 2002; Treinen-Molsen & Kanz, 2006). NSAIDs can undergo multiple cycles of biliary secretion, as elucidated by pharmacokinetic studies that demonstrated a second, delayed peak of drug detectable in the bile (Peris-Ribera *et al*., 1991). While this prolongs the duration of NSAID action, it also delays the elimination of the drug and repeatedly exposes the small intestine to the damaging effects of the drug (Buxton, 2006; Wallace, 2013a).

1.6.2. Interactions between bile and bacteria

Primary bile acids are synthesized in the liver from cholesterol and conjugated to either taurine or glycine before they are secreted into the bile ducts (Floch, 2002). The major primary bile acids in humans and rodents are cholic acid and chenodeoxycholic acid (Floch, 2002). However, rodent livers are also capable of modifying chenodeoxycholic acid to muricholic acids, such as β-muricholic acid (Fisher *et al*., 1976). Conjugated primary bile acids are secreted into the bile and delivered to the small intestine where they are subsequently modified by bacterial enzymatic, bio-transforming reactions. Conjugated bile acids first undergo a deconjugation step to liberate the bile acid and then often undergo bacterial dehydroxylation and dehydrogenation reactions (Begley *et al*., 2005). These reactions produce secondary bile acids such as deoxycholic acid, lithocholic acid, and ursodeoxycholic acid (Floch, 2002). Without enteric bacteria, secondary bile acids cannot be produced (Duboc *et al*., 2013). Conjugated and unconjugated bile acids are absorbed by passive diffusion along the entire GI tract, but the majority of bile acids are reabsorbed in the distal ileum by active transporters (Begley

et al., 2005). Reabsorbed bile acids enter the portal bloodstream and are rapidly taken up by the liver, reconjugated and resecreted into the bile. The intestinal absorption of bile acids is 95% efficient, thus the majority of bile acids are retained and re-circulated many times (Floch, 2002).

Since bacteria play a major role in the modification and production of bile acids, dramatic shifts in intestinal bacteria populations can alter both the quantity and type of bile acids absorbed in the intestinal lumen (Begley *et al*., 2005). Conversely, bile acids have anti-microbial properties and can influence the bacteria strains present within the GI tract (Begley *et al*., 2005). For instance, it has been demonstrated that bile acids regulate the composition of the rat cecal microbiota (Islam *et al*., 2011). As a result of the interactions between bacteria and bile, alterations in the intestinal microbiota can result in significant changes to bile composition and vice versa.

1.8. Pathogenesis of NSAID-induced Enteropathy and Preventative Strategies

1.8.1. Overview of the clinical problem

The pathogenesis of NSAID-induced damage in the upper GI tract is well appreciated and can be effectively managed through co-therapy with gastroprotective drugs, such as antisecretory drugs (Scheiman *et al.*, 2006; Wallace, 2008). Unfortunately, the ability of NSAIDs to induce significant damage and bleeding in the small intestine (i.e., enteropathy), where the majority of NSAID damage occurs, has historically been under-recognized (Adebayo & Bjarnason, 2006; Lanas *et al*., 2009; Wallace, 2013a). Indeed, it was almost 50 years after the first report of NSAID gastropathy that it became

clear that NSAID use was also associated with significant damage to the distal regions of the small intestine (Bjarnason *et al*., 1987). However, improved methods for detecting the damage, such as video capsule endoscopy and double-balloon enteroscopy, have refocused much of the recent research to the small intestine (Wallace, 2013a). It has been reported that significant small intestinal damage, inflammation, and bleeding can be observed in about 70% of patients chronically taking NSAIDs (Bjarnason *et al*., 1993; Graham *et al*., 2005). Furthermore, McCarthy reported that the majority of GI bleeds in patients taking rofecoxib or naproxen originated from lesions in the small intestine (McCarthy, 2009). Even low-dose ASA administration for 2 weeks was found to cause significant small intestinal damage in 80% of patients (Endo *et al*., 2009). Table 1.2 lists the frequency of major adverse events that occur in the small intestine due to NSAID use.

Table 1.2 Frequency of major adverse effects of NSAIDs in the lower gastrointestinal tract

Adverse effect	Frequency (%)
Increase gut permeability	40 to 70
Gut inflammation	60 to 70
Blood loss and anemia	30
Malabsorption	40 to 70
Mucosal ulceration	30 to 40

[Table adapted from Sostres et al., 2013]

Clinically, it remains a challenge to diagnose NSAID enteropathy in patients despite the improved ability to explore (e.g., video capsule endoscopy) the distal regions

of the small intestine. Due to a lack of specificity and/or sensitivity, there remains no gold standard for detecting and quantifying enteropathy besides directly visualizing the damage via scoping techniques (Wallace, 2013a). An additional layer of complexity to the diagnosis of NSAID-induced enteropathy is the time it takes for NSAID-enteropathy to manifest. Unlike gastroduodenal damage, small intestinal damage and bleeding occurs over a much longer period of time and the analgesic properties of NSAIDs themselves often mask the patient's symptoms (Wallace, 2012). Indeed, there is a poor correlation between NSAID-induced enteropathy and clinical symptoms. A troubling trend has recently emerged; while the incidence of NSAID-induced upper GI events is declining, the incidence of small intestinal adverse events is increasing (Lanas *et al*., 2009; Scarpignato & Hunt, 2010; Sostres *et al*., 2013). This trend is both very dangerous and costly, as lower GI adverse events result in significantly higher rates of recurrence, death, and days of hospitalization as compared to upper GI adverse events (Figure 1.4) (Lanas *et al*., 2009).

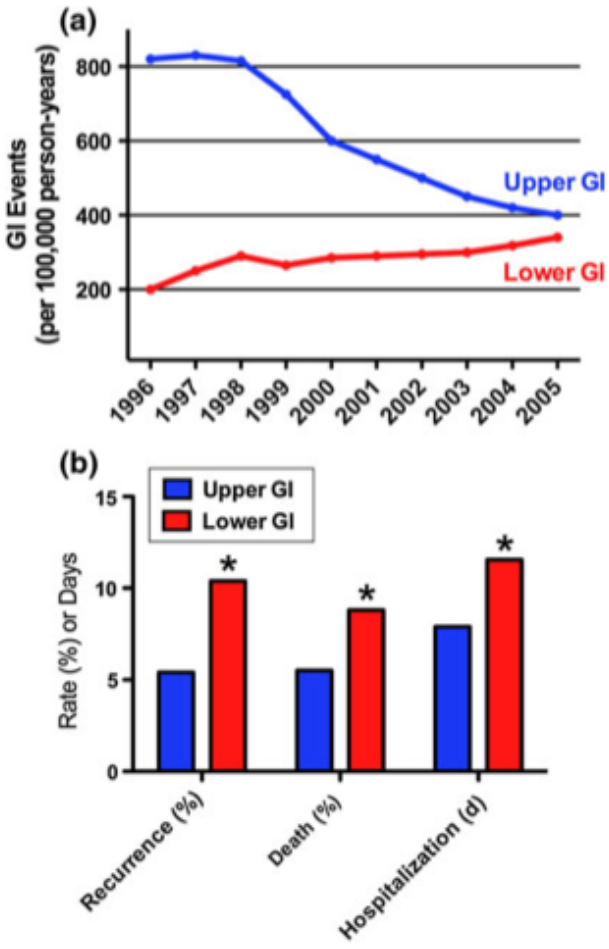

Figure 1.4. The increasing incidence of adverse lower GI events caused by NSAIDs. **Panel A**: Between 1996-2005, the number of upper GI events per 100,000 person-years associated with NSAID use has declined, whereas the number of lower GI events has risen. **Panel B:** Lower GI events have higher rates of recurrence and death, and result in an increase number of hospitalized days as compared to upper GI events. [Figure from Wallace, 2013a and data from Lanas *et al*., 2009]

Unfortunately, there are no preventative or curative treatments for NSAID-enteropathy that have proved effective and many efforts by clinicians and pharmaceutical companies to protect patients from NSAID-induced gastroduodenal and cardiovascular

damage have worsened small intestinal damage (Wallace, 2013b). A better understanding of the pathogenesis of NSAID-enteropathy may eventually lead to proven treatments or ideally, effective NSAIDs that do not cause gastrointestinal injury.

Distal to the ligament of Treitz, the critical mechanisms of NSAID-induced damage and bleeding are quite distinct from the NSAID-induced mechanisms of upper GI damage (Wallace, 2012). For example, the co-administration of antisecretory drugs with NSAIDs reduces the severity of NSAID-gastropathy, where the role of acid in the development of NSAID-induced gastric damage has been clearly demonstrated, but there is no evidence to suggest that antisecretory drugs protect NSAID users from small intestinal damage and bleeding (Lanas & Scarpignato, 2006; Wallace, 2008; Hunt & Scarpignato, 2010). The majority of the insight into the mechanisms of NSAID-enteropathy has been obtained from animal studies. From these studies, it appears that NSAID enteropathy is multifactorial and a clear, unifying hypothesis (akin to NSAID-gastropathy) may not adequately explain the damage process. Figure 1.5 shows the key mechanisms of NSAID-induced enteropathy and bleeding that have been identified, which will be discussed in more detail below. Several studies have demonstrated critical roles for the enterohepatic circulation of the NSAIDs, bile, and enteric bacteria in NSAID-enteropathy (Wax *et al*., 1970; Uejima *et al*., 1996; Seitz & Boelsterli, 1998; Hagiwara *et al*., 2004). However, other contributing mechanisms include COX inhibition, altered intestinal permeability (i.e., barrier disruption), epithelial and mitochondrial injury, neutrophil infiltration, and TNF-α release (Wallace, 2012).

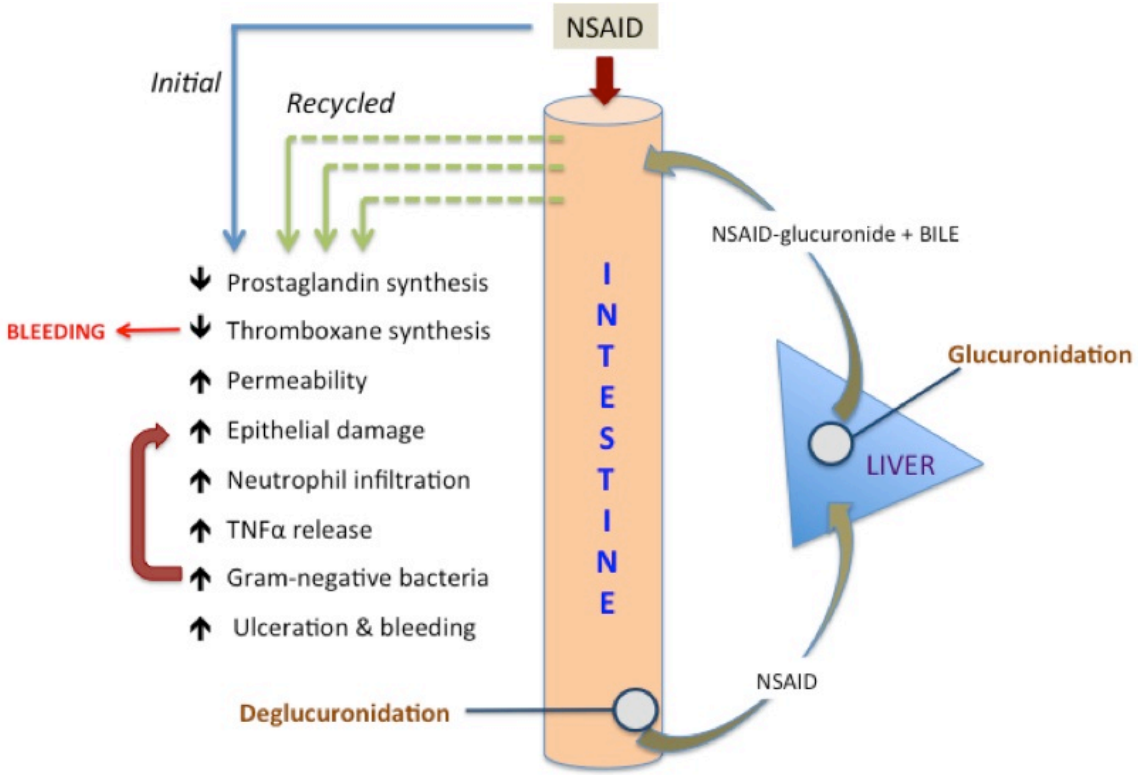

Figure 1.5. The pathogenesis of nonsteroidal anti-inflammatory drug-induced (NSAID)-enteropathy. NSAIDs affect the small intestine upon first exposure and when secreted back into the small intestine (via bile) following reabsorption in the distal intestine and glucuronidation in the liver. The inhibition of prostaglandin (PG) synthesis occurs with all NSAIDs and renders the intestinal mucosa susceptible to damage. NSAIDs can increase intestinal permeability and cause direct epithelial damage. Epithelial damage causes an inflammatory response (e.g., tumor necrosis factor-alpha (TNF-α) release). The resulting neutrophil infiltration contributes to the progression of tissue injury and the suppression of thromboxane synthesis promotes bleeding. Repeated exposure of the small intestine to the combination of NSAIDs and bile promotes further injury, and the damage is likely exacerbated by a shift in enteric bacteria (e.g. elevated gram negative bacteria). [Figure from Wallace, 2013a]

1.8.2. Pathogenesis: inhibition of cyclooxygenase activity

Two observations indicate that cylcooxygenase (COX) inhibition does not appear to be the primary role through which NSAIDs cause enteropathy (Reuter *et al*., 1997; Wallace, 2013a). First, there is a lack of correlation between the extent of inhibition of intestinal PG synthesis and the subsequent degree of intestinal ulceration and bleeding. Secondly, the appearance of NSAID-induced intestinal damage is not temporally synchronized with the suppression of intestinal PG synthesis (Whittle, 1981; Reuter *et al*., 1997). However, the inhibition of mucosal PG synthesis renders the small intestine more susceptible to injury and less conducive to repair following injury (Reuter *et al*., 1997; Tanaka *et al*., 2002; Bukhave & Rask-Madsen, 2004). As in the stomach, the inhibition of COX-1 causes a rapid, compensatory increase in COX-2 expression in the small intestine, and the suppression of both isozymes exacerbates intestinal injury (Tanaka *et al*., 2002). Clinically, it is has been reported that selective COX-2 inhibitors in humans can cause similar rates of small intestinal damage and bleeding as tNSAIDs (Maiden *et al*., 2007; McCarthy, 2009). Maiden *et al.* demonstrated that 62% and 50% of patients on long-term tNSAID or selective COX-2 inhibitor therapy, respectively, exhibited small intestinal damage when evaluated by video capsule endoscopy.

While the suppression of COX activity does not appear to be the primary cause of intestinal damage, the inhibition of PG synthesis in the small intestine still compromises various physiological processes (Mohajer & Ma, 2000). For instance, administration of indomethacin (a tNSAID) prevents small intestinal secretion, but co-treatment with exogenous PGs reestablishes small intestinal fluid secretions (e.g., bicarbonate and chloride) (Roberts *et al*., 1976; Ruwart *et al*., 1979). PGs also mediate

intestinal mucus production, blood flow, and epithelial repair, all of which contribute to mucosal defence (Bukhave & Rask-Madsen, 2004; Wallace, 2013a). Similar to the stomach, suppression of COX activity by NSAID administration can cause a disturbance in the microcirculation of the intestine and promote leukocyte adherence and infiltration (Miura *et al*., 1991; Chmaisse *et al*., 1994). Furthermore, it is likely that the suppression of COX activity contributes to intestinal injury and bleeding by impairing intestinal repair processes, such as angiogenesis, and by inhibiting platelet aggregation (Jones *et al*., 1999; Wallace, 2013a).

1.8.3. Pathogenesis: epithelial and mitochondrial injury

NSAIDs and their metabolites have the ability to directly damage intestinal epithelial cells and cause cell death. Epithelial damage can occur as a result of disruption of the cellular lipid bilayer, induction of oxidative stress, and/or intracellular organelle damage including mitochondrial injury (Zhou *et al*., 2010; Mani *et al*., 2014). Mitochondrial injury (morphological) can be observed within 1 hour after NSAID administration to rats, and *in vitro* studies of the liver have further demonstrated that NSAIDs could rapidly cause the uncoupling of oxidative phosphorylation (Somasundaram *et al*., 1995; Somasundaram *et al*., 1997). Numerous studies have shown that, following NSAID administration, tight junctions were disrupted upon epithelial and mitochondrial injury, leading to an increase in intestinal permeability (Bjarnason *et al*., 1989b; Reuter *et al*., 1997; Mani *et al*., 2014). An increase in intestinal epithelial permeability can be detected both in humans and rats within 12 hours of NSAID administration (Bjarnason *et al*., 1989b; Reuter *et al*., 1997). Bjarnason and colleagues

demonstrated that the exogenous administration of PGs could prevent NSAID-induced increases in intestinal permeability and ulceration in humans (Bjarnason, 1989a). However, it remains unclear whether the increase in intestinal permeability caused by NSAID administration is a result of the inhibition of PG synthesis or direct epithelial injury (Wallace, 2013a).

However, NSAID-induced epithelial and mitochondrial injury with the associated increase in small intestinal permeability, does not appear to play a critical role in NSAID-enteropathy. For example, there is a lack of correlation between the extent of intestinal injury and the increase in small intestinal permeability that occurs following NSAID administration (Choi *et al*., 1995). In addition, the endoscopic evaluation of patients taking diclofenac demonstrated that denuded regions (perhaps due to topical, erosive injury) occurred throughout the small intestine, but ulcers were concentrated in the distal jejunum and ileum, where the largest numbers of small intestinal bacteria are found and where NSAID deconjugation occurs (Fujimori *et al*., 2010). It is likely that as a consequence of epithelial and mitochondrial injury, increased intestinal permeability allows damaging luminal agents (e.g., bile acids and bacteria) to enter the mucosa and subsequently cause the activation of pro-inflammatory cytokines in the lamina propria (Somasundaram *et al*., 1995). The inflammatory signals in the lamina propria contribute to injury by causing neutrophil infiltration in the inflamed mucosa. Activated neutrophils subsequently release damaging levels of reactive oxygen species (ROS) and proteases that cause collateral damage to surrounding cells (Antoon & Perry, 1997). Akin to NSAID-induced gastropathy, there is evidence that TNF-α contributes to NSAID-induced

intestinal damage by driving an acute inflammatory response (Santucci *et al*., 1994; Appleyard *et al*., 1996). For instance, the attenuation of the inflammatory process provides improved resistance to NSAID-induced intestinal injury; as demonstrated in studies where indomethacin-induced small intestinal damage was associated with the expression of TNF-α (likely through toll-like receptor-4 (TLR-4) activation) and administration of antibodies against TNF-α reduced the damage by 67% (Watanabe *et al*., 2008). However, the role of TNF-α in NSAID-enteropathy appears minor and is likely only a consequence of damage (Appleyard *et al*., 1996; Reuter & Wallace, 1999). As discussed below, the majority of observations suggest other key mechanisms in the pathogenesis of NSAID-enteropathy.

1.8.4. Pathogenesis: the role of bile and enterohepatic circulation

Several findings suggest critical roles for both the bile and the enterohepatic circulation of NSAIDs in the pathogenesis of NSAID-enteropathy. For instance, the ligation of the bile duct in rats completely prevents small intestinal ulceration (Kent *et al*., 1969; Wax *et al*., 1970). NSAIDs that undergo extensive enterohepatic circulation, such as indomethacin, exhibit a much greater propensity to cause small intestinal ulceration (Kent *et al*., 1969; Beck *et al*., 1990; Reuter *et al*., 1997). Conversely, NSAIDs that undergo limited enterohepatic circulation (e.g., ASA) often cause limited intestinal injury (Reuter *et al*., 1997; Somasundaram *et al*., 1997). In addition, early experiments with nitric oxide (NO)-releasing NSAIDs, which caused substantially less intestinal injury as compared to the parent NSAID (diclofenac), indicated that less diclofenac could be recovered in bile of rats treated with the NO-NSAID compared to those treated with

diclofenac (Davies *et al*., 1997; Reuter *et al*., 1997). It has also been observed that rats lacking Mrp2, the dominant hepatocanalicular conjugate exporter pump of NSAID glucuronides, were protected from NSAID-induced intestinal injury (Seitz & Boelsterli, 1998). However, following treatment with an orally administered NSAID, bile collected from wild-type rats (thus containing NSAID glucuronides) increased intestinal injury in Mrp2-deficient rats (Seitz & Boelsterli, 1998). Collectively, these studies indicate that biliary drug delivery is an important factor in the severity of NSAID-induced intestinal injury. The reason for this being that the enterohepatic circulation of an NSAID results in the repeated exposure of intestinal epithelial cells to the topical damaging effects of the drug and its metabolites. The enterohepatic circulation of an NSAID also appears necessary to significantly alter the intestinal microbiota and to promote the growth of damaging, gram-negative bacteria (Reuter *et al*., 1997).

A number of *in vitro* studies have indicated that the combination of an NSAID and bile results in toxic micelles that are particularly damaging to intestinal epithelial and non-GI cells (Uchida *et al*., 1997; Petruzzelli *et al*., 2007; Dial *et al*., 2008; Zhou *et al*., 2010). However, *in vivo* reports have suggested that the reactive acyl glucuronide metabolites of NSAIDs are the primary toxic agent exported into the bile. These metabolites are able to covalently bind to enterocytes and are often associated with small intestinal ulceration sites (Seitz & Boelsterli, 1998; Treinen-Moslen & Kanz, 2006). It has also been demonstrated that the administration of NSAIDs to rats can alter the composition of the bile. Indomethacin-administration resulted in increased concentrations of secondary bile acids in the bile which as discussed above, are quite damaging to the

cellular membrane of epithelial cells *in vitro* due to their strong hydrophobic nature (Coleman *et al*., 1979; Yamada *et al*., 1996; Hofmann, 1999). The ability of different types of bile acids to affect NSAID-enteropathy has been highlighted by studies where specific bile acids orally co-administrated with NSAIDs to rats, and either ameliorated or exacerbated intestinal injury (Uchida *et al*., 1997; Ishikawa & Watanabe, 2011).

While the combination of bile acids and NSAIDs (or their metabolites) is particularly toxic to intestinal epithelial cells, the major sites of NSAID-induced ulceration are in the distal regions of the small intestine, where the concentrations of bile and NSAIDs are lower compared to more proximal regions (i.e., near the Sphincter of Oddi). The propensity of NSAID damage to occur in the distal small intestine may be explained by the effects of enteric bacteria. Enteric bacteria rapidly colonize sites of mucosal injury, which delays tissue repair and thus likely prevent the healing of NSAID-induced intestinal injury (Elliot *et al*., 1998). Furthermore, the distal small intestine has an exponentially greater number of bacterial populations than more proximal regions. The major of these populations produce bacterial β-glucuronidase, which is capable of deconjugating bile acids and promoting the reabsorption of the aglycone (i.e., free NSAID) (Treinen-Molsen & Kanz, 2006; LoGuidice *et al*., 2012). Therefore, it has been suggested that small intestinal epithelial cells at the sites of NSAID reabsorption may be exposed to high concentrations of damaging, free NSAID (Boelsterli & Ramirez-Alcantara, 2011).

1.8.5. Pathogenesis: the role of enteric bacteria

The role of bacteria in NSAID-enteropathy is strongly supported by the observations that rodents without enteric bacteria do not develop NSAID-induced intestinal injury. For instance, treatment with broad-spectrum antibiotics can prevent NSAID-enteropathy in rodents and germ-free rodents do not develop intestinal ulcers when treated with NSAIDs (Robert & Asano, 1977; Konaka *et al*., 1999). However, it is unclear whether enteric bacteria induce this damage through a primary mechanism by initiating tissue injury, or through a secondary mechanism by exacerbating NSAID-induced tissue injury and impeding mucosal repair (Uejima et al., 1996; Elliott et al., 1998). Support for a primary mechanism is demonstrated by the observations that germ-free mice colonized with *Escherichia coli (E. coli) or Eubacterium limosum* (both gram-negative bacteria) were rendered susceptible to NSAID-enteropathy, but germ-free mice colonized with *Bifidobacter adolescentis* or *Lactobacillus acidophilus* (both gram-positive bacteria) retained their resistance to NSAID-enteropathy (Uejima *et al*., 1996). Several other studies have documented the role of gram-negative bacteria in the pathogenesis of NSAID-enteropathy (Reuter *et al*., 1997; Hagiwara *et al*., 2004). For instance, NSAID administration causes shifts in the types of bacteria in the small intestine, often resulting in an overall increase in gram-negative bacterial species and a concomitant reduction in gram-positive bacterial species (Kent *et al*., 1969; Konaka *et al*., 1999; Hagiwara *et al*., 2004; Dalby *et al*., 2006). In particular, an enrichment of specific enteric bacteria, such as *Enterococcus faecalis*, *Clostridium*, *Bacteroides* and *E. coli*, has been reported following NSAID administration in rodents. The mechanism of unbalanced gram-negative bacteria growth remains unknown, although it is conceivable that an alteration in luminal bile

acids (e.g., bile acids with less anti-microbial activity) could encourage the shift (Floch *et al*., 1972). The role of gram-negative bacteria in the pathogenesis of NSAID-enteropathy was further supported by a study in which genetically altered mice that lacked toll-like receptor-4 (TLR-4), a receptor stimulated by lipopolysaccharide (LPS) (an outer cell membrane component of gram-negative bacteria), developed 80% less small intestinal damage when administered indomethacin than their wild-type controls (Watanabe *et al*., 2008).

Antibiotics that target gram-negative bacteria have been particularly effective in reducing the extent of NSAID-induced intestinal ulceration (Uejima *et al*., 1996; Koga *et al*., 1999; Watanabe *et al*., 2008). On the other hand, antibiotics that target gram-positive bacteria do not appear to alter the severity of NSAID-induced intestinal damage (Watanabe *et al*., 2008). However, there are two major limitations to most of the existing literature evaluating the co-administration of antibiotics and NSAIDs. First, these studies often administer only a single, large dose of an NSAID to the rodents, which does not adequately represent the clinical scenario of chronic NSAID administration. Secondly, the experiments utilize either indomethacin or diclofenac, two NSAIDs that are exceptionally damaging to the intestine and thus are often not prescribed clinically.

Although an abundance of evidence exists to suggest that bacteria contribute to the development of NSAID-enteropathy, whether the bacteria directly help initiate the damage once the intestine has been rendered susceptible the inhibition of mucosal PG synthesis, or whether bacteria exacerbate tissue injury and impede repair remains unclear (Wallace, 2013a). An additional layer of complexity in determining the role of bacteria in

NSAID-enteropathy is that the intestinal microbiota, enterohepatic circulation, and bile are all closely connected. Thus, multiple variables could influence the resistance to intestinal ulcerations observed in germ-free rodents, or rodents with a ligated bile duct, after NSAID-administration. For instance, the ligation of the bile duct prevents both the enterohepatic circulation of the NSAID and the entry of bile acids (luminal irritants) into the small intestine. In germ-free rodents, the enterohepatic circulation of NSAIDs does not occur, as there is no bacterial β-glucuronidase present to free the NSAID from the glucuronide group for reabsorption. The inability for NSAID reabsorption limits the repeated exposure of the small intestine to the NSAID (Treinen-Molsen & Kanz, 2006). Furthermore, another variable that may influence the resistance of germ-free rodents to intestinal damage is that they do not exhibit any secondary bile acids (Begley *et al*., 2005; Duboc *et al*., 2013).

1.8.6. NSAID-enteropathy and the problem of polypharmacy

The people most commonly taking NSAIDs on a chronic basis are those with chronic illnesses, such as rheumatoid arthritis and osteoarthritis. In some circumstances, these patients may have other comorbid disorders, such as hypertension, obesity, and diabetes, which can increase the risk of adverse GI- and/or CV-events associated with NSAID use (Solomon & Gurwtiz, 1997; Hernandez-Diaz & Rodriguez, 2002; Aro *et al*., 2006). Furthermore, patients taking NSAIDs chronically are often elderly (>65 years of age) which, in itself, is another strong risk factor for adverse NSAID-associated events (Greenwald, 2004). The elderly are most prone to drug toxicity and drug interactions, in part because of the greater prevalence of comorbidities in this population. Therefore,

physicians manage the risk of NSAID-associated GI- and CV-adverse effects through co-therapy with gastro-protective and cardio-protective drugs, respectively (Rostom *et al*., 2009; Hochber *et al*., 2012).

In the case of osteoarthritis (OA), where the incidence increases sharply with age, patients are often managed with chronic NSAID administration since these drugs provide relief of pain and joint swelling (Neogi & Zhang, 2013). NSAIDs remain the principal therapy for OA despite the known GI- and CV-adverse effects caused by this class of drugs (Wallace, 2013b). However, these risks are managed by utilizing a "polypharmacy" approach. Inhibitors of gastric acid secretion (i.e., antisecretory drugs; PPIs or H_2RAs) are commonly co-prescribed with NSAIDs to protect patients from the risk of upper GI injury (Zhao & Encinosa, 2008). In addition, the CV risks associated with NSAID use (in particular selective COX-2 inhibitors) are addressed by recommending low-dose ASA for cardio-protection due to its ability to reduce the incidence of severe cardiovascular events (i.e., myocardial infarction) (Kimmey, 2004; Kearney *et al*., 2006; Wallace, 2013b). Unfortunately, recent data suggests that this practice of "polypharmacy" can result in a synergistic increase in intestinal injury and bleeding (Zhao & Encinosa, 2008; Lanas *et al*., 2009). Indeed, the co-treatment of an NSAID with low-dose ASA and an antisecretory drug has been described as "the perfect intestinal storm", due to the high incidence rates and severity of NSAID-enteropathy observed in these patients (Figure 1.6) (Wallace, 2013b). With aging populations in developed countries, the prevalence of chronic NSAID use and associated co-treatments may become more common, and thus the prevalence and severity of NSAID-enteropathy may increase.

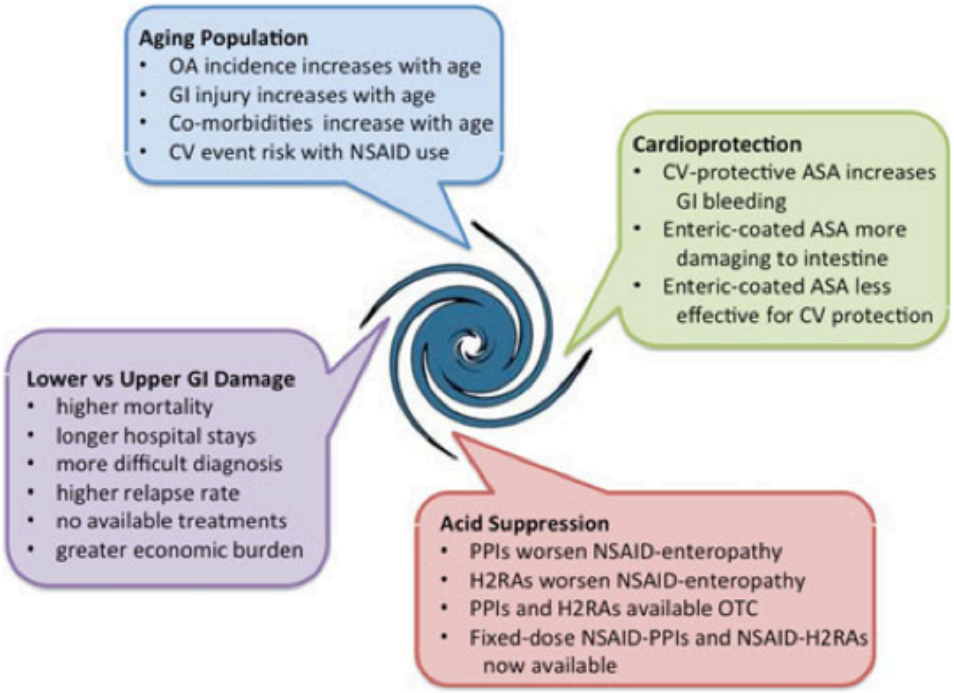

Figure 1.6. The perfect intestinal storm. The risk of non-steroidal anti-inflammatory drug (NSAID)-associated gastrointestinal (GI) and cardiovascular adverse events is increased in individuals with advanced age. To protect at-risk patients, the co-administration of low-dose ASA (for cardio-protection) and antisecretory drugs (for upper GI protection) is often recommended with NSAIDs. However, the simultaneous use of these drugs can significantly worsen intestinal damage and bleeding. A relative increase in lower versus upper GI adverse events is a major clinical concern, since the former is more difficult and expensive to diagnose and teat, and results in higher reoccurrence rates and mortality rates. [Figure from Wallace, 2013b]

The practice of co-administering low-dose ASA to protect patients from the CV-risks associated with NSAIDs increases the incidence and severity of NSAID-enteropathy in multiple ways. The cardio-protective effects of ASA lie in the ability of ASA to inhibit platelet aggregation. Unfortunately, the inhibition of platelet aggregation also impedes

mucosal tissue repair and increases the risk of a GI bleed even when ASA is used at low doses (Endo *et al*., 2009; Zhu *et al*., 2012). Secondly, low-dose ASA administration also increases the risk of a GI bleed. This should not be a surprise, since the rationale for taking low-dose ASA for cardioprotection is to block platelet aggregation. In the United States and Europe, ASA was identified as the precipitator of GI bleeding in over 50% of cases that led to hospital admission (Lanas *et al*., 1992; Stack *et al*., 2002; McCarthy, 2009). A recent clinical trial demonstrated that the majority of GI bleeds caused by low-dose aspirin occur in the lower GI tract (Casado Arroyo *et al*., 2012; Zhu *et al*., 2012). Lastly, physicians often recommend that low-dose ASA be taken in an enteric-coated form, based on the false concept that reduced contact of the active drug with the gastric mucosa would lessen upper GI ulceration. Ironically, enteric-coated NSAIDs increase the exposure of the small intestine to the NSAID, and thus exacerbate enteropathy (Davies, 1999; Endo *et al*., 2012).

To reduce the upper GI toxicity associated with the combination of an NSAID and low-dose ASA, an antisecretory drug (usually PPIs) is frequently co-prescribed. However, it has been apparent for several years that these drugs offer no protection to the distal regions of the small intestine. In fact in recent years, several studies have reported that patients who were prescribed antisecretory drugs and NSAIDs exhibited an exacerbation of intestinal injury and bleeding compared to patients receiving NSAIDs alone (Goldstein *et al*., 2005; Maiden *et al*., 2005; Zhao & Encinosa, 2008; Watanabe *et al*., 2013). The incidence of small intestinal damage in healthy volunteers taking both an NSAID and a PPI over a 2-week period has been reported between 55-75% (Goldstein *et*

al., 2005; Fujimora *et al*., 2010). Some clinical studies have also reported a significant elevation of a marker of intestinal inflammation (i.e., calprotectin) in healthy patients taking only PPIs (Poullis *et al*., 2003). In fact, a recent cross-sectional study identified PPI usage as the greatest independent risk factor for severe ulceration and bleeding in patients who were being treated with NSAIDs for the management of with rheumatoid arthritis (Watanabe *et al*., 2013). The authors further reported that the use of H_2RAs was also a significant risk factor for NSAID-enteropathy. However, the conclusions drawn from this study have limitations, as the number of patients included was relatively small and quite homogenous (i.e., rheumatoid arthritis patients and single centre recruitment) (Watanabe *et al*., 2013).

Similar to the evidence from human studies, animal models of NSAID-enteropathy have demonstrated an exacerbation of intestinal damage and bleeding when gastric acid secretion was suppressed by the co-administration of an antisecretory drug (Wallace *et al*., 2011; Blackler *et al*., 2012; Satoh *et al*., 2012). Satoh and colleagues reported that antisecretory drug administration exacerbated NSAID-enteropathy in part due to enhanced intestinal motility (Satoh *et al*., 2012). However, a major underlying mechanism for the exacerbation of NSAID-enteropathy appears to be the intestinal dysbiosis that results from the suppression of acid (Wallace *et al*., 2011). For instance, the administration of a PPI to rats caused a marked decrease in intestinal colonization by *Bifidobacteria.* Correction of the dysbiosis, by replenishing the diminished *Bifidobacteria,* resulted in the prevention of NSAID-enteropathy in the rats (Wallace *et al*., 2011). It remains unclear how PPI-induced dysbiosis contributes to NSAID-induced

intestinal damage and bleeding, but it is possible that dybiosis triggers changes in bile metabolism that could contribute to intestinal injury.

Numerous clinical reports and studies have reported alterations in the GI tract following the chronic use of an antisecretory drug. Most notably, chronic antisecretory drug use can cause small intestinal bacterial overgrowth (SIBO) and bile acid dysmetabolism in patients (Lombardo *et al*., 2010; Williams & McColl, 2006; Shindo & Fukumura, 1995; Shindo *et al*., 1998). The development of SIBO is associated with the suppression of gastric acid since a reduced amount of gastric acid cannot adequately serve as an immune barrier to sterilize what we consume. The ensuing bile acid dysmetabolism in SIBO patients is likely the result of an excessive growth of microbes that are capable of deconjugating bile acids (Shindo & Fukumura, 1995; Shindo *et al*., 1998). The deconjugation of bile acids by bacterial enzymes increases bile acid hydrophobicity, which subsequently enhances the ability of the bile acid to interact with the cellular membranes of enterocytes and cause deleterious topical damage (Hofmann, 1999; Begley *et al*., 2005; Petruzzelli *et al*., 2007). As a result, an altered bile acid composition may be a consequence of PPI administration and contribute to the PPI-induced susceptibility to NSAID-enteropathy.

The combination of NSAIDs, low-dose ASA, and antisecretory drugs is meant to protect the upper GI tract and CV system. However, the combination also results in a dangerous clinical scenario. Indeed, this combination of drugs creates the 'perfect intestinal storm' (Wallace, 2013b). In order to limit intestinal damage, it will be important

to gain a better understanding of how these combinations of drugs affect the mechanisms of NSAID-enteropathy.

1.8.7. Preventative strategies for NSAID-enteropathy

In contrast to NSAID-induced gastroduodenal damage, which can be minimized by available therapies such as antisecretory drugs and misoprostol (a PGE_1 analogue), there are currently no curative or preventative treatments for NSAID-enteropathy that have proven to be effective (Wallace, 2013a). Although the evidence is limited, PG administration may offer some benefit to the prevention and treatment of NSAID-enteropathy. For instance, Bjarnason and colleagues demonstrated that co-treatment with misoprostol could reduce indomethacin-induced increases in intestinal permeability. However, whether this would impact the development of intestinal injury remains unclear (Bjarnason *et al*., 1989a). Some small clinical trials have suggested that misoprostol use may be favourable for protecting against NSAID-enteropathy in humans, but these studies had limited sample sizes and/or design flaws (e.g., open-label) (Morris *et al*., 1994; Fujimori *et al*., 2009).

Similar to misoprostol, there is evidence to suggest that treatment with metronidazole may protect against NSAID-enteropathy. Metronidazole is a nitroimidazole compound that has selective antiparasitic and antimicrobial effects on anaerobic and microaerophilic pathogens (Shapiro & Goldberg, 2006). The administration of metronidazole to rats has been shown to reduce intestinal injury and bleeding (Yamada *et al*., 1993). In addition, an open-label study of patients using NSAIDs chronically suggested that the co-administration of metronidazole could limit

intestinal bleeding and inflammation, although the extent of ulceration was not elevated (Bjarnason *et al*., 1992).

The observation from animal studies that enteric bacteria are critical to the pathogenesis of NSAID-enteropathy has prompted the evaluation of many therapies that target bacteria (Scarpignato, 2008). Despite the reported success of certain antibiotics (i.e., broad spectrum or gram-negative targeting) in preventing NSAID-enteropathy in rodents, no clinical trials have been formally carried out in humans in order to evaluate the effect of antibiotics in the prevention of NSAID-enteropathy (Koga *et al*., 1999; Watanabe *et al*., 2008). The potential for adverse effects and for the development of antibiotic-resistance precludes this approach. Furthermore, many intestinal bacteria are integral to metabolism and to the maintenance of health. However, the enteric microbiota can be manipulated by the use of probiotics or prebiotics. In studies using rats, the administration of *Lactobacillus acidophilus* and *Bifidobacteria adolescentis* significantly reduced the severity of NSAID-induced ulceration in the distal small intestine (Kinouchi *et al*, 1998). Only a few clinical trials evaluating probiotic use as a preventive therapy for NSAID-enteropathy have been performed. Montalto and colleagues conducted a randomized control trial in which volunteers were administered indomethacin for four days and also received either a probiotic formulation consisting of 8 different live bacteria termed VSL#3, or placebo. Volunteers who received the placebo demonstrated elevated fecal calprotectin levels (a marker of large intestine inflammation) after indomethacin treatment, while fecal calprotectin levels remained normal in VSL#3-treated volunteers (Montalto *et al*., 2010). In a study by Endo *et al*, patients who received *Lactobacillus*

casei while on low-dose enteric-coated ASA and omeprazole for 3 months had significantly reduced mucosal injury (via VCE evaluation) compared to patients who received placebo (Endo *et al*., 2011). However, more studies are required to evaluate the benefits of specific strains, dose responses, and treatment duration, if probiotic administration is to become an effective therapy for NSAID-enteropathy.

Another promising approach to preventing NSAID-enteropathy is the use of bacteria-selective inhibitors of β-glucuronidase. These inhibitors must be selective for bacterial β-glucuronidase, since mammalian β-glucuronidase is required for proper lysosomal storage (Mani *et al*., 2014). The inhibition of bacterial β-glucuronidase prevents the enterohepatic circulation of many NSAIDs, since this enzyme releases the parent NSAID and corresponding metabolites from the respective glucuronide conjugates and thus liberates the aglycone for reabsorption by the enterocytes (LoGuidice *et al*., 2012). As previously discussed, reducing the number of times the small intestine is exposed to the NSAID or its metabolites diminishes the extent of intestinal injury. Indeed, mice that were administered a bacterial-selective β-glucuronidase inhibitor exhibited significantly reduced intestinal damage as compared to mice that received an ulcerogenic dose of diclofenac alone (LoGuidice *et al*., 2012). Interestingly, bacterial β-glucuronidase appears to be nonessential for bacteria and thus the inhibition of this enzyme is nonlethal for bacteria.

While targeting the intestinal microbiota can influence NSAID-enteropathy, much of the evidence indicates that bacteria play a secondary role in intestinal injury.

Therefore, enhancing mucosal defence during NSAID administration or designing novel NSAIDs that do not cause GI damage are approaches that will likely be more successful.

1.9. The Protective Role of Hydrogen Sulfide in the Gastrointestinal Tract and Hydrogen Sulfide-Releasing NSAIDs

1.9.1. Gastrointestinal cytoprotection by hydrogen sulfide

The endogenous, gaseous mediator hydrogen sulfide (H_2S) is one of the most important mediators of mucosal defence (Wallace *et al*., 2014). The critical role of H_2S in mucosal defence is evident from studies that demonstrated that the suppression of mucosal H_2S synthesis rendered the mucosa much more susceptible to NSAID damage (Wallace *et al*., 2010), stress (Aboubakr *et al*., 2013), ischemia-reperfusion (Liu *et al*., 2009), and colitis (Flannigan *et al*., 2013). On the other hand, the administration of H_2S-releasing compounds has shown to increase the resistance of the GI mucosa to injury (Fiorucci *et al*., 2005; Wallace, 2010; Blackler *et al*., 2012). There are multiple mechanisms of action through which H_2S likely protects the GI mucosa, as summarized in Table 1.3. For instance, a constant level of mucosal blood flow is important for the minimization of tissue damage and the facilitation of rapid repair of incurred damage, as it enables an immediate buffering of the acid that contacts the mucosa epithelial cells (Wallace & McKnight, 1990). H_2S is a strong vasodilator and is thus important for the maintenance of mucosal blood flow (Fiorucci *et al*., 2005; Mard *et al*., 2012). Furthermore, H_2S has been shown to mediate bicarbonate secretions in the duodenum and mucus production in the intestine, which help to protect mucosal tissue from the

damaging effects of luminal agents (e.g., acid, bile, and bacteria) (Ise *et al*., 2011; Motta *et al*., 2014). H_2S can also inhibit the release of the damaging myeloperoxidase enzyme from activated neutrophils and can scavenge oxygen-derived free radicals, thus preventing further damage to mucosal tissue (Whiteman *et al*., 2004; Palinkas *et al*., 2014). It has also been demonstrated that the administration of H_2S-releasing compounds accelerated the healing of pre-existing ulcers (Wallace *et al*., 2007), perhaps due to the ability of H_2S to increase COX-2-derived PGs and promote angiogenesis, two important contributors to ulcer healing (Ma *et al*., 2002; Papapetropoulos *et al*., 2009; Wallace *et al*., 2014). H_2S is also capable of exhibiting potent anti-inflammatory effects (Figure 1.7) (Zanardo *et al*., 2006; Wallace *et al*., 2012). For instance, it can reduce pro-inflammatory cytokine expression and release in the stomach through the inhibition of the NF-κB pathway (Bai *et al*., 2005; Li *et al*., 2007; Lee *et al*., 2013). H_2S is also a potent anti-oxidant and is capable of dampening inflammation by inducing neutrophil apoptosis (Wallace & Vong, 2008; Wallace *et al*., 2012).

Table 1.3 Mechanisms of Gastrointestinal-Protective Actions of Hydrogen Sulfide

Action	References
Inhibits leukocyte adherence/extravasation	Zanardo et al., 2006
Stimulates mucus and bicarbonate secretion	Ise et al., 2011; Motta et al., 2014
Prevents mitochondrial damage	Elrod et al., 2007; Kimura et al., 2010
Enhances anti-microbial defence	Motta et al., 2014
Scavenges oxygen-derived free radicals	Whiteman et al., 2004
Inhibits neutrophil-mediated tissue injury (myeloperoxidase activity)	Palinkas et al., 2014
Reduces damage-associated tumor necrosis factor release	Fiorucci et al., 2007

[Table adapted from Wallace et al., 2014]

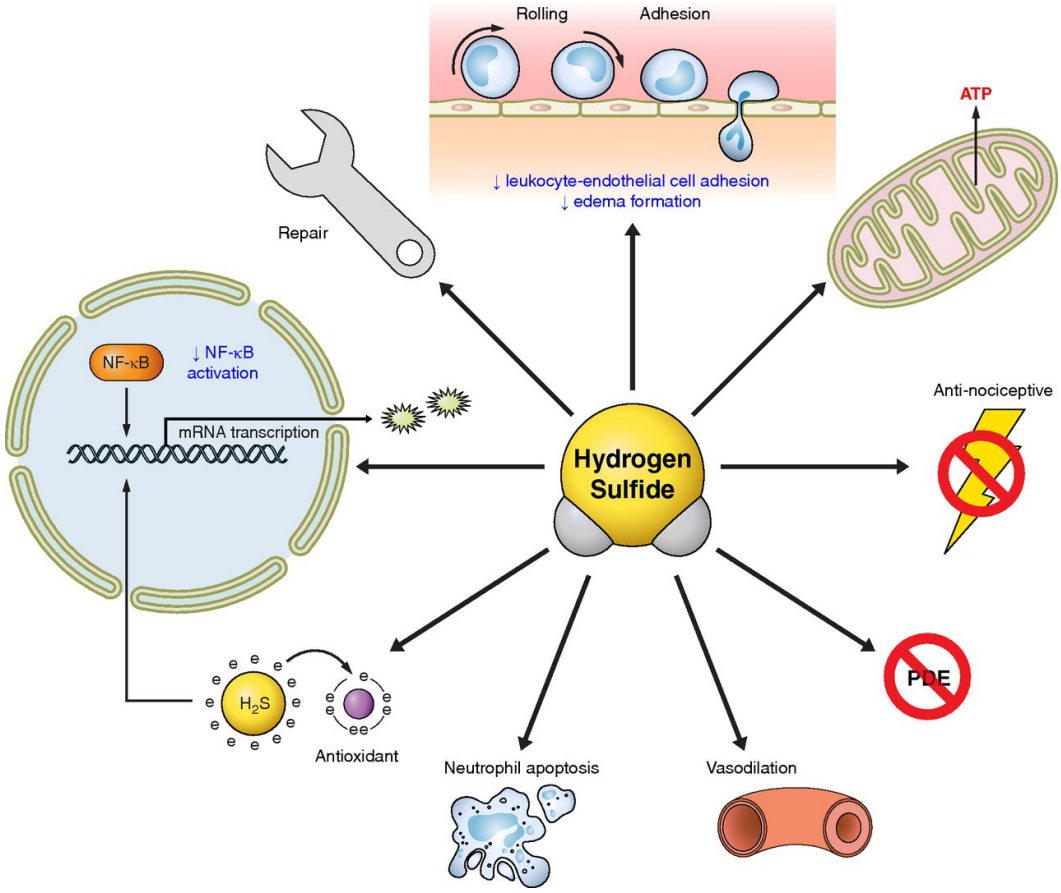

Figure 1.7. The anti-inflammatory effects of hydrogen sulfide (H_2S). H_2S suppresses leukocyte adherence to the vascular endothelium and infiltration into the inflamed tissue. H_2S likely promotes mucosal repair through the combined ability to upregulate cyclooxygenase (COX)-2 expression, induce vasodilation, and promote angiogenesis. In addition, it can reduce the expression and release of pro-inflammatory cytokines and chemokines, most likely through the suppression of NF-κB activity. H_2S is also an antioxidant and can induce neutrophil apoptosis. The inhibition of phosphodiesterases (PDE) may also contribute to the anti-inflammatory effects of H_2S. H_2S is an analgesic in the viscera and can substitute for oxygen in mitochondrial respiration, allowing hypoxic tissues to continue to produce adenosine triphosphate (ATP). [Figure from Chan & Wallace, 2013].

1.9.2. Hydrogen sulfide-releasing NSAIDs

It is now clear that H_2S is capable of eliciting many of the same physiological effects as PGs. This has fueled extensive research into the ability of H_2S to protect the GI tract from NSAID-induced injury (Wallace & Vong, 2008). Indeed, many of the protective actions of H_2S counteract the detrimental effects of the inhibition of PG synthesis by NSAIDs (Figure 1.8) (Chan & Wallace, 2013). The strong protective actions of H_2S prompted the design of H_2S-releasing NSAIDs. Similar to the NO-releasing NSAIDs which preceded them, the design of H_2S-releasing NSAID derivatives has involved the covalent linkage of a current, GI-damaging NSAID to an H_2S-releasing moiety, creating a co-drug that slowly releases protective H_2S while allowing the NSAID to inhibit its target enzymes (COX-1 and -2). In theory, the released H_2S would compensate for the reduction of PG synthesis inhibition and thus maintain mucosal integrity (Wallace & Vong, 2008). For example, H_2S prevents two critical mechanisms of NSAID-gastropathy by maintaining mucosal blood flow and inhibiting leukocyte adherence to the vascular endothelium, a very early and critical event (McCafferty *et al*., 1995; Zanardo *et al*., 2006; Wallace, 2007; Wallace & Vong, 2008).

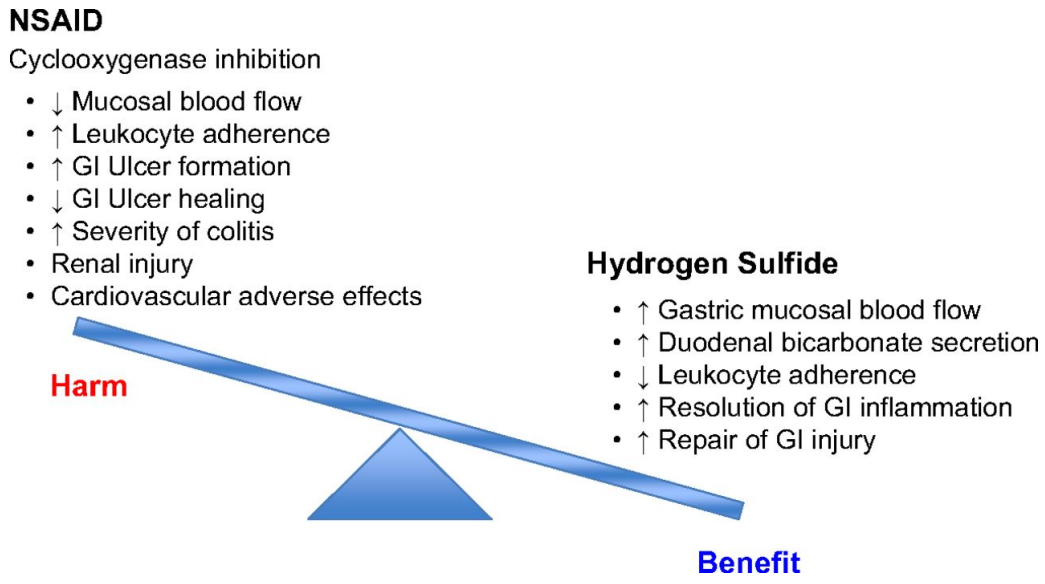

Figure 1.8. Hydrogen sulfide (H_2S) can counteract numerous harmful effects of nonsteroidal anti-inflammatory drugs (NSAIDs). The inhibition of the cyclooxygenase (COX) enzymes by NSAIDs prevents the synthesis of protective PGs, leading to impaired mucosal defence and repair, exacerbation of inflammation, and detrimental effects on the renal and cardiovascular systems. The delivery of H_2S along with an NSAID can counteract the detrimental effects of the NSAID on mucosal defence and also promote both the repair of existing ulcers and the resolution of inflammation. [Figure from Chan & Wallace, 2013]

The therapeutic potential of H_2S has indeed been exploited in the design of H_2S-releasing NSAID-derivatives. These novel drugs produce considerably less GI damage than their parent NSAIDs in animal models (Wallace *et al*., 2010; Blackler *et al*., 2012). The GI safety of H_2S-releasing NSAID-derivatives was maintained in rats even after administration at doses many times greater than those required for anti-inflammatory effects, in models of compromised mucosal defence, and in circumstances in which

NSAIDs were co-administered with other pharmaceuticals (i.e., low-dose ASA and PPIs) (Wallace *et al*., 2010; Blackler *et al*., 2012). H_2S-releasing NSAIDs also exhibit enhanced anti-inflammatory activity compared to their respective parent derivatives due to the potent anti-inflammatory actions of H_2S (Wallace *et al*., 2010).

In recent years, an H_2S-releasing naproxen derivative, ATB-346 [2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester], has proved particularly promising. ATB-346 consists of a molecule of naproxen linked to an H_2S-releasing moiety (i.e., 4-hydroxythiobenzamide (TBZ); via an ester bond). Unlike naproxen, the administration of an anti-inflammatory dose of ATB-346 (equimolar to naproxen) causes negligible gastroduodenal and intestinal damage (Wallace *et al*., 2010; Blackler *et al*., 2012). ATB-346 also promotes the repair of pre-existing gastric ulcers, whereas naproxen administration hinders the healing process (Wallace *et al*., 2010). In terms of CV safety, ATB-346 may also be superior to naproxen. NSAIDs can significantly exacerbate hypertension, which likely contributes to the increase in severe CV adverse evens in chronic NSAID users (Grosser *et al*., 2006). Rats administered a single, high dose of naproxen elicited a significant increase in systemic blood pressure, whereas an equimolar dose of ATB-346 did not significantly affect blood pressure (Wallace *et al*., 2010).

The mucosal-protective effects of the H_2S likely contribute to the superior GI safety ATB-346 over naproxen. For instance, H_2S inhibits leukocyte adherence to the vascular endothelium and can promote bicarbonate secretions in the duodenum (Zanardo *et al*., 2006; Wallace & Vong, 2008; Takeuchi *et al*., 2012). However, there is also emerging evidence that suggests that H_2S can modulate the microbiota, an important

contributor to NSAID-enteropathy (Motta *et al*., 2014). It has also been observed that there were very low levels of naproxen in the bile following the administration of ATB-346 to rats, relative to biliary naproxen concentrations following the administration of naproxen itself (Blackler *et al*., 2012). In light of these findings and the intestinal tolerability of ATB-346, further studies are needed to determine the extent to which ATB-346 differs from naproxen in terms of how it affects critical mechanisms in NSAID-enteropathy. In particular, chapter 5 discusses the roles of bile, enterohepatic circulation, and enteric bacteria in NSAID-enteropathy and mechanisms through which ATB-346 may influence these roles and thus be particularly tolerant in the small intestine compared to naproxen.

– CHAPTER 2 –

THESIS SCOPE AND OBJECTIVES

2.1. Thesis Scope

The pathogenesis of non-steroidal anti-inflammatory drug (NSAID)-enteropathy is incompletely understood and has largely been ignored for decades as a result of the focus on NSAID-gastropathy. NSAID-enteropathy occurs more frequently than NSAID-gastropathy is more dangerous, and is more difficult to detect. Unfortunately, there are no proven-effective prevention strategies or treatment options for NSAID-enteropathy. Moreover, drugs that are often co-prescribed with NSAIDs to limit gastropathy or adverse cardiac events exacerbate the incidence of NSAID-enteropathy and the severity of small intestinal damage. Several studies have demonstrated the critical role of bile, enterohepatic circulation, and enteric bacteria in the pathogenesis of NSAID-enteropathy. However, it is not clear which of these mechanisms is/are the primary instigator(s) of intestinal damage and injury. Hydrogen sulfide (H_2S) is an important mediator of gastrointestinal (GI) mucosal defence and contributes significantly to the repair of damage and the resolution of inflammation. A variety of H_2S-donating agents protect the GI tract from injury, and H_2S-releasing NSAID derivatives do not cause appreciable GI injury.

*Therefore, the **central aim of this thesis** was to evaluate the roles of bile, enteric bacteria, and the enterohepatic circulation of NSAIDs in the pathogenesis of NSAID-enteropathy, and investigate the ability of H_2S to protect the small intestine from NSAID-induced damage.*

The animal models of NSAID-enteropathy are reflective of NSAID-enteropathy in humans, reproducible and simple. For example, ulcerations that develop in rats following

NSAID administration are predominately in the distal jejunum and ileum, similar to what is observed in humans (Reuter *et al*., 1997; Fujimori *et al*., 2010). For experiments in rats, I used a chronic NSAID-administration protocol, which resembles the common clinical scenario of individuals taking NSAIDs chronically. Furthermore, I administered a commonly prescribed NSAID, naproxen, that is also available over-the-counter.

I first examined whether an inhibitor of endogenous H_2S synthesis would exacerbate NSAID-enteropathy. Conversely, I hypothesized that the co-administration of diallyl disulfide (DADS), an H_2S-releasing garlic derivative, would protect against naproxen-induced enteropathy in rats (**Chapter 3**). I also evaluated whether DADS co-administration would alter bile toxicity, the enterohepatic circulation of naproxen, and the composition of enteric bacteria.

It is important to evaluate NSAIDs in combination with the drugs that are often co-prescribed (e.g., PPIs and low-dose ASA), given that these drugs can exacerbate NSAID-enteropathy. This approach is more predictive of the clinical scenario, and therefore may provide improved insight to the pathogenesis of NSAID-enteropathy. Therefore, I investigated why the co-administration of an antisecretory drug with an NSAID exacerbates NSAID-enteropathy. Specifically, I examined the effects of PPI and H_2RA administration on the bile and enteric bacteria in naproxen-treated rats (**Chapter 4 and 5**).

ATB-346 is a unique NSAID, as it does not induce significant GI damage, even in models of impaired mucosal defence and in models of polypharmacy. On the other hand, the parent NSAID of ATB-346, naproxen, can cause substantial GI injury and bleeding. I

explored why ATB-346 exhibits superior GI safety compared to naproxen (**Chapter 5**). In particular, I compared the effects of each drug on bile toxicity and on the small intestinal microbiota.

To study the causative role of bile in NSAID-induced small intestinal injury, I designed a novel model of *in vitro* rat epithelial cell injury that evaluated bile cytotoxicity. I established this model since it is more informative of the physiological effects of bile and NSAIDs on enterocytes that occur *in vivo*, compared to the commonly used practice of mixing commercial bile acids and NSAIDs and exposing epithelial cells to the solution (Petruzzelli *et al*., 2007; Dial *et al*., 2008; Zhou *et al*., 2010). By using bile collected from rats following NSAID administration, our model of NSAID, bile-induced cytotoxicity includes a variety of bile acids, endogenous bile constituents (e.g., phospholipids, cholesterol, and glutathione) as well as NSAID metabolites (i.e., NSAID glucuronides), all of which may be important *in vivo* to the role of bile in the pathogenesis of NSAID-enteropathy. Therefore, our model involved dosing rats with NSAIDs and/or other drugs, and collecting the bile via a bile duct cannulation procedure. The collected bile was then incubated on rat epithelial, intestinal cells and the quantity of cell death was measured following incubation. I believe that this novel approach to evaluating NSAID-induced bile toxicity is more predictive of the *in vivo* role of bile in the pathogenesis of NSAID-enteropathy.

Finally, I explored the ability of antibiotic (with varying bacterial specificity) administration to affect the severity of naproxen-induced enteropathy (**Appendix I**). Numerous studies have demonstrated a protective effect of gram-negative-targeting

antibiotic administration and a non-protective effect of gram-positive antibiotic administration on NSAID-enteropathy (Kent *et al*., 1969; Koga *et al*., 1999; Watanabe *et al*., 2008). However, the influence of antibiotics on NSAID-enteropathy is highly variable and the majority of studies were not reflective of a clinical scenario as they evaluated the effects of antibiotics after the administration of a single, large dose of indomethacin or diclofenac.

2.2. Thesis Objectives

The major findings in this thesis are organized into three chapters, each of which contributes to the overall goal of the thesis. The specific objectives of the thesis that are addressed in **Chapters 3-5** are described below:

Objective 1: *To investigate whether hydrogen sulfide-releasing agents would protect against NSAID-induced small intestinal damage and bleeding.* Results pertaining to this objective have been submitted for publication in the following manuscript:

Chapter 3 Blackler RW, Motta JP, Manko A, Workentine M, Bercik P, Surette MG, Wallace JL. (2014). Hydrogen sulfide protects against NSAID-enteropathy: role of bile and bacteria. [Submitted to *British Journal of Pharmacology*].

Objective 2: *To clarify the role of bile and enteric bacteria in the pathogenesis of NSAID-enteropathy.* This was a complicated objective to address due to the strong

interactions between bile and bacteria. Therefore, data pertaining to this objective is a component of each manuscript and Appendix I. The following manuscript helped address this objective and included novel data that suggested that antisecretory drug-induced changes in bile and enteric bacteria contribute to the exacerbation of NSAID-enteropathy:

Chapter 4 Blackler RW, Gemici B, Manko A, Wallace JL. (2014). NSAID-gastroenteropathy: new aspects of pathogenesis and prevention. *Curr Opin in Pharmacol*. [Epub ahead of print]

Objective 3: *To explore why ATB-346, a hydrogen sulfide-releasing derivative of naproxen, exhibits superior gastrointestinal safety compared to naproxen, the parent NSAID.* The manuscript in Chapter 5 addressed this objective by exploring whether the administration of naproxen (an NSAID associated with GI toxicity) differed from ATB-346 (a GI-safe NSAID) in the ability to affect the cytotoxic properties of bile. Furthermore, alterations in enteric bacteria were evaluated following the administration of naproxen or ATB-346.

Chapter 5 Blackler RW, Manko A, De Palma G, Da Silva GJ, Bercik P, Surette MG, Wallace JL. (2014). Pathogenesis of NSAID-Induced Enteropathy: Elucidation of Roles of Bile, Bacteria and Enterohepatic Circulation via Use of a GI-Safe NSAID. [In preparation for submission to *FASEB J*].

The data included in each manuscript required a collaborative effect with numerous institute colleagues and laboratory members. The specific details regarding authorship and contribution can be found in the **Preface** of each chapter.

– CHAPTER 3 –

HYDROGEN SULFIDE PROTECTS AGAINST NSAID-ENTEROPATHY: ROLE OF BILE AND BACTERIA

Hydrogen sulfide protects against NSAID-enteropathy: role of bile and bacteria

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The material presented in this chapter was submitted to *BJP*

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Preface: *The research presented in this manuscript was conducted from December 2012 to February 2014. I am the primary author of the paper. Dr. John L. Wallace and I designed the experiments, analyzed the data, wrote the manuscript and addressed the reviewers' concerns. I performed the majority of the experiments with assistance from the following people: W. McKnight (technician) assisted in the animal work and sample collection, Y. Deng (technician) assisted in preparing and running the gels for the denaturing gel gradient-electrophoresis (DGGE) experiments, J.P. Motta and A. Manko assisted in the animal work and bile cytotoxicity assays, M. Workentine assisted in the*

microbiota analysis, and P. Bercik and M.G. Surette contributed valuable scientific input and critically appraised the manuscript.

Title: Hydrogen sulfide protects against NSAID-enteropathy: role of bile and bacteria

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Conflict of Interest: Dr. Wallace is founder and a director of Antibe Therapeutics Inc., a company developing novel anti-inflammatory drugs.

Short Title: H_2S Protects Against NSAID-enteropathy

Keywords: Hydrogen sulfide; non-steroidal anti-inflammatory drug; enteropathy; inflammation; gastrointestinal; bile; dysbiosis; naproxen; diallyl disulfide; cyclooxygenase

Abbreviations: BCA, β-cyano-L-alanine; BHI, brain heart infusion; CBA, Columbia blood agar; CFU, colony-forming units; COX, cyclooxygenase; DADS, diallyl disulfide; DGGE, denaturing gradient gel electrophoresis; DMEM, Dulbecco's modified eagle

medium; GI, gastrointestinal; H_2S, hydrogen sulfide; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drugs; PG, prostaglandin; PPI, proton pump inhibitor; TLR, toll-like receptor; TX, thromboxane; UPGMA, unweighted-pair-group method with arithmetic mean.

3.1. Abstract

Background and purpose: Hydrogen sulfide 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 sulfide donor to protect against NSAID-induced enteropathy.

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

Key results: Suppression of endogenous hydrogen sulfide synthesis by β-cyano-L-alanine exacerbated naproxen-induced enteropathy. Diallyl disulfide co-administration dose-dependently reduced the severity of naproxen-induced small intestinal damage, inflammation and bleeding. Diallyl disulfide administration attenuated naproxen-induced

increases in the cytotoxicity of bile on cultured enterocytes, and prevented naproxen-induced changes in the intestinal microbiota.

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

3.2. 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 insidious small intestinal damage that can also occur (Wallace, 2013b). This is concerning 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 that suppression of gastric acid secretion has any benefit in terms of reducing damage or bleeding distal to the ligament of Treitz (i.e., beyond the duodenum). On the contrary,

there is emerging evidence that PPIs and histamine H2 receptor antagonists exacerbate the small intestinal damage and bleeding caused by NSAIDs (Zhao & 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 H2 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). Correction of this dysbiosis, through administration of *Bifidobacteria*, prevented the PPI-induced exacerbation of NSAID-induced intestinal damage (Wallace *et al.*, 2011).

The damaging effects of NSAIDs in the GI tract, particularly in the stomach, are directly related to their ability to suppress mucosal synthesis of prostaglandins (Wallace, 2008). Suppression of prostaglandin 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 sulfide (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; Wallace *et al.*, 2009; Wallace, 2010). Suppression 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). Prevention of gastric injury by H_2S is partly due to its inhibitory effect 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 beneficial effects of H_2S in the GI tract have prompted the design of a new class of H_2S-releasing NSAID derivatives. These H_2S-releasing NSAIDs have been shown to produce negligible gastroduodenal damage compared to 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; Wallace *et al.*, 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; Wallace *et al.*, 2010), even when co-administered with PPIs and/or ASA, 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 has distinct mechanisms from 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 & Boelsterli, 1998). There is also a wealth of evidence that the enteric flora contributes to the pathogenesis of NSAID-enteropathy

(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 disulfide (DADS), could prevent NSAID-induced enteropathy. We also attempted to determine if effects on enteric flora, bile and/or enterohepatic circulation of NSAIDs might explain any observed beneficial effects of the H_2S donor.

3.3. Methods

Animals. Male, Wistar rats weighing 220-250 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.

Effects of a Hydrogen Sulfide Donor on Naproxen-Induced Enteropathy. Rats ($n \geq 6$ per group) were treated orally, twice daily, with naproxen (20 $mg \cdot kg^{-1}$) or vehicle (dimethylsulfoxide and 1% carboxymethylcellulose; 5:95 ratio) for 4.5 days (9 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 hematocrit (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 B_2 (TXB_2) synthesis, as an index of systemic COX-1 activity (Wallace *et al.*, 1998). The small intestine was then blindly evaluated for hemorrhagic damage. This 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).

Immediately prior to each administration of naproxen or vehicle, rats were treated with DADS (10, 30, or 60 $mmol \cdot kg^{-1}$ p.o.) or an equivalent volume of vehicle (1% carboxymethylcellulose). Damage was assessed and samples were taken, as described above.

Effects of Inhibition of Hydrogen Sulfide Synthesis. Rats (*n*=6 per group) were treated orally, twice daily, with a lower dose of naproxen (10 $mg \cdot kg^{-1}$) 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 $mg \cdot kg^{-1}$ i.p.), or with vehicle (phosphate-buffered saline; PBS) immediately prior to naproxen administration. Three hours after the final dose, the small intestine was blindly evaluated for damage and samples were collected, as described above.

Prostaglandin 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 prostaglandin (PG) E_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 $mmol \cdot L^{-1}$; pH 7.4). Using scissors, the tissue sample was minced for 30 sec then placed in a shaking water bath (37°C) for 20 min. The samples were centrifuged (9000 *g*) for 30 sec 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. Specimens of jejunal tissues were collected, fixed and processed by routine techniques for light microscopy (H&E staining) (Wallace *et al.*, 2009).

Intestinal Epithelial Cell Culture. Rat intestinal epithelial (IEC-6) cells 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 Dulbecco's modified eagle medium (DMEM) containing 5% ($vol \cdot vol^{-1}$) fetal bovine serum, 4 $mmol \cdot L^{-1}$ glutamine, 50 $U \cdot mL^{-1}$ penicillin, and 50 $\mu g \cdot mL^{-1}$ streptomycin

(complete medium) at 37°C and 5% (vol·vol^{-1}) CO_2. 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 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 to 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% (vol·vol^{-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 were blindly 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 (with 5% sheep blood) (CBA) 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 analyzed to determine bacterial numbers, and the results expressed as CFU per gram of tissue.

DNA Extraction and Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis. Bacterial DNA was extracted from cecal 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 DNA gene was amplified using polymerase chain reaction with universal bacterial primers (HDA-1 and HDA-2) (Mobixlab, McMaster University core facility, Hamilton, Ontario, Canada). Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode universal

mutation system (Bio-Rad, Mississauga, Ontario, 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 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, 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 multi-dimensional 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.

Materials. Naproxen sodium and diallyl disulfide were purchased from Sigma-Aldrich (St. Louis, MO, USA). B-cyano-L-alanine, and the PGE_2 and TXB_2 ELISA kits were purchased from Caymen Chemical (Ann Arbor, MI, USA). Columbia and BHI agar media plates were purchased from Becton-Dickinson (Mississauga, ON, CA). DMEM, fetal bovine serum, penicillin, and streptomycin were purchased from Life Technologies Inc. (Burlington, ON, CA).

3.4. Results

Suppression of H_2S Synthesis Exacerbated Naproxen-induced Ulceration and Bleeding. Administration of naproxen at 10 $mg \cdot kg^{-1}$ resulted in a low level of intestinal damage (Figure 3.1 A). 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 3.1 A) and a small, but significant decrease in hematocrit (Figure 3.1 B). Jejunal granulocyte infiltration (MPO activity) in naproxen-treated rats was not affected by BCA co-treatment (data not shown).

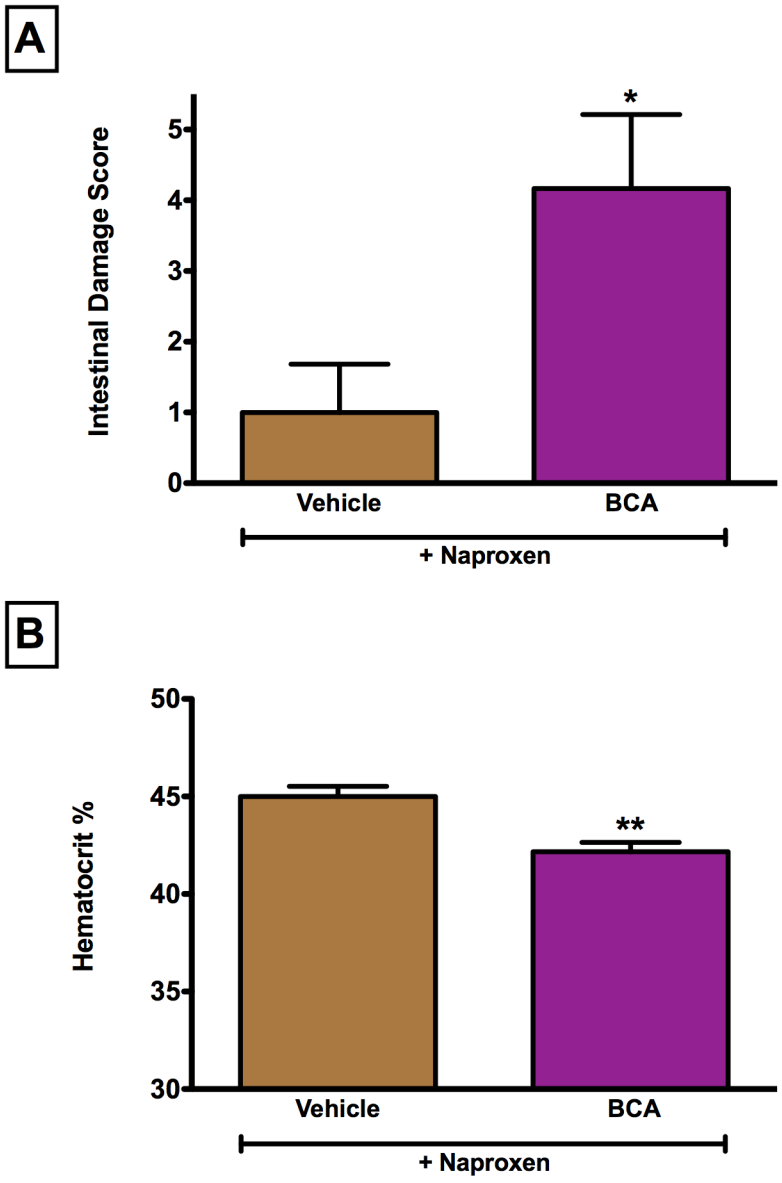

Figure 3.1. Inhibition of hydrogen sulfide (H_2S) synthesis by cystathionine γ-lyase (β-cyano-L-alanine (BCA) exacerbated naproxen-induced intestinal damage and bleeding. Panel A: Administration of naproxen (10 $mg \cdot kg^{-1}$) twice daily over 4.5 days resulted in marginal intestinal damage. Co-treatment with BCA significantly worsened naproxen-induced intestinal erosions (*$p<0.05$). Panel B: Rats co-treated with BCA and naproxen had significantly reduced hematocrit compared to rats treated with vehicle and naproxen

(**$p<0.01$). Results are shown as mean ± SEM ($n\geq6$ per group). The data were analyzed by an unpaired, two-tailed Student's t-test.

DADS Dose-Dependently Reduced Enteropathy and Bleeding. Administration of naproxen (20 $mg \cdot kg^{-1}$) twice daily for 4.5 days resulted in severe intestinal ulceration and bleeding (Figure 3.2 A). 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 3.2 A). Naproxen treatment resulted in a 35% decrease in hematocrit ($p<0.001$), whereas rats treated with DADS at doses of 30 or 60 $mmol \cdot kg^{-1}$ did not exhibit a significant change in hematocrit (Figure 3.2 B). Co-administration of DADS (30 or 60 $mmol \cdot kg^{-1}$) also significantly reduced weight loss in naproxen-treated rats ($p<0.01$; Figure 3.2 C).

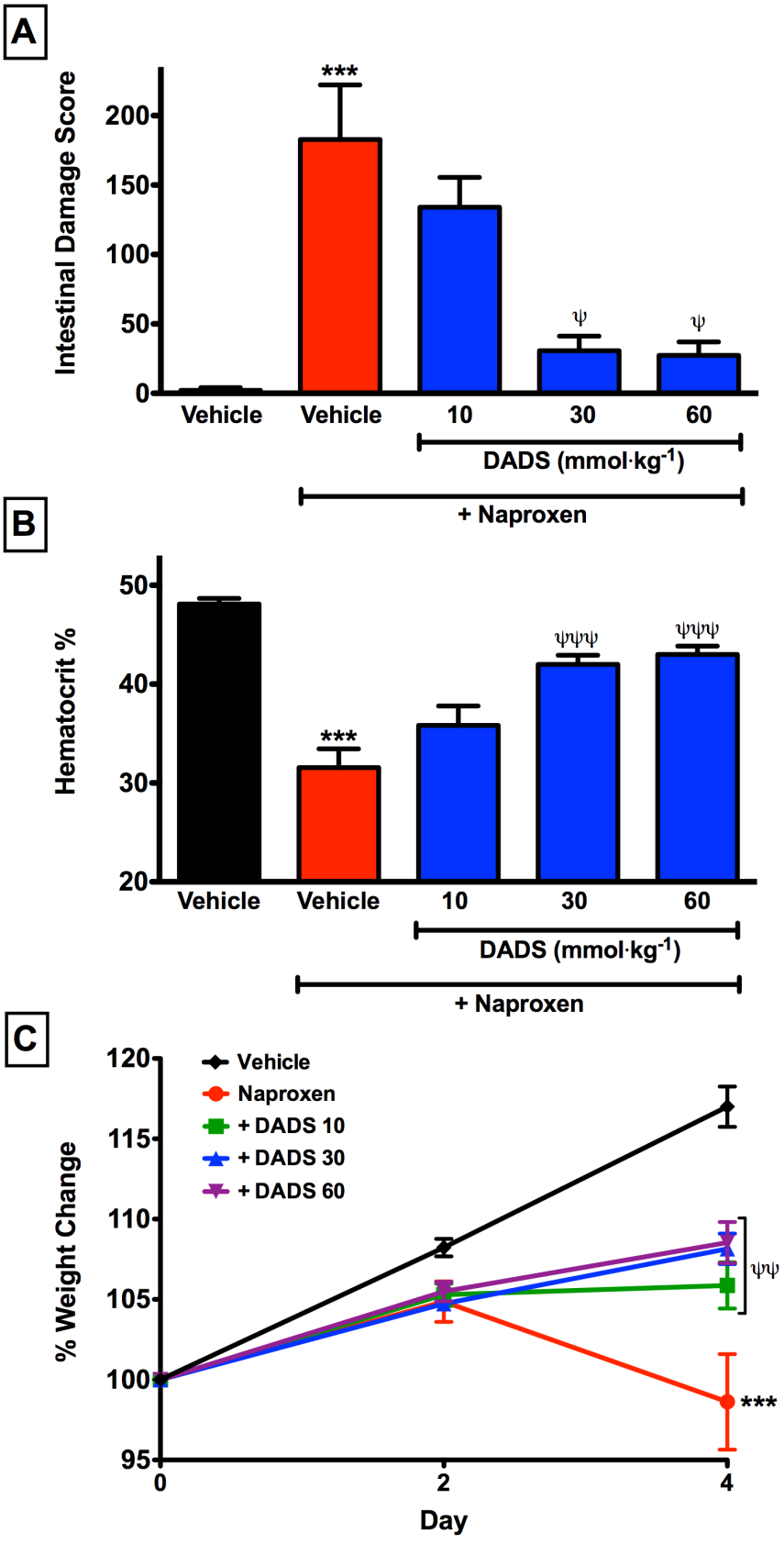
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Figure 3.2. Dose-dependent reduction of naproxen-induced intestinal ulceration by diallyl disulfide (DADS). Rats were co-treated, twice daily, with naproxen (20 $mg \cdot kg^{-1}$) and vehicle or DADS (10, 30, or 60 $mmol \cdot kg^{-1}$) for 4.5 days. Panel A: Naproxen-induced small intestinal damage was significantly reduced by co-treatment with DADS at doses of 30 and 60 $mmol \cdot kg^{-1}$ ($^{\Psi}p<0.05$). Panel B: Naproxen administration caused significant bleeding compared to vehicle treatment (***$p<0.001$), but co-treatment with DADS at doses of 30 or 60 $mmol \cdot kg^{-1}$ significantly reduced the naproxen-induced decrease in hematocrit ($^{\Psi\Psi\Psi}p<0.001$). Panel C: Weight loss caused by naproxen administration (***$p<0.001$) was significantly reduced by co-treatment with DADS at doses of 30 or 60 $mmol \cdot kg^{-1}$ ($^{\Psi\Psi}p<0.01$). Results are shown as mean ± SEM ($n \geq 6$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's and Bonferroni post-hoc tests.

Effects of DADS on Suppression of Cyclooxygenase Activity. Naproxen administration profoundly suppressed systemic COX-1 activity (whole blood thromboxane synthesis; by 99%) (Figure 3.3 A) and intestinal PGE_2 synthesis (by 64%) after twice daily dosing for 4.5 days. A similar degree of suppression of thromboxane 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 $mmol \cdot kg^{-1}$ exhibited an increase (~1.8-fold) in intestinal PGE_2 synthesis as compared to naproxen-treated rats ($p<0.05$) (Figure 3.3 B). Similar effects were observed in gastric tissue (Supplemental Figure 3.7). Naproxen significantly inhibited gastric PGE_2 synthesis (by 81%) when compared to vehicle-treated rats. However, co-treatment with DADS at 30 or 60 $mmol \cdot kg^{-1}$ resulted in significantly elevated gastric PGE_2 synthesis as compared to that in naproxen-treated rats ($p<0.05$).

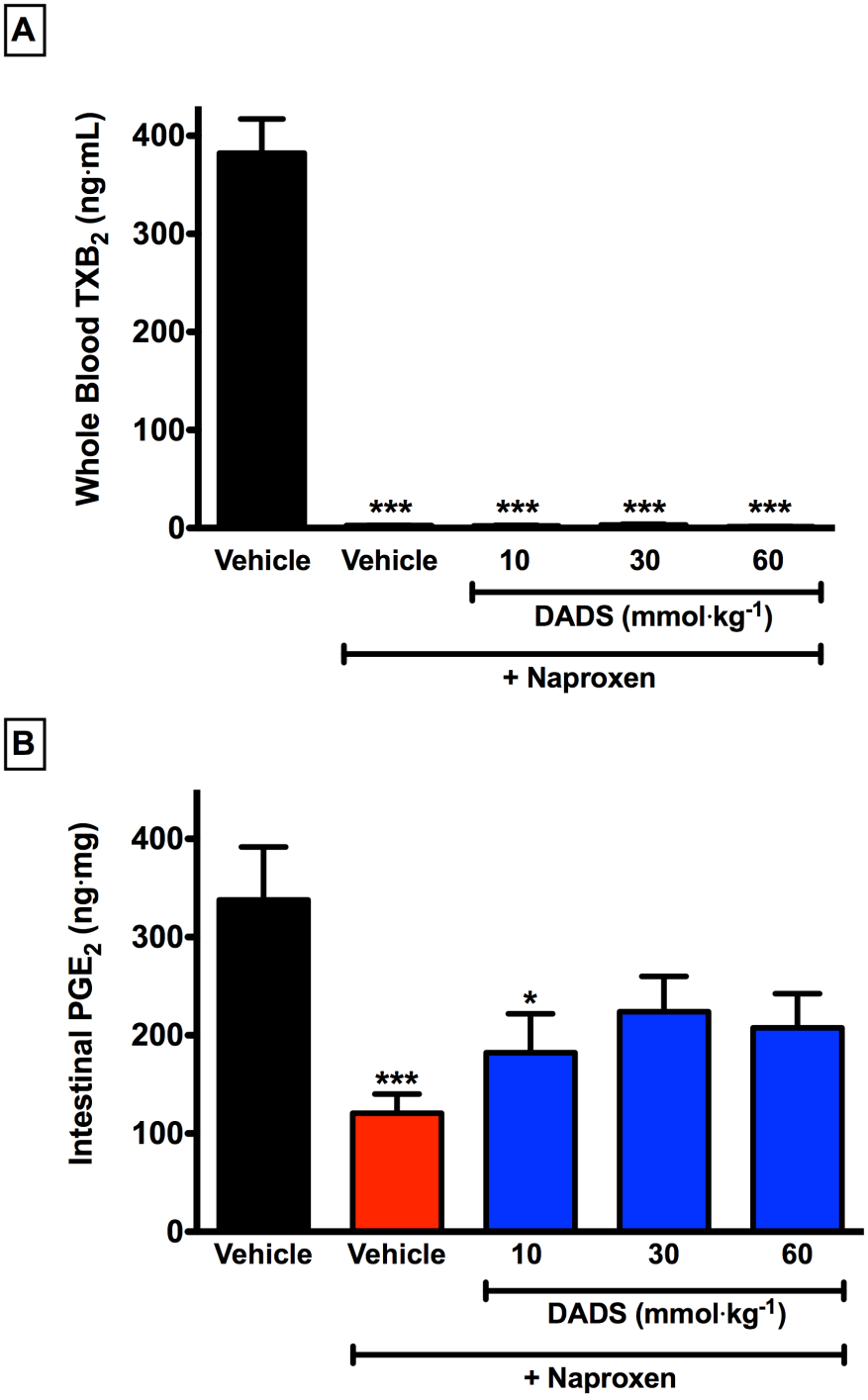

Figure 3.3. Diallyl disulfide (DADS) did not prevent systemic cyclooxygenase inhibition by naproxen. Naproxen administration significantly suppressed (by 99%) whole blood synthesis of thromboxane (TXB_2) (***$p<0.001$, panel A), and this was not affected by

co-administration of DADS (10, 30, or 60 mmol·kg^{-1}). Three hours after the final dose, naproxen administration also significantly inhibited (by 64%) (***$p<0.001$) intestinal prostaglandin (PG) E_2 synthesis compared to 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 to vehicle treated rats. Results are shown as mean ± SEM ($n\geq6$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's and Bonferroni post-hoc tests.

DADS Dose-dependently Reduced Intestinal Inflammation. Naproxen administration resulted in a significant (~4-fold) increase in jejunal granulocyte infiltration (MPO activity) as compared to 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 3.4 A). 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 edema, as compared to vehicle-treated rats (Figure 3.4 B and 3.4 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 3.4 D).

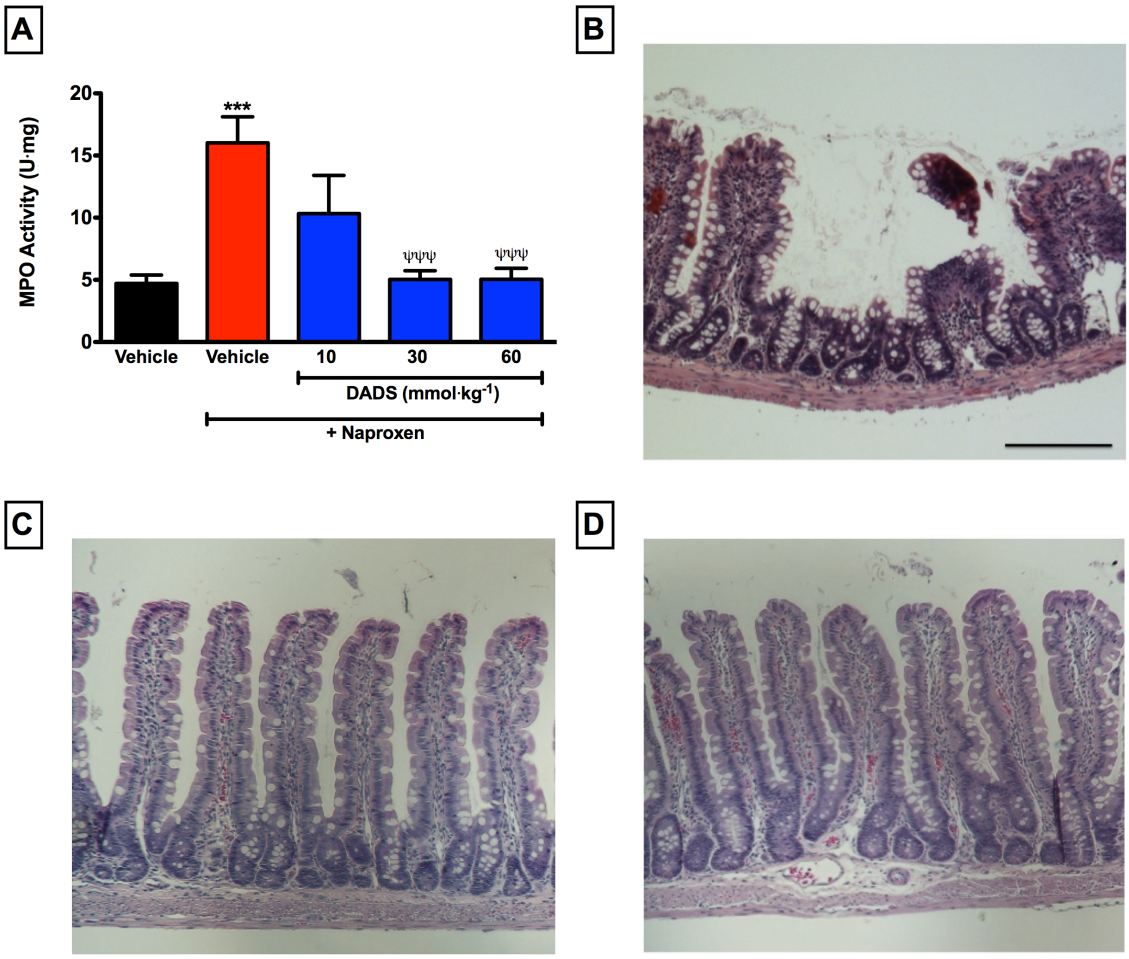

Figure 3.4. Diallyl disulfide (DADS) dose-dependently prevented naproxen-induced mucosal inflammation and structural damage. Panel A: Naproxen administration significantly increased intestinal myeloperoxidase (MPO) activity compared to vehicle-treated rats (***$p<0.001$). However, co-treatment with DADS at doses of 30 or 60 mmol·kg^{-1} significantly diminished the naproxen-induced increase in MPO activity ($^{\psi\psi\psi}p<0.001$). 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 ($n\geq6$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's and Bonferroni post-hoc tests. *Scale bar*, 100 μm (applicable to each panel).

Cytotoxic Effects of Bile were Enhanced by Naproxen. When tested at dilutions of 1:6 or 1:12, bile collected from rats administered naproxen (20 $mg \cdot kg^{-1}$) over 4.5 days exhibited significantly increased cytotoxic effects on IEC-6 intestinal epithelial cells as compared to bile from rats administered vehicle. Results for the 1:6 dilutions are shown in Figure 3.5. Thus, exposure of the cells to a 1:6 dilution of bile from naproxen-treated rats for 3 h resulted in 58% cytotoxicity, as compared to 26% cytotoxicity ($p<0.001$) for bile from vehicle-treated rats (Figure 3.5). 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 3.5). Indeed, bile collected from rats co-treated with naproxen and DADS at 60 $mmol \cdot kg^{-1}$ 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 (Supplementary figure 3.8).

The severity of naproxen-induced intestinal damage correlates well with the concentrations of naproxen in the bile after administration of naproxen to rats (unpublished 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 $mg \cdot kg^{-1}$) 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 (Supplementary figure 3.9).

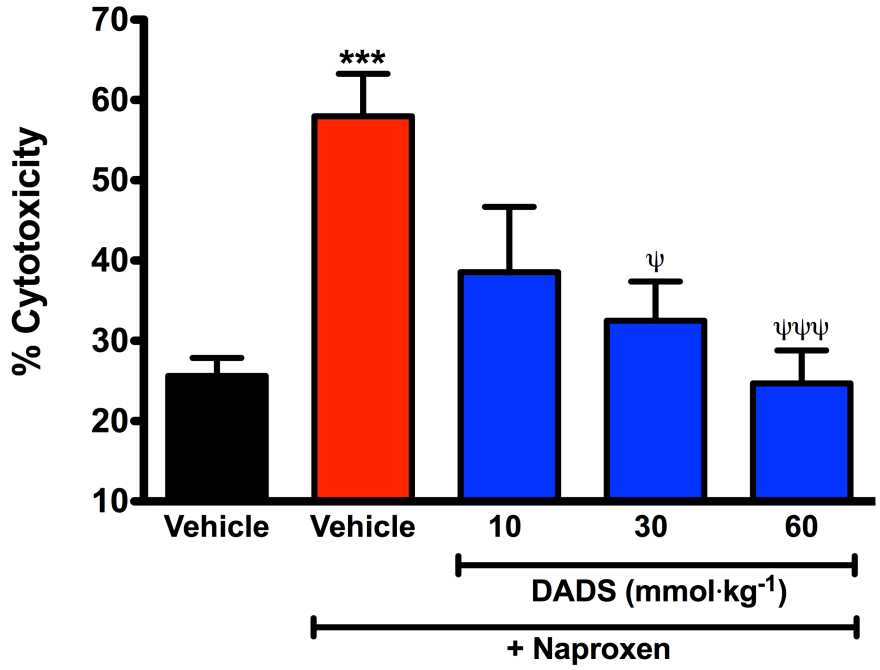

Figure 3.5. Diallyl disulfide (DADS) dose-dependently reduced naproxen-induced bile cytotoxicity. Bile collected from rats treated with naproxen (20 mg·kg^{-1}) twice daily for 4.5 days was significantly more cytotoxic to cultured rat intestinal epithelial (IEC-6) cells than bile collected from vehicle-treated rats (***$p<0.001$). Co-treatment with DADS at 30 mmol·kg^{-1} significantly reduced the naproxen-induced increase in cytotoxicity of bile (*$p<0.05$). 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 ($^{\Psi\Psi\Psi}p<0.001$). Data shown are from the 1:6 dilutions of bile samples, and are expressed as the mean ± SEM of at least 6 rats per group. The data were analyzed by a one-way analysis of variance followed by Dunnett's and Bonferroni post-hoc tests.

DADS Administration Altered the Composition of the Microbiota. We examined whether DADS administration would alter the composition of the intestinal microbiota. DGGE analysis of cecal 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 $mg \cdot kg^{-1}$) plus vehicle, or naproxen plus DADS at a protective (30 $mmol \cdot kg^{-1}$) and a non-protective (10 $mmol \cdot kg^{-1}$) dose. Naproxen administration caused cecal dysbiosis in rats and co-treatment with a non-protective dose of DADS (10 $mmol \cdot kg^{-1}$) did not correct this shift, as analyzed by the Dice coefficient and Ward algorithm to determine similarities (Figure 3.6 A). Interestingly, the microbiota of naproxen-administered rats co-treated with a protective dose of DADS (30 $mmol \cdot kg^{-1}$) clustered with that of rats not treated with naproxen. Construction of a UPGMA tree demonstrated similar clustering and each branch had 100% resampling support (Figure 3.6 B). The total number of aerobes in the jejunum of rats treated with naproxen or naproxen plus DADS (10, 30, or 60 $mmol \cdot kg^{-1}$), was not significantly different from that in vehicle-treated rats (whether plated on CBA or BHI media) (Figure 3.6 C).

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 $mmol \cdot kg^{-1}$). Similar to the above-mentioned results, the total number of aerobes and anaerobes in the jejunum of rats treated with DADS at doses of 10 or 30 $mmol \cdot kg^{-1}$was not significantly different from that in vehicle-treated rats (whether plated on CBA or BHI media) (Supplementary figure 3.10 C).

DGGE analysis of the cecal 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 as compared to 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 versus that in rats treated with vehicle (Supplementary figure 3.10 A and 3.10 B).

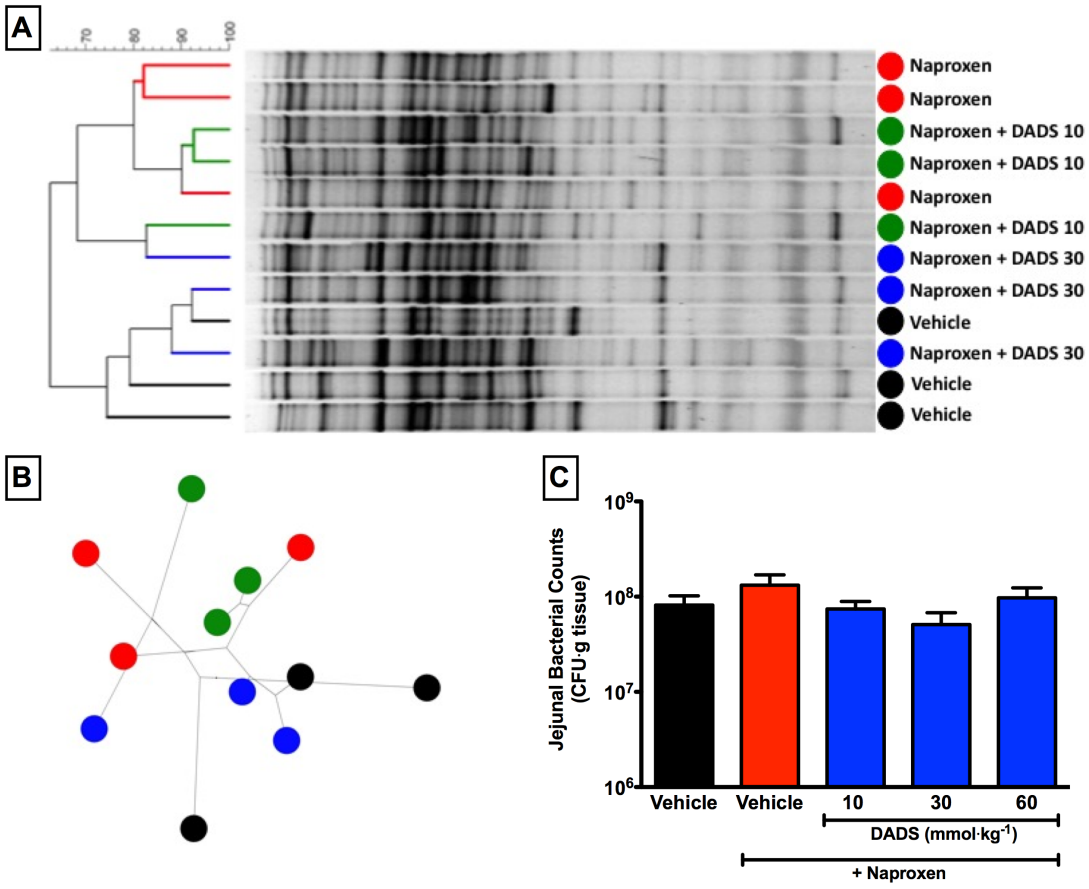

Figure 3.6. Co-treatment with diallyl disulfide (DADS) prevented naproxen-induced dysbiosis. Panel A: Denaturing gradient gel electrophoresis analysis revealed that naproxen (20 mg·kg^{-1}) administration to rats caused dysbiosis of the cecal microbiota,

with distinct clustering from vehicle-treated rats. Co-treatment with DADS at 30 $mmol{\cdot}kg^{-1}$ shifted the microbiota of naproxen-treated rats back to being similar to that of vehicle-treated rats. Using a resampling technique (majority unweighted-pair-group method with arithmetic mean 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 $mmol{\cdot}kg^{-1}$) twice daily for 4.5 days. Results in panel C are from samples plated on Columbia blood agar (CBA) and shown as mean ± SEM ($n \leq 5$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison test.

3.5. 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, 2013). 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.

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 likely more complex (Wallace, 2012). COX inhibition contributes to the injury that develops in the intestine following NSAID administration, but 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; Somasunduram *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 *et al*., 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). 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. 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 enterhepatic 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.

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*., 2007). 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 (Goulbern *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 statistically significant changes in intestinal prostaglandin synthesis. As expected, naproxen administration resulted in a marked inhibition of intestinal PGE_2 synthesis and of whole blood thromboxane synthesis (the latter is almost entirely derived from platelets) (Wallace *et al*., 1998). In rats pre-treated 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 pre-treated 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 above, 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. ATB-346 is an H_2S-releasing derivative of naproxen. At a dose equivalent to the 20 $mg \cdot kg^{-1}$ dose of naproxen used in the present study, ATB-346 produced no gastric or

small intestinal damage (Wallace *et al*., 2010; Blackler *et al*., 2012), despite having the capacity to deliver less than 1% of the H_2S that is released by the protective doses of DADS. Further studies are required to better understand the mechanisms for the GI-sparing effects of ATB-346, and why it appears to differ mechanistically from that of an H_2S donor such as DADS.

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 above, 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.

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, since changes in enteric bacteria can lead to altered bile metabolism, and in turn to an alteration of cytotoxicity.

3.6. Supplementary Material

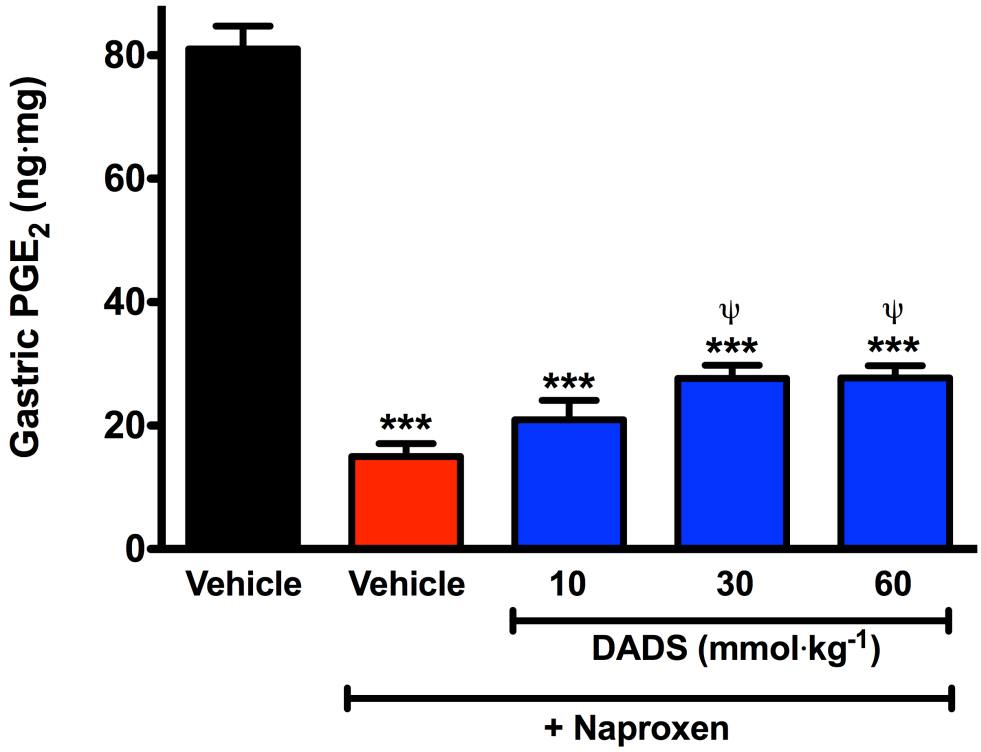

Figure 3.7. Effects of diallyl disulfide (DADS) on inhibition of gastric prostaglandin (PG) E_2 synthesis by naproxen. Three hours after the final dose, naproxen significantly

suppressed gastric prostaglandin (PG) E_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 ($n\geq6$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's and Bonferroni post-hoc tests.

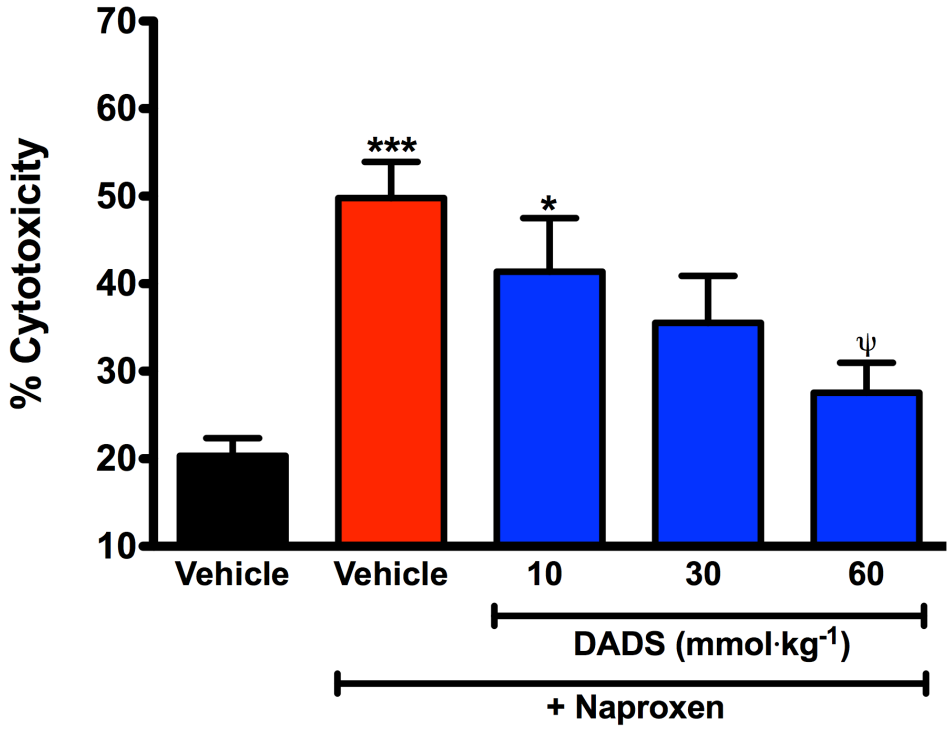

Figure 3.8. Diallyl disulfide (DADS) dose-dependently reduced naproxen-induced bile cytotoxicity on HT-29 cultured cells. Similar to the bile cytotoxicity results from IEC-6 cell cultures, bile collected from rats treated with naproxen (20 mg·kg^{-1}) twice daily for 4.5 days was significantly more cytotoxic 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 phosphate buffer saline (DPBS) prior to being added to the cultured cells. Results are shown as mean ± SEM (n=6 per group). The data

were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison test.

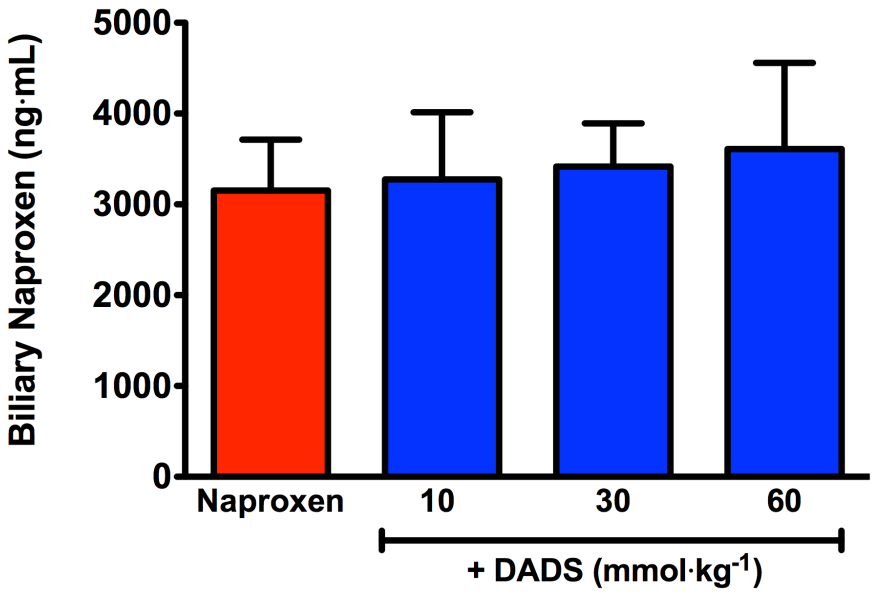

Figure 3.9. Biliary concentrations of naproxen were unaltered by co-administration of diallyl disulfide (DADS). Naproxen (20 mg·kg^{-1}) was co-administered with vehicle or with DADS (10, 30 or 60 mmol·kg^{-1}) twice daily for 4.5 days. Bile was collected 1 h after the final drug administration. Biliary naproxen concentrations were measured by liquid chromatography/mass spectrometry. Results are shown as mean ± SEM (*n*=6 per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's and Bonferroni post-hoc tests.

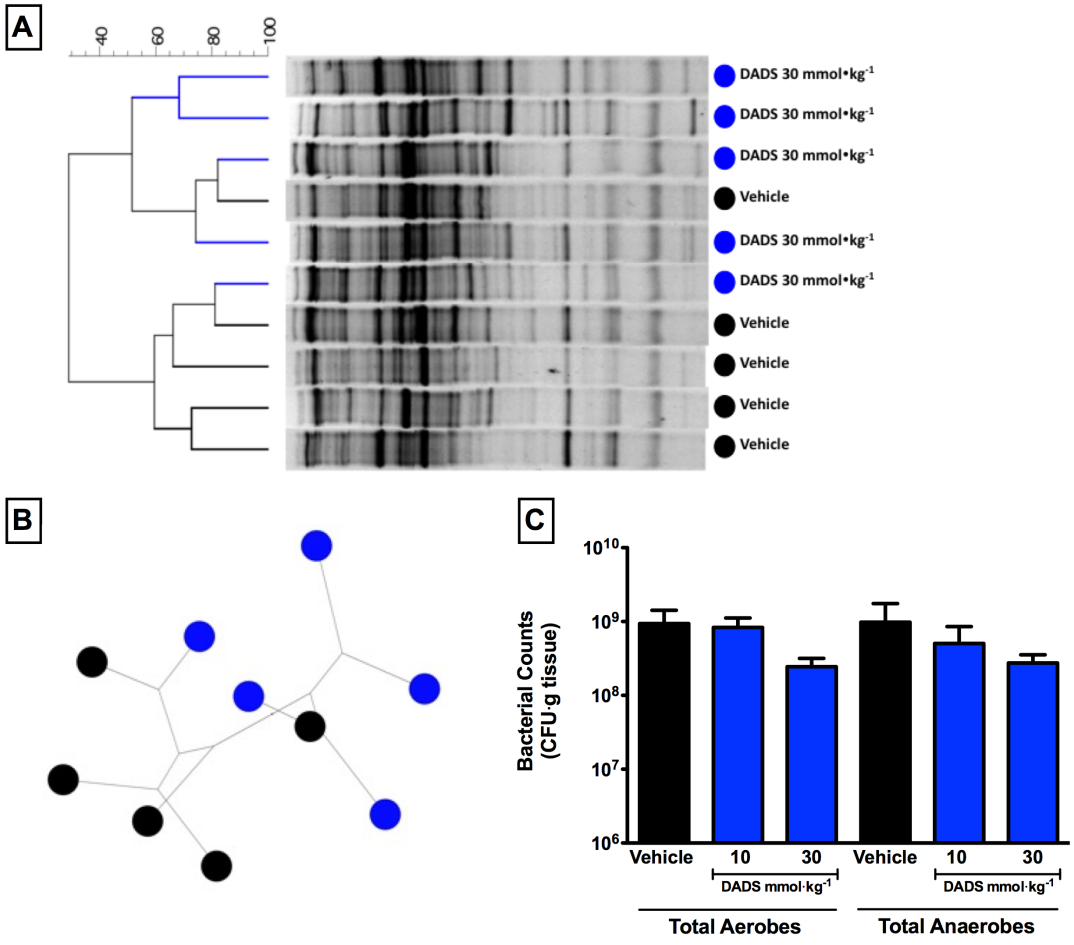

Figure 3.10. Treatment with diallyl disulfide (DADS) caused a shift in the microbiota composition. Panel A: Denaturing gradient gel electrophoresis analysis revealed that there were significant differences in the composition of cecal microbiota in vehicle-treated and DADS-treated (30 mmol·kg^{-1}) rats. The clustering observed in the dendrogram constructed using the Dice coefficient and Ward algorithm in Panel A was confirmed using majority unweighted-pair-group method with arithmetic mean 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 Columbia blood agar (CBA) and shown as mean ± SEM (n≤5 per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison test.

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– CHAPTER 4 –

NSAID-GASTROENTEROPATHY: NEW ASPECTS OF PATHOGENESIS AND PREVENTION

NSAID-Gastroenteropathy: New Aspects of Pathogenesis and Prevention

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Preface: *The research presented in this manuscript was conducted from April 2013 to December 2013. I am the primary author of the paper, along with Burcu Gemici. John L. Wallace, Burcu Gemici, and I designed the experiments, analyzed the data, wrote the manuscript and addressed the reviewers' concerns. I performed the famotidine and naproxen co-administration experiments and Burcu Gemicic conducted the gastric acid experiments with assistance from the following people: W. McKnight (technician) assisted in the animal work and sample collection and A. Manko assisted in the animal work and bile cytotoxicity assays.*

Title: NSAID-gastroenteropathy: new aspects of pathogenesis and prevention

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Conflict of Interest: Dr. Wallace is founder and a director of Antibe Therapeutics Inc., a company developing novel anti-inflammatory drugs.

Abbreviations: GI, gastrointestinal; H_2RA, histamine H_2 receptor antagonist; H_2S, hydrogen sulfide; NSAID, nonsteroidal anti-inflammatory drug; PPI, proton pump inhibitor; SIBO, small intestinal bacterial overgrowth

Keywords: Hydrogen sulfide; non-steroidal anti-inflammatory drug; enteropathy; inflammation; gastrointestinal; bile; dysbiosis; enterohepatic circulation; naproxen; cyclooxygenase

Highlights:

- NSAID-induced enteropathy may be more clinically significant than upper GI damage
- NSAID-enteropathy is significantly worsened by inhibitors of gastric acid secretion
- Changes in enteric bacteria and bile are critical in the pathogenesis of NSAID-enteropathy
- Hydrogen sulfide is a potent protective factor throughout the GI tract.

4.1. Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) remain among the most commonly used medications because of their effectiveness at reducing pain and inflammation. The damaging effects of NSAIDs in the stomach and duodenum can be substantially reduced by inhibitors of gastric acid secretion. However, there are no proven-effective preventative or curative treatments for NSAID-induced enteropathy. In recent years, substantial progress has been made in better understanding the pathogenesis of NSAID-enteropathy, and in particular the interplay of enteric bacteria, bile and the enterohepatic recirculation of the NSAID. Moreover, it is becoming clear that suppression of gastric acid secretion significantly worsens NSAID-enteropathy.

4.2. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) remain a mainstay for treatment of numerous inflammatory diseases, including osteoarthritis, despite significant untoward effects on the gastrointestinal (GI) tract. The damage produced by these drugs in the stomach and duodenum can be greatly reduced by co-administration of inhibitors of gastric acid secretion, such as proton pump inhibitors (PPIs) and histamine H_2 receptor antagonists (H_2RAs). As discussed below, these drugs are not without adverse effects, so there continues to be a search for improved approaches for preventing NSAID-gastroduodenopathy.

Much recent research has been focused on the damage caused by NSAIDs in the lower intestine. NSAID-enteropathy has a distinct pathogenesis from the damage

produced in the upper GI tract [1]. Suppression of cyclooxygenase activity contributes to NSAID-enteropathy, but the roles of bile and bacteria appear to be much more significant (Figure 4.1). There is a growing interest in NSAID-enteropathy largely because of improved methods for detecting the damage, through video capsule endoscopy and double-balloon enteroscopy. Moreover, the emerging evidence that use of agents that suppress gastric acid secretion are causing a significant worsening of the small intestinal damage caused by NSAIDs [2-5] is stimulating further research into the pathogenesis of this condition.

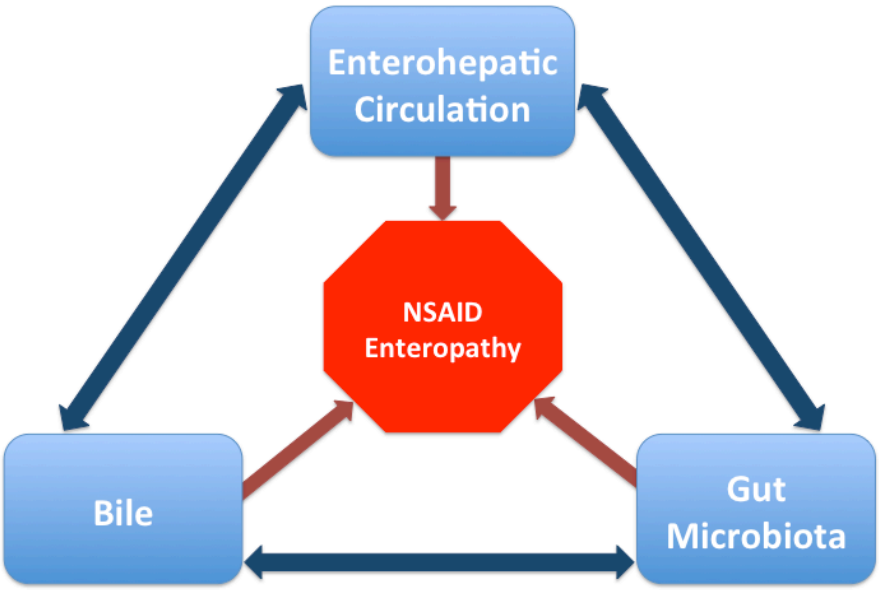


Figure 4.1. Interactions among bile, intestinal microbes and enterohepatic circulation of NSAIDs contribute significantly to NSAID-induced intestinal damage. The cytotoxicity of bile in increased following NSAID administration, and also by conversion from primary to secondary bile acids (catalyzed by bacterial enzymes). Bacteria also contribute to enterohepatic recirculation of NSAIDs, as bacterial β-glucuronidase is necessary for NSAID re-uptake in the ileum. Suppression of gastric acid secretion leads

to significant changes to the intestinal microbiota and increases the cytotoxicity of bile, but the temporal relationship of these effects is unclear.

4.3. Gastroduodenal Protection

Secretion of bicarbonate by gastric and duodenal epithelial cells is an important component of mucosal defence. Hydrogen sulfide has been shown to markedly reduce the severity of gastric damage induced by NSAIDs [6] or by ischemia-reperfusion [7]. Maintenance of gastric blood flow [8] and inhibition of leukocyte-endothelial adhesion [9] contribute to the protective effects of H_2S, but stimulation of bicarbonate secretion may be another important mechanism. Takeuchi *et al*. [10] demonstrated a key role of endogenous H_2S in the secretion of bicarbonate in the rat duodenum that is stimulated by mucosal acidification. Duodenal bicarbonate secretion was increased following administration of an H_2S donor, and reduced by an inhibitor of endogenous H_2S synthesis. The latter also led to enhanced acid-induced duodenal damage. Acid-induced duodenal bicarbonate secretion was also shown to be mediated by two other gaseous mediators, nitric oxide and carbon monoxide [10].

Regulation of bicarbonate secretion by H_2S also extends to the stomach and may contribute significantly to the gastroprotective effects of this mediator [11]. As shown in Figure 4.2, administration of an H_2S-releasing derivative of naproxen (ATB-346) resulted in a marked (~50%) decrease in gastric acidity, with an increase in mean pH of gastric juice from 1.48 to 2.11, and a ~82% decrease in the volume of secretion. This was most likely attributable to a combination of increased gastric bicarbonate secretion and decreased gastric acid secretion. Mard *et al*. [12] recently reported that H_2S could inhibit

gastric acid secretion, but it is possible that at least some of the decrease in titratable acidity that they observed was actually due to stimulation of bicarbonate secretion. ATB-346 did not affect gastric emptying rates (Fig. 4.2).

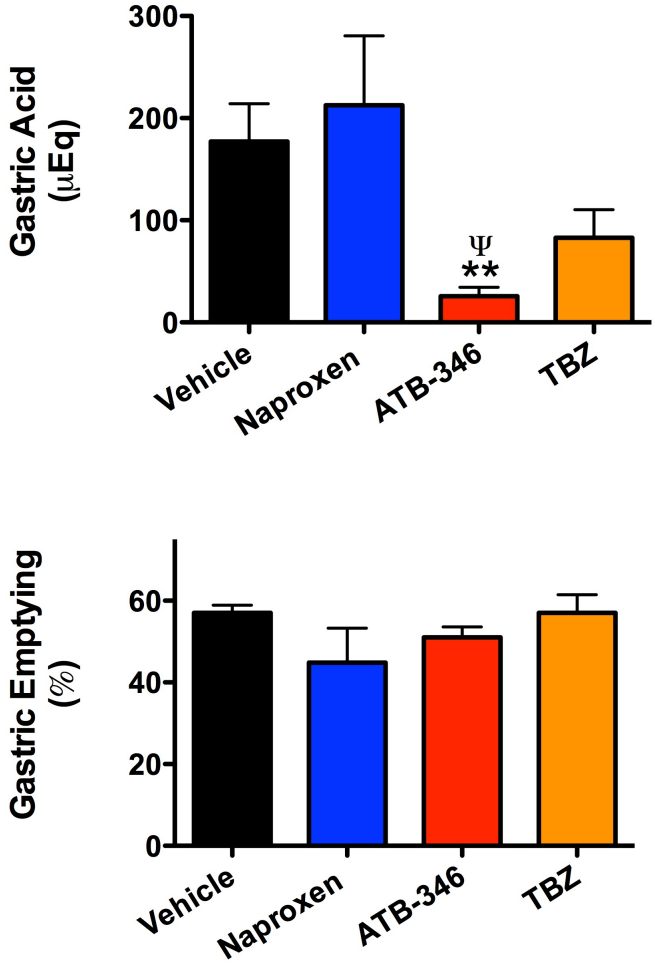


Figure 4.2. ATB-346, a hydrogen sulfide-releasing derivative of naproxen, significantly reduced gastric acidity (upper panel), but did not affect gastric emptying rates (lower panel). In contrast, naproxen itself did not affect gastric acidity, and the effect of the H_2S-releasing moiety of ATB-346 (4-hydroxy-thiobenzamide; TBZ) did not reach statistical significance. Naproxen was administered at 20 mg/kg, and the other drugs were administered at equimolar doses to that of naproxen. *$p<0.05$ versus the

vehicle-treated group; $^{\Psi}p<0.05$ versus the TBZ-treated group. Data are shown as the mean ± standard error of the mean of at least 5 rats per group. The data were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison.

4.4. NSAID-enteropathy

4.4.1. Exacerbation by acid suppressing drug. A number of studies have confirmed the ability of NSAIDs, including low-dose ASA, to cause significant ulceration and bleeding in the small intestine [13-15]. Indeed, co-use of an NSAID with low-dose ASA and inhibitors of gastric acid secretion is now commonplace, but can result in a synergistic increase in intestinal injury and bleeding [16,17], that has been described as "*the perfect intestinal storm*" [5]. As is the case in humans, the most severe ulceration caused by NSAIDs is found in the ileum [15]. Recent human data are consistent with what has been reported in animal studies [2-5,16]. Thus, using video capsule endoscopy, Watanabe *et al*. examined the small intestine of rheumatoid arthritis patients taking NSAIDs [18]. Using multivariate regression analysis, they identified risk factors associated with severe intestinal damage and significantly decreased hemoglobin levels [18]. The three statistically significant relative risk factors (RR) were: use of a PPI (RR: 5.22), age over 65 (RR: 4.16), and use of a H_2RA (RR: 3.95).

4.4.2 Intestinal protection through inhibition of NSAID re-absorption. Enterohepatic circulation of NSAIDs is a key component of the mechanism of damage these drugs produce in the small intestine (Figure 4.1). After absorption, NSAIDs can undergo glucuronidation in the liver, and are then secreted into bile. Bacterial β-D-

glucuronidase can deconjugate the NSAID-glucuronides, facilitating re-absorption of the NSAID in the ileum. Inhibition of this enzyme with a novel inhibitor has been shown to prevent enterohepatic circulation of NSAIDs, and to reduce the intestinal injury caused by these drugs [19]. Saitta *et al*. reported that pretreatment of rats with an inhibitor of β-D-glucuronidase markedly protected against diclofenac-induced damage in the small intestine [20]. A similar effect was observed when indomethacin or ketoprofen were the NSAIDs used to induce intestinal damage. Delaying administration of the β-D-glucuronidase inhibitor until 3 hours after NSAID administration resulted in a diminished protective effect, which was consistent with pharmacokinetic data suggesting a short half-life of the inhibitor [20].

4.4.3. Hydrogen sulfide prevents NSAID-enteropathy. An H_2S-releasing derivative of naproxen (ATB-346) was previously shown not to produce gastric damage, even at exceptionally high doses [21]. Administration of the drug to rats with compromised gastric mucosal defence also did not result in significant damage, while the comparator drug, naproxen (and sometimes celecoxib), caused extensive hemorrhagic damage [21]. This drug also did not produce intestinal damage when administered twice-daily over several days [16]. Blackler *et al*. examined the effects of this drug in several models of clinical conditions in which susceptibility to NSAID-induced GI damage is markedly increased: e.g., arthritis, obesity, hypertension [16]. In each case, ATB-346 did not cause significant GI damage. Moreover, when administered together with low-dose ASA and a proton pump inhibitor (over several days), ATB-346 did not cause detectable small

intestinal damage, whereas naproxen and celecoxib at comparable anti-inflammatory doses caused severe intestinal ulceration and bleeding [16].

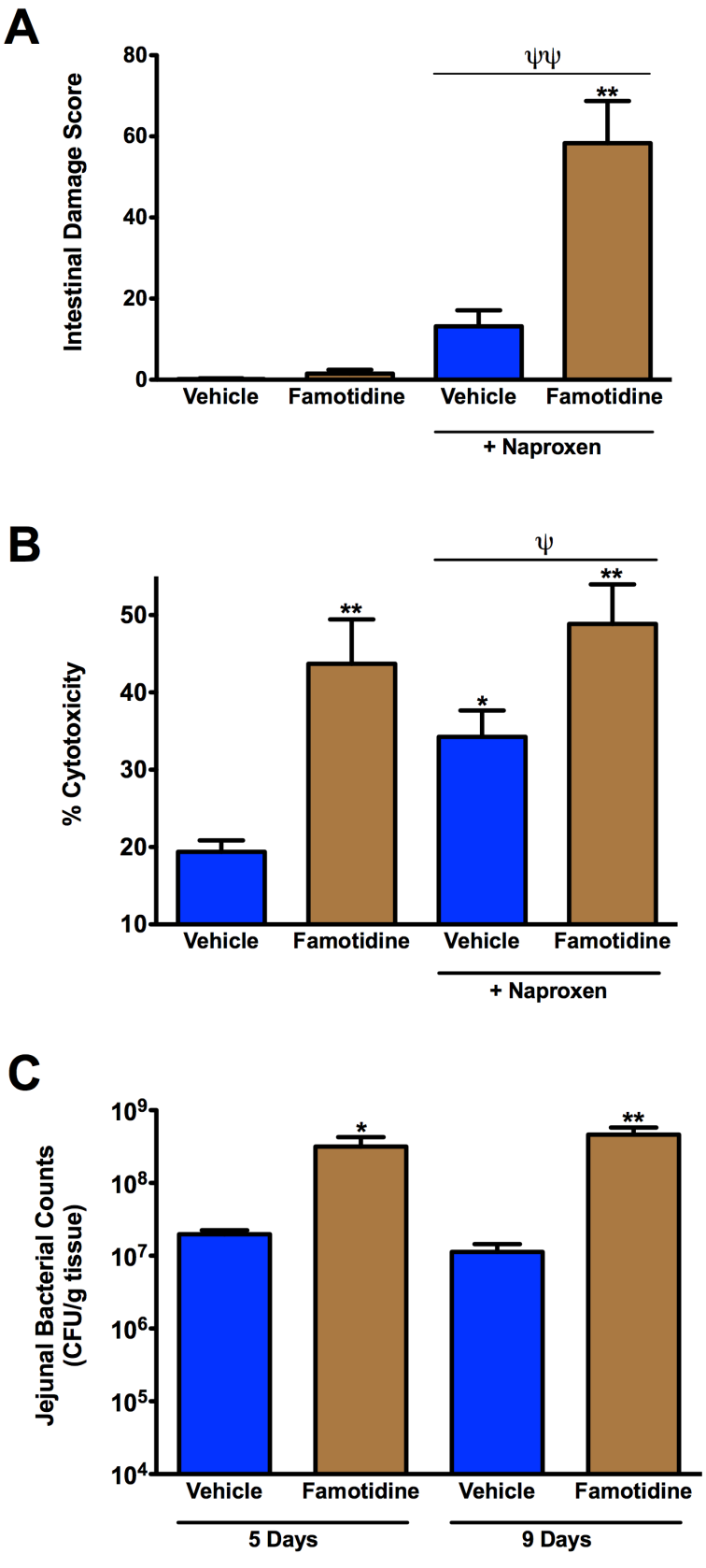
4.4.4. Acid suppression and enteropathy. The most commonly used agents for preventing or treating NSAID-induced damage in the stomach and duodenum are antisecretory drugs, such as H_2RAs antagonists and PPIs. However, it has been clear for many years that these drugs offer no protection to the lower small intestine, and in recent years it has been reported that they exacerbate NSAID-induced small intestinal lesions in rats [2-5]. There are no proven-effective preventative or therapeutic regiments for NSAID-enteropathy [1]. Satoh *et al*. [22] examined the effects of three agents with proven protective effects in the upper GI tract (misoprostol, irsogladine, and rebamipide) on diclofenac-induced intestinal lesions, as well as on the exacerbation of those lesions by ranitidine or omeprazole. Pretreatment with misoprostol, irsogladine, or rebamipide inhibited the formation of intestinal lesions caused by a high dose of diclofenac alone. These agents also prevented the exacerbation of diclofenac-induced lesions that was caused by ranitidine and omeprazole. These studies involved only acute administration of diclofenac, so it remains to be seen if these potential protective agents are effective in a model where the NSAID is administered repeatedly over several days [22].

4.4.5. Bile, bacteria and enterohepatic circulation in NSAID-enteropathy. Several studies have demonstrated critical roles for the bile, enteric bacteria and the enterohepatic circulation of the NSAIDs in the pathogenesis of NSAID-induced enteropathy [23-27]

(Figure 4.1). A critical role for bacteria was recently reinforced by a study demonstrating that the exacerbation of NSAID-enteropathy in rats by treatment with a PPI was attributable to the dysbiosis that occurred following PPI administration [2]. Specifically, there was a marked loss of *Bifidobacter* following PPI treatment. The increased susceptibility to NSAID-enteropathy caused by the PPI could be reversed if intestinal levels of *Bifidobacter* were replenished [2]. It remains unclear *why* the PPI-induced dysbiosis resulted in greater NSAID-induced intestinal damage and bleeding, but it is possible that this triggered changes in bile that contributed to intestinal injury.

Numerous clinical reports and animal studies have associated chronic use of PPIs or H_2RAs with alterations in the GI tract. Among the reported changes are small intestinal bacterial overgrowth (SIBO) and bile acid dysmetabolism [28-31]. The development of SIBO is a direct consequence of suppression of gastric acid secretion. The ensuing bile acid dysmetabolism in patients with SIBO is likely the result of the disproportionate increase in numbers of microbes capable of deconjugating bile acids and/or of converting primary bile acids to secondary bile acids [28,29,32]. The deconjugation of bile acids by bacterial enzymes increases bile acid hydrophobicity, thereby increasing the ability of the bile acid to disrupt the cellular membranes of enterocytes [33]. Bacterial enzymatic conversion of primary bile acids to secondary bile acids may also contribute to ulceration, because secondary bile acids are particularly damaging to intestinal epithelial cells [17,34]. Therefore, we hypothesized that bile acid dysmetabolism as a consequence of treatment with an inhibitor of gastric acid secretion would exacerbate NSAID-enteropathy. Using a rat model, we modeled the common clinical scenario of co-use of

an NSAID and an inhibitor of gastric acid secretion, and we explored how this would affect the cytotoxic properties of bile. The results are summarized in Figure 4.3. Rats treated twice-daily with famotidine (an H_2RA) for 9 days did not develop significant small intestinal damage (Fig. 4.3 A), but the bile collected from those rats was significantly more cytotoxic when added to cultured intestinal epithelial cells (IEC-6) than the bile collected from vehicle-treated rats (Fig. 4.3 B). Co-treatment of rats with both famotidine (9 days) and an NSAID (naproxen, for the final 4.5 days) resulted in extensive ulceration and bleeding in the small intestine (Fig. 4.3 A), and bile from rats receiving these treatments was significantly more cytotoxic *in vitro* than that from rats treated only with naproxen (Fig. 4.3 B; $p<0.05$). Therefore, these data suggest that a marked increase in the cytotoxicity of bile (seen with famotidine treatment) is not sufficient to produce overt intestinal damage, but is likely to be a contributing factor to the exacerbation of enteropathy when co-administered with an NSAID. Consistent with clinical reports of SIBO in patients treated with inhibitors of acid secretion [30,31], treatment of rats with famotidine resulted in significant increases (>16-fold) in the number of aerobes in the jejunum (Fig. 4.3 C).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5 Days
9 Days

Figure 4.3. Treatment with an inhibitor of gastric acid secretion (famotidine) exacerbates NSAID-induced intestinal damage, increases bile toxicity, and alters the intestinal microbiome in rats. Panel A: Twice-daily administration of famotidine (30 mg/kg, po) and naproxen (10 mg/kg, po) caused extensive small intestinal damage that was significantly more severe than that induced by naproxen alone ($^{\Psi\Psi}p<0.01$) or famotidine alone (**$p<0.01$). Famotidine alone did not cause significant intestinal damage. Famotidine was administered for 9 days, while naproxen was administered only on the final 4.5 days. Panel B: *In vitro* exposure of rat intestinal epithelial (IEC-6) cells to bile (diluted 1:6 in buffer) that had been collected from rats treated with vehicle resulted in a low level of cytotoxicity as measured by lactate dehydrogenase release. However, when the bile was collected from rats treated with famotidine for 9 days, there was a ~120% increase in cytotoxicity (**$p<0.01$ vs. vehicle-treated). While treatment with naproxen for 4.5 days markedly increased the cytotoxicity of bile, it was significantly greater when the rats were treated with both famotidine and naproxen ($^{\Psi}p<0.05$). Panel C: Twice-daily treatment with famotidine for 5 or 9 days significantly increased the number of total aerobes in the jejunum (*$p<0.05$, **$p<0.01$ versus the vehicle-treated group). For all panels, results are shown as mean ± SEM (n=6 rats per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison test.

4.5. Conclusions

Damage induced in the stomach and duodenum by NSAIDs can be reduced substantially be co-administration of a PPI, and to a lesser extent by co-administration of an H_2RA. However, these agents are ineffective in preventing NSAID-induced damage in the more distal small intestine, and there is growing evidence that by altering the intestinal microbiota, they worsen NSAID-enteropathy. Hydrogen sulfide is a particularly potent cytoprotective agent in the GI tract. H_2S-releasing NSAIDs produce negligible

upper GI damage even at very high doses and in animal models where mucosal defence is significantly impaired. In the small intestine, H_2S-releasing NSAIDs produce negligible damage, even when co-administered with a PPI and low-dose ASA.

Development of effective preventative or curative therapies for NSAID-enteropathy requires a better understanding of the complicated pathogenesis of this disorder. Three inter-related factors appear to be of paramount importance: the nature of bile, the enterohepatic circulation of the NSAID, and the nature of the intestinal microbiota. PPIs can alter these factors, leading to increased intestinal damage. Bacterial enzymes are important for deconjugation of NSAIDs in bile, allowing their reabsorption, as well as for conversion of primary bile acids to more cytotoxic secondary bile acids. These bacterial enzymes themselves may be targets for novel therapies for NSAID-enteropathy.

4.6. Acknowledgements

This work was supported by a grant from the Canadian Institutes of Health Research.

4.7. References

At least 10% of the references were selected and annotated as being papers of special interest (*) or outstanding interest (**). Annotated references must have been published in the past two years. A brief description of the major findings and the importance of the study was provided for each annotated reference.

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– CHAPTER 5 –

PATHOGENESIS OF NSAID-INDUCED ENTEROPATHY: ELUCIDATION OF ROLES OF BILE, BACTERIA, AND ENTEROHEPATIC CIRCULATION VIA USE OF GI-SAFE NSAID

Pathogenesis of NSAID-induced Enteropathy: Elucidation of Roles of Bile, Bacteria and Enterohepatic Circulation via Use of a GI-Safe NSAID

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Preface: *The research presented in this manuscript was conducted from June 2013 to June 2014. I am the primary author of the paper. John L. Wallace and I designed the experiments, analyzed the data, wrote the manuscript and addressed the reviewers' concerns. The animal work and sample collection performed in experiments with naproxen, ATB-346, and/or proton pump inhibitors were conducted with assistance from the following people: W. McKnight (technician) assisted in the animal work and sample collection and A. Manko assisted in the animal work, bile cytotoxicity assays, and MPO assays. G.J. Da Silva, G. De Palma, Y. Deng (technician), and M.G. Surette assisted in*

the microbiota sample collection and analysis. P. Bercik and M.G. Surette contributed valuable scientific input and critically appraised the manuscript.

Title: Pathogenesis of NSAID-induced Enteropathy: Elucidation of Roles of Bile, Bacteria and Enterohepatic Circulation via Use of a GI-Safe NSAID

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Conflict of Interest: Dr. Wallace is founder and a director of Antibe Therapeutics Inc., a company developing novel anti-inflammatory drugs.

Abbreviations: GI, gastrointestinal; H_2S, hydrogen sulfide; MPO, myeloperoxidase; NSAID, nonsteroidal anti-inflammatory drug; PPI, proton pump inhibitor; SIBO, small intestinal bacterial overgrowth

Keywords: Hydrogen sulfide; non-steroidal anti-inflammatory drug; enteropathy; inflammation; gastrointestinal; bile; dysbiosis; enterohepatic circulation; naproxen; cyclooxygenase

5.1. Abstract

Background & Aims: The pathogenesis of non-steroidal anti-inflammatory drug (NSAID)-induced small intestinal damage remains incompletely understood. The aim of this study was to explore the roles of enteric bacteria, bile, and enterohepatic circulation in NSAID-enteropathy, using a gastrointestinal (GI)-safe NSAID or by administering inhibitors of gastric acid secretion (i.e., proton pump inhibitors (PPIs)), which can significantly exacerbate NSAID-enteropathy.

Methods: Rats received chronic, oral doses of naproxen, a GI-toxic NSAID, or equimolar doses of ATB-346, a GI-safe naproxen derivative, and intestinal ulceration and inflammation was evaluated. Bile was collected after *in vivo* naproxen, ATB-346, and/or PPI administration for evaluation of cytotoxicity *in vitro* using cultured intestinal epithelial cells and to determine the biliary excretion of naproxen and ATB-346. The impact of naproxen, ATB-346, and/or PPI administration on the composition of the intestinal microbiota was examined by deep sequencing of 16s rRNA.

Results: Naproxen administration caused a dose-dependent increase in intestinal damage, inflammation, and the cytotoxicity of bile on cultured enterocytes, whereas equimolar doses of ATB-346 did not significantly increase these outcomes. Unlike naproxen administration, ATB-346 did not undergo extensive enterohepatic recirculation. The enteric microbiota of naproxen-treated rats was distinct from vehicle- or ATB-346-treated rats. PPI administration caused intestinal dysbiosis and increased bile cytotoxicity.

Conclusions: The bile, enteric bacteria and enterohepatic circulation of NSAIDs appear to contribute significantly to NSAID-induced intestinal damage. ATB-346 undergoes limited enterohepatic recirculation, which contributes to the substantially reduced intestinal toxicity of this NSAID. PPI administration can alter the intestinal microbiota and nature of the bile, and these alterations likely contribute to the exacerbation of NSAID-enteropathy.

5.2. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs, due to 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 these drugs in the stomach and duodenum can be significantly diminished by the co-administration of inhibitors of gastric acid secretion, such as proton pump inhibitors (PPIs) and histamine H_2 receptor antagonists (H_2RAs) (Scheiman *et al.*, 2006). However, it has been clear for many years that these drugs offer no protection to the small intestine, where the majority of NSAID-induced damage and bleeding occurs (Adebayo & Bjarnason, 2006; Lanas *et al*., 2009). In fact, emerging evidence indicates that inhibitors of gastric acid secretion significantly worsen the small intestinal damage caused by NSAIDs (Zhao & Encinosa, 2008; Lanas *et al.*, 2009; Watanabe *et al.*, 2013; Wallace *et al.*, 2011; Blackler *et al.*, 2012; Satoh *et al.*, 2012). 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 (Wallace, 2013a). 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 (Lanas *et al*., 2009).

The mechanisms of NSAID-enteropathy are in many ways distinct from NSAID-induced gastroduodenal damage (Wallace, 2012). The damaging effects of NSAIDs in the stomach and proximal duodenum are closely related to their ability to suppress cyclooxygenase (COX) activity. In contrast, the pathogenesis of NSAID-enteropathy is less linked to COX suppression, and more related to bile, enteric bacteria and enterohepatic circulation of the NSAID (Wax *et al*., 1970; Beck *et al*., 1990; Uejima *et al*., 1996; Seitz & Boelsterli, 1998; Hagiwara *et al*., 2004; Jacob *et al*., 2004; Wallace, 2013). The critical role of bile in the pathogenesis of NSAID-enteropathy is evident from studies that demonstrated bile duct ligation in rats prevented NSAID-induced intestinal damage (Wax *et al*., 1970; Somasundaram *et al*., 1997; Jacob *et al*., 2007; Lichtenberger *et al*., 2011), and studies that demonstrated that NSAIDs that do not undergo enterohepatic circulation do not cause small intestinal damage (Kent *et al*., 1969; Wax *et al*., 1970; Reuter *et al*., 1997; Somasundaram *et al*., 1997). A number of *in vitro* experiments have also demonstrated that the combination of bile and an NSAID is particularly damaging to intestinal epithelial cells (Reuter *et al*., 1997; Somasundaram *et al*., 1997; Petruzzelli et al., 2007), which may explain reports that suggest NSAIDs that do not undergo enterohepatic recirculation do not cause small intestinal damage. The role

of bacteria was recently highlighted in a study by Wallace *et al.* (2011) that demonstrated that the exacerbation of NSAID-enteropathy in rats by co-treatment with a PPI was a consequence of the dysbiosis that resulted following PPI administration.

While it remains unclear why the PPI-induced dysbiosis resulted in an exacerbation of NSAID-induced intestinal damage and bleeding, some evidence suggests that changes in bile may be responsible (Blackler *et al*., 2014). Several clinical reports have associated chronic use of PPIs with alterations in the GI tract, such as small intestinal bacterial overgrowth (SIBO) and bile acid dysmetabolism (Shindo *et al*., 1995; Williams & McColl, 2006; Lombardo *et al*., 2010). It is possible that PPI-induced dysbiosis triggers bile acid dysmetabolism, as many intestinal microbes are capable of enzymatic modification of bile acids (Begley *et al*., 2004).

Hydrogen sulfide (H_2S), 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 (Wallace *et al*., 2014). A new class of H_2S-releasing NSAID derivatives has demonstrated vastly improved GI safety, producing negligible damage in both the stomach and small intestine (Wallace *et al*., 2010; Blackler *et al*., 2012). In particular, ATB-346, a H_2S-releasing naproxen derivative, has demonstrated superior GI safety compared to its parent NSAID (naproxen) in circumstances in which mucosal defence is significantly impaired (Wallace *et al*., 2010; Blackler *et al*., 2012), and even at doses many times greater than those required for anti-inflammatory effects (Wallace *et al*., 2010). 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 (Wallace, 2013b). The GI-safety of ATB-346 is seen despite this drug causing comparable suppression of GI and systemic COX activity as equimolar doses of naproxen (Wallace *et al*., 2010; Blackler *et al*., 2012; Elsheikh *et al*. 2014).

In the present study, we utilized ATB-346 and naproxen to help to determine the roles of cytotoxicity of bile, intestinal microbiota and enterohepatic circulation in the pathogenesis of NSAID-induced enteropathy. Specifically, we explored whether administration of naproxen vs. ATB-346 would affect the cytotoxic properties of bile, and what effects they would have on the intestinal microbiota, and the extent of their excretion in bile. In addition, we modeled the common clinical scenario of co-use of an NSAID with a PPI, and explored if changes in the microbiota and bile as a consequence of treatment with a PPI, contribute to the exacerbation of NSAID-enteropathy.

5.3. 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 Toxicity. 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 (4 administrations in total). Naproxen was administered at 3, 10, or 30 mg/kg. ATB-346 was given at doses equimolar to those of naproxen. 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 (Reuter *et al.*, 1997). The bile was stored at -80°C until used in the cytotoxicity assay. Anaesthetized rats then had blood drawn from the aorta for measurement (by ELISA) of whole blood thromboxane B_2 (TXB_2) synthesis, as an index of systemic COX-1 activity (Wallace *et al.*, 1998). After blood collection, the small intestine was blindly evaluated for hemorrhagic damage. This 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). Samples of jejunum were collected for the measurement of prostaglandin (PG) E_2 synthesis, as described previously (Wallace *et al.*, 2000). The concentrations of PGE_2 in the supernatants were determined by ELISA.

Effects of PPIs on NSAID-induced Bile Toxicity. Rats (*n*=5-6/group) were treated for a total of 9 days with one or more drugs. The rats received twice-daily omeprazole, lansoprazole (both at 10 mg/kg intraperitoneally), or vehicle (DMSO) for a total of 9

days. 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 of PPI/vehicle administration. One hour after the final administration of drug or vehicle, rats were anaesthetized with sodium pentobarbital and underwent bile duct cannulation to collect bile, as described above. The small intestine was then evaluated for damage, as described above. Doses of naproxen and ATB-346 were selected based on previous studies that demonstrated these two drugs 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). At the doses tested, naproxen elicits a low level of damage in the small intestine, whereas ATB-346 produces no detectable damage (Blackler *et al.*, 2012). Previously we demonstrated that the dose of omeprazole and lansoprazole administered in this study resulted in 99% suppression of gastric acid secretion by the 5th day of administration, when NSAID treatment was initiated (Wallace *et al*., 2011).

Effects of PPIs on Intestinal pH. Rats (n=5/group) were treated twice daily with omeprazole, lansoprazole (each at 10 mg/kg intraperitoneally) or vehicle (DMSO) for a total of 9 days. The pH of intestinal contents was assessed. Beginning 15 cm distal to the pyloric sphincter, the contents of the intestine in each subsequent section of 10 cm of intestine (3 in total) were collected into sterile tubes. The pH recordings for each tube were obtained for analysis, as measured by a micro pH glass combination electrode (Fisher Scientific Accumet, Ottawa, ON, Canada).

Intestinal Inflammation. Intestinal inflammation was quantitatively assessed in jejunal samples by the measurement of myeloperoxidase (MPO) activity, a biochemical marker of granulocyte infiltration (Boughton-Smith *et al.*, 1988).

Intestinal Epithelial Cell Culture. Rat intestinal epithelial (IEC-6) cells 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 (Quaroni *et al.*, 1979). 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) CO_2. 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 10^4 cells per well in 24-well plates and allowed to reach confluence (grown for 1-2 days post-seeding). In a series of separate experiments, bile cytotoxicity assays were also carried out using HT-29 epithelial cells, which were cultured as previously described (Jobin *et al.*, 1998).

Bile Cytotoxicity Assay. Bile samples were diluted with Dulbecco's PBS (DPBS) (pH 7.4) immediately prior to incubation with IEC-6 or HT-29 cells. A range of dilutions that encompass the physiological range of concentrations of bile acids present in the small intestinal tract of rats (1:3 to 1:12; Dietschy, 1968) were assessed for their cytotoxic

effects. Cells were washed with warm DPBS prior incubation with diluted rat bile. Solutions of bile were incubated with IEC-6 or HT-29 cells for 3 h at 37°C and 5% (vol/vol) CO_2. Following the incubation period, the cells were centrifuged at 250 x*g* 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). The assay quantitatively measures LDH, a stable cytosolic enzyme, which is released upon cell lysis.

Pharmacokinetics of Naproxen. Concentrations of naproxen in bile collected from naproxen- or ATB-346-treated rats were measured by mass spectrometry/liquid chromatography, as described previously (Blackler *et al*., 2012). These measurements were carried out blindly by Nucro-Technics (Scarborough, ON, Canada).

DNA Extraction and Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis. Bacterial DNA was extracted from jejunal and cecal contents 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 DNA gene was amplified using polymerase chain reaction with universal bacterial primers (HDA-1 and HDA-2) (Mobixlab, McMaster University core facility, Hamilton, Ontario, Canada). Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode universal mutation system (Bio-Rad, Mississauga, Ontario, 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 and Ward algorithm. 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®. Jejunal bacterial DNA was extracted using a phenol/chloroform/isoamyl method, paired with a Clean and Concentrator kit (Zymo Research Corp., Irvine, California, USA). The V3 region of the 16S rRNA gene was amplified as previously described (Bartram *et al*., 2011). 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, Ontario, Canada). Custom, in-house Perl scripts were developed to process the sequences after Illumina sequencing. Cutadapt (Martin, 2011) was used to trim any over-read, and paired-end sequences were aligned with PANDAseq (Masella *et al*., 2012). 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+ (Ye *et al*., 2010) and taxonomy assigned using the Ribosomal Database Project (RDP) classifier (Caporaso *et al*., 2010) against the Greengenes reference database. The graphics in Fig 5.6 and 5.7 were produced using the phyloseq package (version 1.8.2) implemented in R (version 3.1.0) (McMurdie & Holmes, 2013) with the following functions: plot_heatmap() function and plot_bar() function (McMurdie & Holmes, 2013), after subsetting the data to remove low occurring OTUs (< 3). The ordination method used in the plot_heatmap() function was the principal coordinates analysis (PCA) and the metric used was Bray Curtis. The scripts used in order to create the heatmap plots was: plot_heatmap(data, "PCoA", "bray", "Samples", "Family", low = "#000033", high = "#FF3300"), and the script used to create the bar graphs was: plot_bar(data, fill = "Family") + facet_wrap(~Samples, scales = "free"). Statistical analysis was performed using SPSS 20.0 software for Windows (SPSS Inc, Chicago, IL, USA). Statistical comparisons were performed by Mann-Whitney and Kruskal-Wallis tests. Correlations were done applying the Spearman's test. A p value of <0.05 was considered statistically significant.

***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). The PGE_2 and TXB_2 ELISA kits were purchased from Caymen Chemical (Ann Arbor, MI, USA). DMEM, fetal bovine serum, penicillin, and streptomycin were purchased from Life Technologies Inc. (Burlington, ON, CA).

5.4. Results

Dose-dependent Naproxen-induced Enteropathy. Rats treated with naproxen (30 mg/kg) twice daily for 2 days resulted in severe intestinal ulceration (Figure 5.1 A). At 10 mg/kg, naproxen induced a low level of intestinal damage, whereas in rats treated with naproxen at 3 mg/kg there was no macroscopically visible damage. In sharp contrast to the effects of naproxen, rats treated with ATB-346 did not result in significant small intestinal damage, regardless of the naproxen equivalent of ATB-346 administered.

Consistent with the small intestinal damage, naproxen administration twice daily for 2 days resulted in a dose-dependent increase in jejunal granulocyte infiltration (MPO activity; Figure 5.1 B). In contrast, at all doses tested, ATB-346 administration did not alter MPO activity. In fact, treatment with ATB-346 at the two higher doses resulted in significantly lower MPO activity compared to rats treated with an equivalent dose of naproxen ($p<0.01$) (Figure 5.1 B).

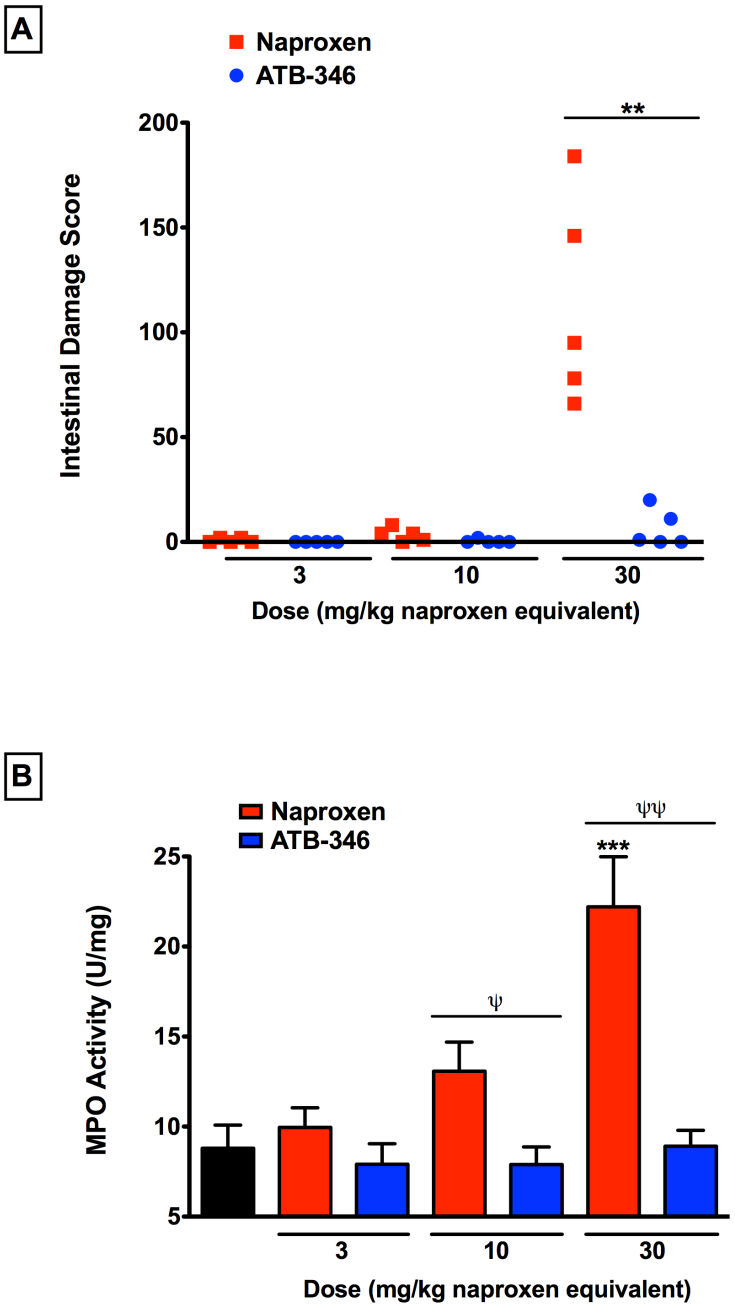


Figure 5.1. Effects of naproxen and a hydrogen sulfide-releasing naproxen derivative (ATB-346) on small intestinal damage and inflammation. **Panel A**: Oral administration of naproxen twice-daily for two days caused small intestinal damage that increased in severity in a dose-dependent manner. In contrast, ATB-346 administration caused

markedly less intestinal damage at all doses tested; the intestine appeared normal with doses equimolar to naproxen at 3 or 10 mg/kg. 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, there was significantly more intestinal MPO activity with naproxen than 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.

Suppression of Cyclooxygenase Activity. Naproxen (10 mg/kg) administration effectively suppressed systemic COX-1 activity (whole blood thromboxane synthesis; by 98%) (Supplementary Figure 5.6) and intestinal PGE_2 synthesis (by 96%) after twice daily dosing for 2 days. ATB-346 administration achieved comparable suppression of whole blood thromboxane synthesis and intestinal PGE_2 synthesis. Extension of the twice-daily dosing protocol to 4 days resulted in a similar degree of suppression (>99% whole blood thromboxane synthesis and >97% intestinal PGE_2 synthesis) of COX activity in rats administered naproxen (10 mg/kg) or an equimolar dose of ATB-346.

Naproxen Administration Enhanced Bile Toxicity. When tested at 1:6 or 1:12 dilutions, bile collected from rats that had been treated with naproxen twice-daily over 2 days exhibited a dose-dependent enhancement of cytotoxic effects on IEC-6 intestinal epithelial cells compared to bile collected from vehicle-treated rats. Exposure of IEC-6 cells to bile collected from vehicle-treated rats for 3 h resulted in 19% cytotoxicity,

whereas bile collected from rats given naproxen orally at doses of 3, 10, or 30 mg/kg exhibited 23%, 34% ($p<0.001$) and 42% ($p<0.001$), respectively (Figure 5.2 A). However, bile collected from rats treated with ATB-346 at equimolar doses to naproxen, did not exhibit enhancement of cytotoxic effects. At the two higher doses tested, bile collected from ATB-346-treated rats was significantly less cytotoxic compared to bile collected from rats treated with an equivalent dose of naproxen ($p<0.05$ at 10 mg/kg; $p<0.001$ at 30 mg/kg) (Figure 5.2 A). Similar to twice daily dosing for 2 days, naproxen (10 mg/kg) administration for 4 days resulted in enhanced ($p<0.05$) cytotoxic effects of bile on IEC-6 cells, whereas the equivalent dose of ATB-346 did not alter bile cytotoxicity (Figure 5.4 A and 5.4 C, respectively); comparable results were also observed evaluating the cytotoxic effects of bile on cultured HT-29 cells (Supplementary Figure 5.7).

Pharmacokinetics. When the concentration of naproxen was measured 1 h after the final administration of naproxen or ATB-346, some dramatic differences were apparent in rat bile. Naproxen levels in bile collected from naproxen-treated rats after 2 days of dosing increased in a dose-dependent manner (Figure 5.2 B). In the naproxen-treated rats, biliary naproxen concentrations from rats treated with doses of 3, 10, or 30 mg/kg averaged 360.4 ± 90.6, 1431 ± 303.4, and 2432 ± 340.1 ng/mL, respectively. In sharp contrast, treatment of rats with equimolar doses of ATB-346 resulted in significantly lower levels of naproxen in bile, with no dose-response relationship (Figure 5.2 B).

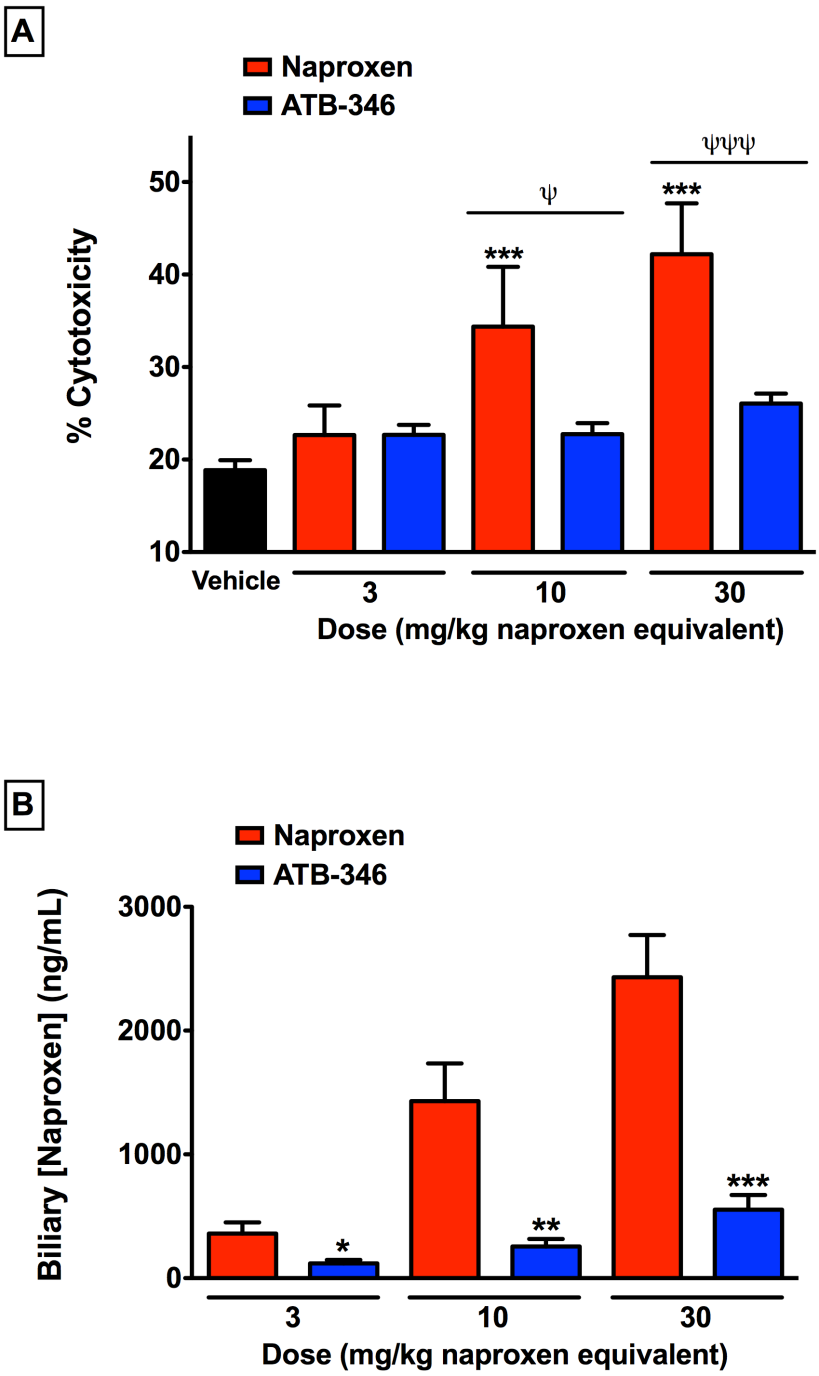


Figure 5.2. Naproxen administration dose-dependently increased the biliary concentration of naproxen and the cytotoxic effects of bile. **Panel A**: Bile collected from rats treated with naproxen (10 or 30 mg/kg) twice-daily for 2 days was significantly more

cytotoxic to cultured rat intestinal epithelial (IEC-6) cells 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 shown are combined from the 1:6 and 1:12 dilutions of bile samples, and are expressed 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). Results are shown as mean ± SEM (*n*=5 per group). The data were analyzed by an unpaired, two-tailed Student's t-test.

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. Administration of naproxen resulted in an increase in abundance of *γ-proteobacteria*, a class of gram-negative bacteria, compared to vehicle-treated rats. More specifically, naproxen-treated rats exhibited a significant decrease in abundance of the *Lachnospiraceae* family (from the gram-positive *Clostridia* class) and increased abundance of the *Bacteroidaceae* family (from the gram-negative *Bacteroidetes* class) (Figure 5.3). The intestinal microbiota of ATB-346 administered rats also exhibited a decrease in abundance of *Lachnospiraceae* compared to vehicle-treated rats, but had significantly (p=0.034) lower abundance of *Bacteroidaceae* compared to naproxen-treated rats (Figure 5.3). On the other hand, ATB-

346 administration resulted in a significant ($p=0.028$) increase in the abundance of the *Lactobacillaceae* family compared to naproxen-treated rats.

In a separate experiment, rats were treated with a higher dose of naproxen (20 mg/kg) or ATB-346 (equimolar dose) twice daily for 2 days to explore whether changes in particular bacteria correlate with the development of intestinal injury. Naproxen administration caused significant intestinal injury, resulting in a mean damage score of 90.2 ± 22.3, whereas ATB-346 did not cause intestinal injury (mean damage score of 0.4 ± 0.4; $p<0.001$). Increased intestinal injury from naproxen treatment positively correlated with higher abundances of specific microbial families (Table 5.1). Naproxen administration also caused a significant increase ($p=0.026$) in the abundance of *Bilophilia*, a pathogenic bacterial genus, compared to vehicle-treated rats. ATB-346 administration, which did not result in intestinal injury, resulted in a higher abundance of the *Rikenellaceae* and *Ruminococcaceae* families ($p=0.028$ and $p=0.031$, respectively) compared to vehicle-treated rats. ATB-346-treated rats also exhibited lower abundances of *Bacteroidaceae* and *Enterobacteriaceae* ($p=0.028$ and $p=0.028$, respectively) versus naproxen-treated rats, similar to the microbiota profiles from rats treated with naproxen or ATB-346 for 4 days.

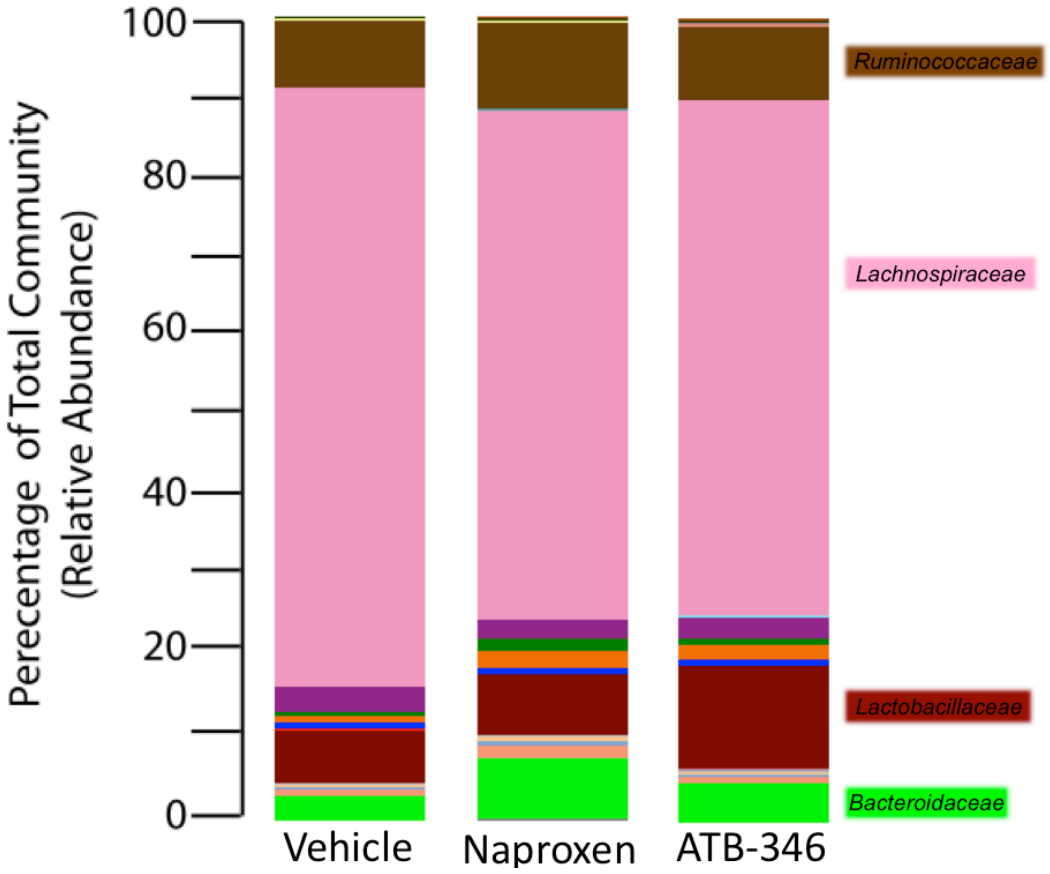


Figure 5.3. Treatment with naproxen or ATB-346 altered the taxonomic composition of the intestinal microbiota. Deep sequencing analysis of 16S rRNA via Illumina revealed changes in the taxonomic composition of the jejunal microbiota following twice-daily administration of naproxen (10 mg/kg) or ATB-346 (14.5 mg/kg) for 4 days in rats. Treatment with naproxen or ATB-346 diminished the relative abundance of *Lachnospiraceae* compared to vehicle-treated rats. Treatment with naproxen increased the relative abundance of *Bacteroidaceae,* whereas treatment with ATB-346 increased the abundance of *Lactobacillaceae*. Taxonomic summaries are resolved to the family level. Each bar represents the relative abundance of bacteria present within each group.

Table 5.1 Association between abundance of bacterial family and naproxen-induced intestinal injury

Bacterial Family	r^2	p-value
Positive Correlation		
Bacteroidaceae	0.362	0.018
Porphyromonadaceae	0.314	0.029
Enterococcaceae	0.271	0.046
Enterobacteriaceae	0.267	0.049
Negative Correlation		
Lachnospiraceae	0.585	0.025

PPIs Exacerbated Bile Toxicity. Bile collected from rats administered naproxen (10 mg/kg) twice daily for 4 days had enhanced ($p<0.05$) cytotoxic effects on IEC-6 cells compared to bile collected from rats treated with vehicle (Figure 5.4 A). In rats co-treated with naproxen and omeprazole or lansoprazole, an exacerbation of the cytotoxic effects of bile was observed compared to bile from rats treated with naproxen and vehicle ($p<0.01$ and $p<0.05$, respectively). Likewise, bile collected from rats co-treated with ATB-346 (14.5 mg/kg) and omeprazole was more cytotoxic to IEC-6 cells than bile collected from rats that received ATB-346 and vehicle (Figure 5.4 C). The cytotoxic effects of bile collected from rats treated with ATB-346 and vehicle was similar to that of bile collected from vehicle- treated rats.

We then examined whether PPI administration alone would alter the cytotoxic effects of bile. Bile collected from rats treated with a PPI (omeprazole or lansoprazole)

for 9 days exhibited enhanced ($p<0.001$) bile cytotoxicity compared to bile collected from rats treated with vehicle (Figure 5.5 A). Similar results were also observed when bile collected from PPI-treated rats was incubated on HT-29 cells (48 ± 6.8, 45 ± 4.4, and 17 ± 3.2% cytotoxicity; omeprazole-, lansoprazole-, or vehicle-treatment, respectively). Enhancement of bile cytotoxicity by omeprazole or lansoprazole administration did not result in macroscopic intestinal damage or inflammation *in vivo*. Tissue MPO levels were similar in vehicle-, omeprazole-, and lansoprazole-treated rats (6.3 ± 3.4, 6.3 ± 2.7, 7.7 ± 1.4 U/mg, respectively).

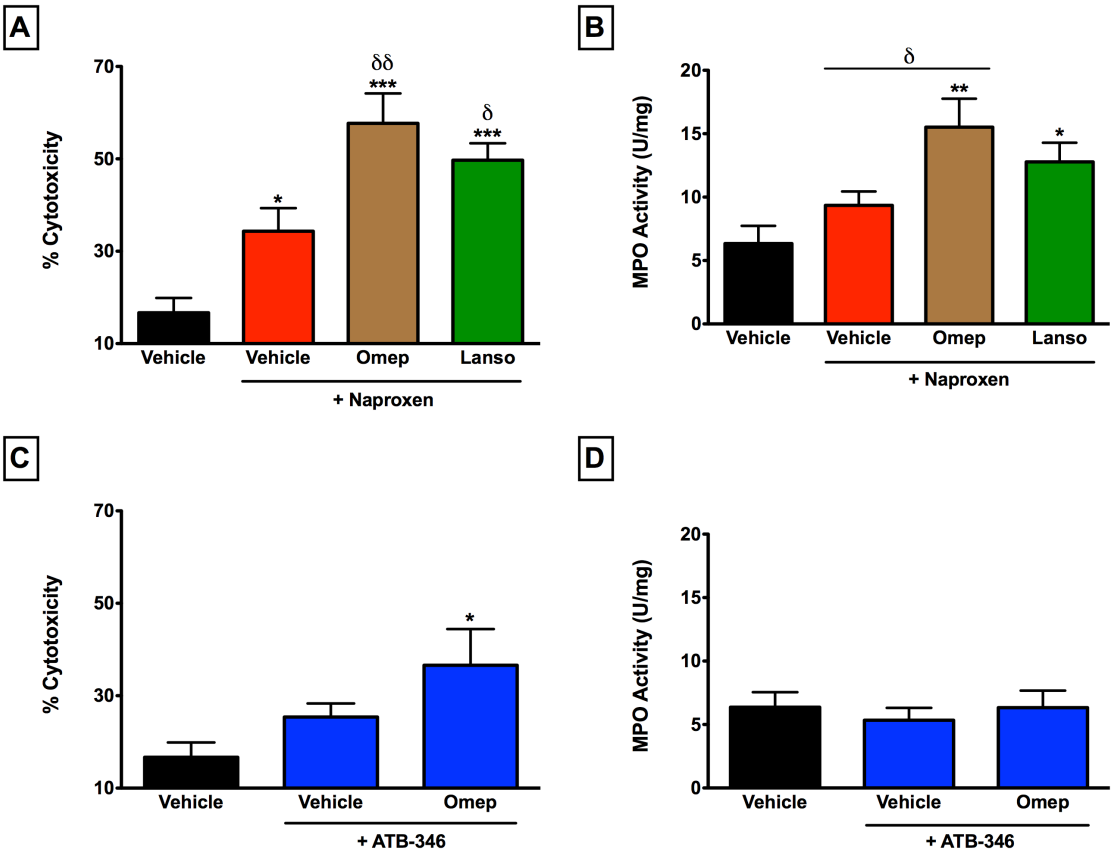


Figure 5.4. Treatment with a proton pump inhibitor (PPI) increased bile cytotoxicity and intestinal inflammation. **Panel A:** Bile collected from vehicle-treated rats co-treated

with naproxen (10 mg/kg, twice-daily for 4 days) was significantly more cytotoxic to cultured rat intestinal epithelial (IEC-6) cells 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 ($^{\delta}p<0.05$, $^{\delta\delta}p<0.01$). Likewise, bile from PPI + naproxen co-treated rats was significantly more cytotoxic than bile from rats treated with vehicle alone (***$p<0.001$). **Panel B**: 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 ($^{\delta}p<0.05$). **Panel C**: In contrast to naproxen, the cytotoxic effects of bile collected from rats treated with ATB-346 (14.5 mg/kg) were similar to that of bile collected from vehicle-treated rats. Co-administration of ATB-346 to omeprazole-treated rats enhanced (*$p<0.05$) bile cytotoxicity as compared to vehicle-treated rats. **Panel D:** Co-administration of ATB-346 to vehicle- or omeprazole-treated rats did not increase intestinal MPO activity. Data shown in bile cytotoxicity graphs are from the 1:6 and 1:12 dilutions of bile samples. 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.

PPI Treatment Caused Intestinal Dysbiosis. Treatment of rats with omeprazole or lansoprazole for 9 days resulted in a marked shift in the composition of the intestinal bacterial community. DGGE analysis of jejunal contents demonstrated that vehicle-treated rats clustered separately from omeprazole- and lansoprazole-treated rats, as analyzed by the Dice coefficient and Ward algorithm to determine similarities (Figure 5.5

B). Construction of a majority UPGMA tree demonstrated similar clustering with nearly 100% resampling support for each branch (Figure 5.5 C). In contrast, DGGE analysis of cecal contents did not exhibit clustering between groups that was observed in intestinal samples, suggesting that PPI-induced dysbiosis does not extend beyond the small intestine (data not shown). Interestingly, the intestinal content of omeprazole- and lansoprazole-treated rats was significantly ($p<0.001$ and $p<0.01$, respectively) more acidic than vehicle-treated controls (Figure 5.5 D). The mean pH in the omeprazole- and lansoprazole-treated rats was 5.8 and 5.9, respectively, while that in the vehicle-treated group was 6.4.

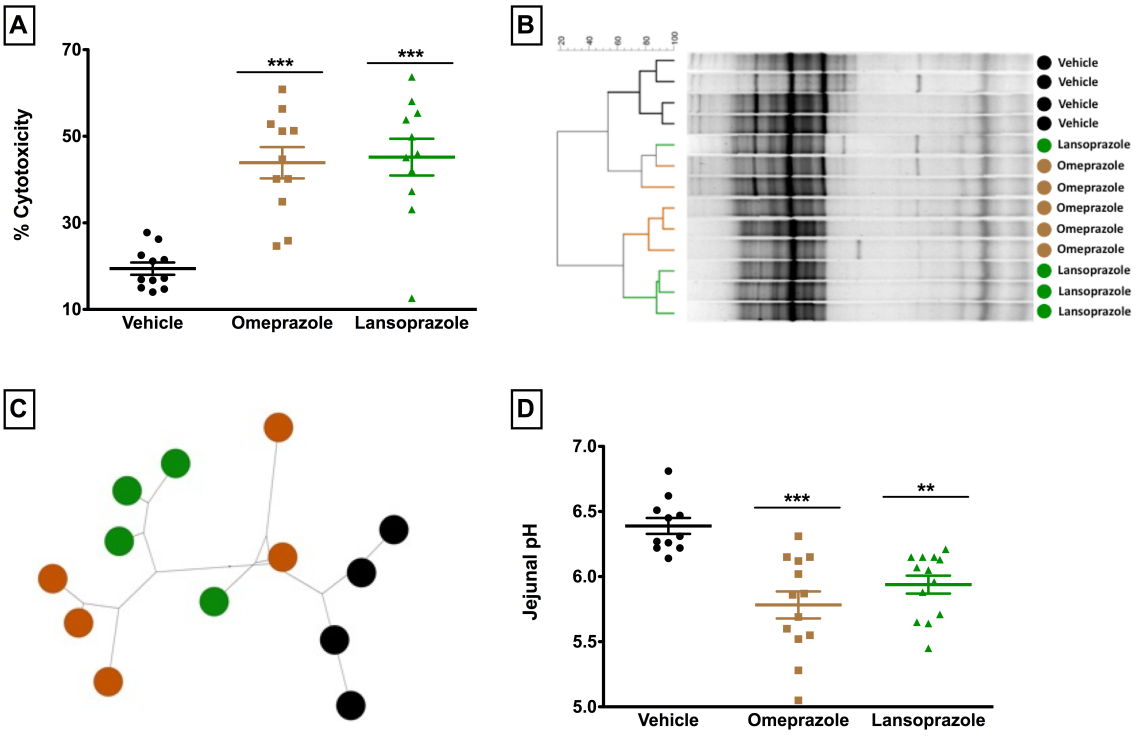


Figure 5.5. Treatment with proton pump inhibitors enhanced bile cytotoxicity and caused intestinal dysbiosis. **Panel A**: Bile collected from rats treated with omeprazole or

lansoprazole, each at 10 mg/kg twice daily for 9 days, resulted in significantly enhanced cytotoxicity when incubated for 3 h on rat intestinal epithelial (IEC-6) cells (***$p<0.001$; versus vehicle-treated group). Data shown are combined from the 1:6 and 1:12 dilutions of bile samples. **Panel B**: Denaturing gradient gel electrophoresis analysis revealed that omeprazole and lansoprazole administration to rats caused dysbiosis of the jejunal microbiota, with distinct clustering from vehicle-treated rats. **Panel C**: Using a resampling technique (majority unweighted-pair-group method with arithmetic mean algorithm), the Ward dendrogram clustering observed in panel B was confirmed and represented in a multi-dimensional layout. **Panel D**: Omeprazole and lansoprazole administration to rats significantly lowered the pH of intestinal contents collected from the jejunum as compared to vehicle-treated rats (**$p<0.05$, ***$p<0.001$). For panels A and D, each group consisted of at least five 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 tests.

To further investigate how PPI administration altered the intestinal microbiota in rats, we analyzed the taxonomic composition of the jejunal microbiota in rats via deep sequencing of 16S rRNA with Illumina. The *Firmicutes* phylum, specifically the *Lactobacillaceae* family, represented the vast majority (<95%) of bacterial abundance in jejunal microbiota samples in both PPI- or vehicle-treated rats. However, administration of omeprazole or lansoprazole (each at 10 mg/kg) twice-daily for 9 days resulted in a significant ($p<0.05$) increase in *γ-Proteobacteria* abundance, including the families *Pseudomonadaceae* and *Enterobacteriaceae,* compared to vehicle-treated rats (Figure 5.6). On the other hand, PPI-treated rats exhibited a significant ($p<0.05$) decrease in the abundance of *Actinobacteria* and several families of *Firmicutes*, including *Lachnospiraceae* and *Ruminococcaceae,* compared to vehicle-treated rats (Figure 5.7A

and 5.7B, respectively). There were no significant differences in the taxonomic composition of the jejunal microbiota between omeprazole and lansoprazole-treated rats.

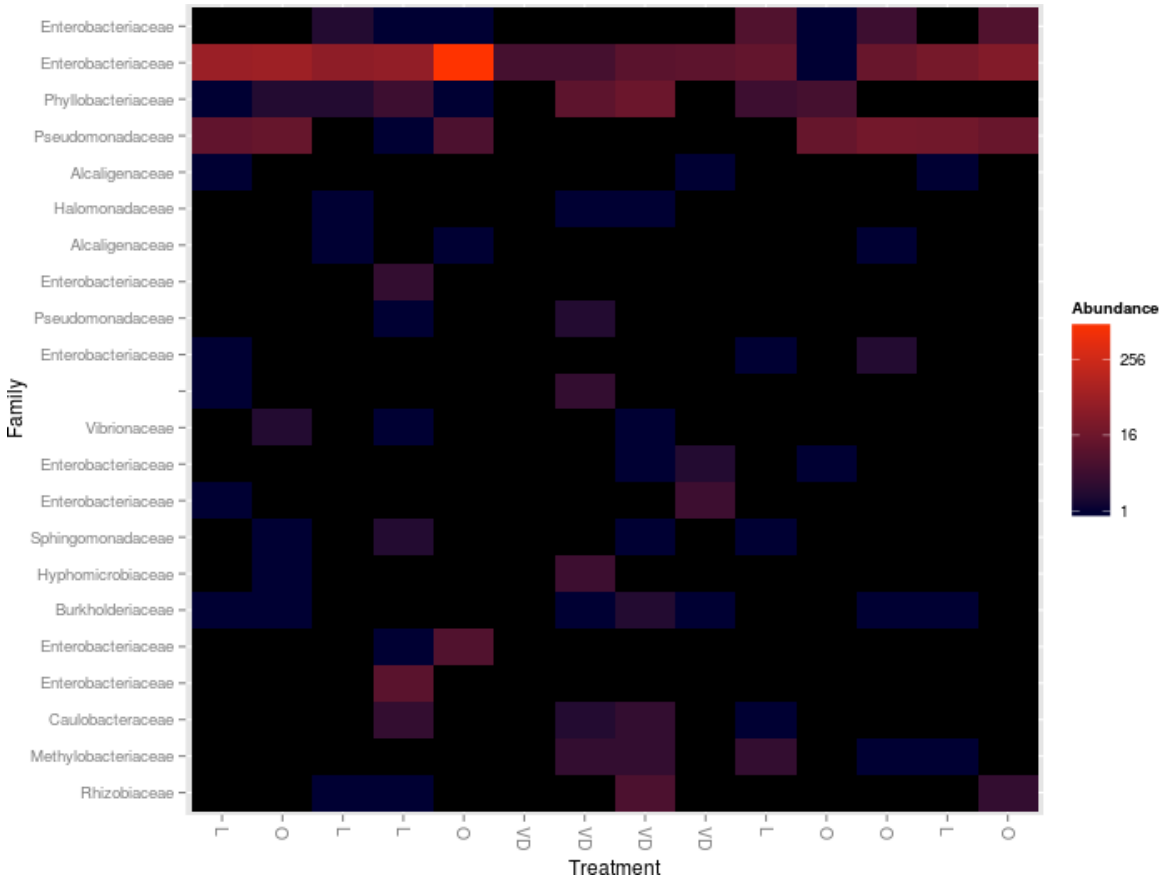
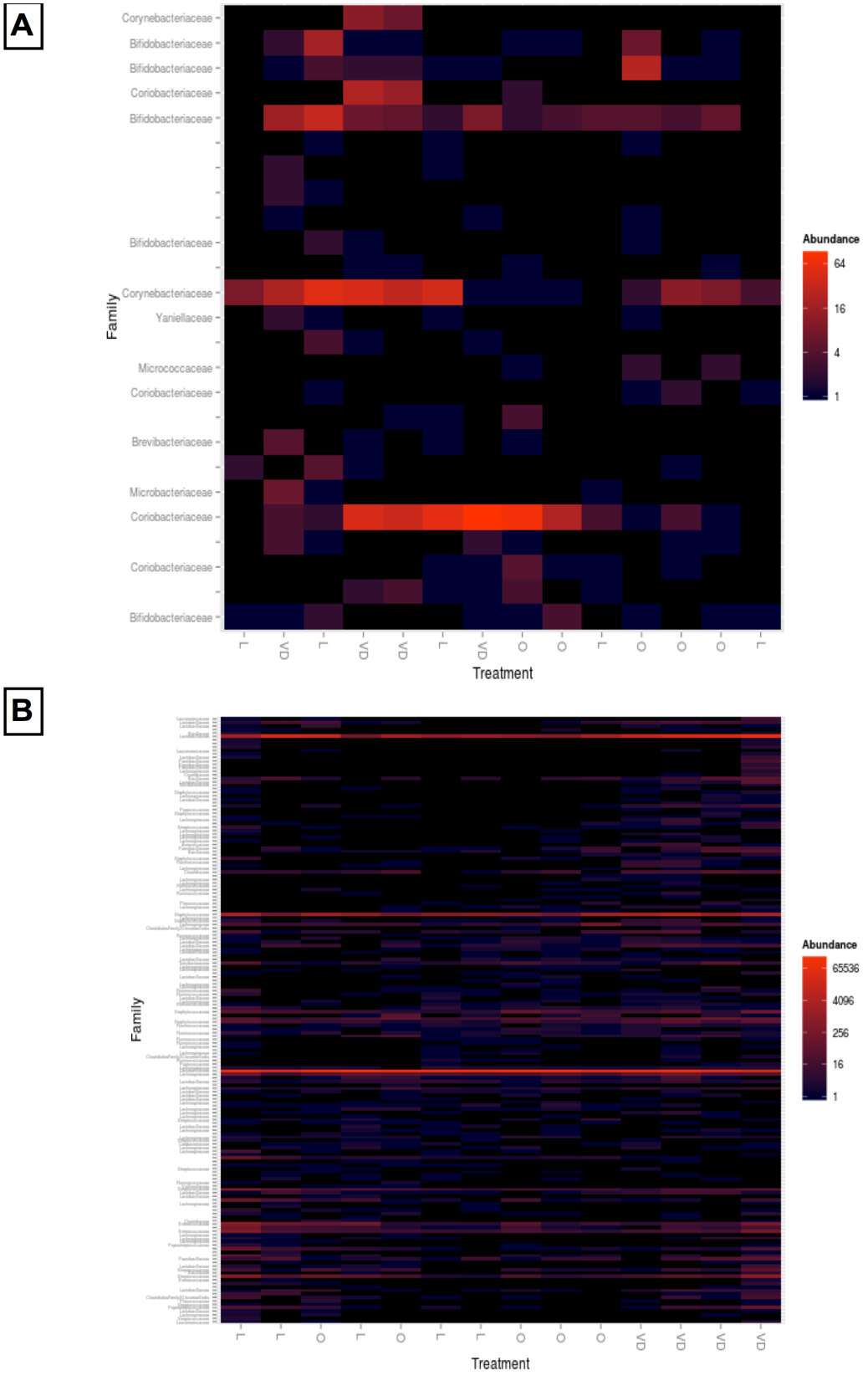


Figure 5.6. PPI-administration increased the abundance of jejunal γ-Proteobacteria. In particular, the abundance of *Pseudomonadaceae* and *Enterobacteriaceae* was significantly increased in PPI-administered rats. Vehicle-treated jejunal samples clustered separately from omeprazole- or lansoprazole-treated rats. The graphic was produced by the plot_heatmap() function in the phyloseq package implemented in R after subsetting the data to remove low occurring OTUs (< 3). The ordination method used in the plot_heatmap() function was the Principal coordinates analysis (PCoA) and the metric used was Bray Curtis. The horizontal axis represents samples (L= lansoprazole-, O= omeprazole-, and VD= vehicle-treated rats), while the vertical axis represents OTUs,

labeled by families of *γ-Proteobacteria.* The red-shade colour scale indicates the abundance of each OTU in each sample, from red (highly abundant) to dark blue, then black (zero, not observed).


A
Corynebacteriaceae
Bifidobacteriaceae
Bifidobacteriaceae
Coriobacteriaceae
Bifidobacteriaceae
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Corynebacteriaceae
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Micrococcaceae
Coriobacteriaceae
Brevibacteriaceae
Microbacteriaceae
Coriobacteriaceae
Coriobacteriaceae
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65536
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256
16
1
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Treatment

Figure 5.7. PPI-administration decreased the abundance of jejunal Actinobacteria and several families of Firmicutes. **Panel A**: The abundance of *Actinobacteria* was significantly decreased in the jejunum of PPI-treated rats. **Panel B:** The abundance of *Lachnospiraceae* and *Ruminococcaceae* was significantly decreased in the jejunum of PPI-treated rats. However, the majority of *Firmicutes* phylum was represented by a high abundance of *Lactobacillaceae,* regardless of treatment. Vehicle-treated jejunal samples clustered separately from omeprazole- or lansoprazole-treated rats. The graphic was produced by the plot_heatmap() function in the phyloseq package implemented in R after subsetting the data to remove low occurring OTUs (< 3). The ordination method used in the plot_heatmap() function was the Principal coordinates analysis (PCoA) and the metric used was Bray Curtis. The horizontal axis represents samples (L= lansoprazole-, O= omeprazole-, and VD= vehicle-treated rats), while the vertical axis represents OTUs, labeled by families of *γ-Proteobacteria.* The red-shade colour scale indicates the abundance of each OTU in each sample, from red (highly abundant) to dark blue, then black (zero, not observed).

5.5. Discussion

The substantial GI-toxicity of NSAIDs remains a major limitation to the use of this class of drugs. The mechanisms of NSAID-gastropathy are well understood and the damage produced in the upper GI tract can largely be prevented by the co-administration of inhibitors of gastric acid secretion, such as PPIs (Scheiman *et al.*, 2006; Lanas *et al*., 2009). Conversely, the complex pathogenesis of NSAID-enteropathy remains incompletely understood and there are currently no proven effective preventative or curative treatments (Wallace *et al*., 2013a). Moreover, it is becoming apparent that inhibitors of gastric acid secretion (e.g., PPIs) worsen NSAID-enteropathy (Zhao & Encinosa, 2008; Wallace *et al*., 2011; Satoh *et al*., 2012; Watanabe *et al*., 2013).

Considerable evidence indicates that interactions among bile, intestinal microbes and enterohepatic circulation of NSAIDs are important to the development of significant NSAID-induced intestinal damage (Blackler *et al*., 2014). In recent years, it has been demonstrated that H_2S is a particularly potent cytoprotective agent in the GI tract (Wallace *et al*., 2014). Indeed, an H_2S-releasing derivative of naproxen (ATB-346) was previously shown to not produce gastrointestinal damage, while the comparator drug, naproxen, resulted in extensive mucosal damage when administered to rats (Wallace *et al*., 2010; Blackler *et al*., 2012).

In the present study, the administration of a commonly prescribed NSAID (naproxen) with known GI toxicity dose-dependently caused intestinal ulceration and inflammation in rats. Naproxen underwent extensive enterohepatic recirculation and the bile collected from rats treated with naproxen was particularly cytotoxic to cultured intestinal epithelial cells. Conversely, when ATB-346 was given at doses equimolar to naproxen, the intestine was spared of damage. ATB-346 exhibited reduced enterohepatic circulation and did not significantly enhance the cytotoxicity of bile, which likely contribute to the remarkable intestinal safety of ATB-346. Naproxen-induced intestinal damage was also associated with specific alterations to the intestinal microbiota, which were not evident in ATB-346-treated rats. PPI-administration led to significant changes in the intestinal microbiota and enhancement of bile cytotoxicity, which could account for the significant exacerbation of NSAID-enteropathy observed in rats co-administered PPIs (Wallace *et al*., 2011).

Numerous reports indicate that NSAIDs that do not undergo enterohepatic recirculation do not cause significant intestinal damage (Wax *et al*., 1970; Reuter *et al*., 1997; Somasunduram *et al*., 1997). When NSAIDs are recirculated, the intestinal epithelial cells are repeatedly exposed to the topical damaging effects of the NSAID, the metabolites of the NSAID, and the bile itself (Treinen-Molsen & Kanz, 2006). As shown in the present study, the substantially reduced enterohepatic circulation of ATB-346, or of naproxen liberated from this drug, appears to be significant contributor to the intestine-sparing effect of ATB-346. The *in vitro* bile cytotoxicity results demonstrate that bile containing increased levels of naproxen, or naproxen-glucuronides, is more damaging to intestinal epithelial cells. NSAID-glucuronides are particularly damaging to intestinal epithelial cells (Seitz & Boelsterli, 1998) and it was previously shown that naproxen administration results in higher levels of naproxen-glucuronides in the bile than ATB-346 administration (Blackler *et al*., 2012). Nevertheless, the enhancement of bile cytotoxicity following naproxen administration may also be related to the composition of the bile. Interestingly, NSAID administration to rats has been shown to increase the concentration of secondary bile acids in the bile (Yamada *et al*., 1996), and secondary bile acids are particularly toxic to intestinal epithelial cells (Hofmann, 1999; Zhou *et al*., 2010). Further studies are required to determine whether naproxen or ATB-346 administration can alter the biliary composition of bile acids and other constituents. As discussed below, the varying effects of naproxen or ATB-346 administration on the intestinal microbiota may also contribute to the intestine-sparing effect of ATB-346.

The enteric bacteria can contribute to the pathogenesis of NSAID-enteropathy in numerous ways. It has been previously demonstrated that PPIs exacerbate NSAID-induced intestinal damage in rats at least in part due to significant shifts in enteric microbial populations (Wallace *et al*., 2011). However, it remained unclear why PPI-induced dysbiosis exacerbates NSAID-enteropathy. As shown in the present study, PPI-administration caused intestinal dybiosis in rats, specifically enhancing the abundance of the *γ-Proteobacteria* and decreasing the abundance of *Actinobacteria* and *Firmicutes*. Moreover, the *Enterobacteriaceae* family of *γ-Proteobacteria* phylum was enriched in PPI-administered rats and this family includes many familiar enteric pathogens, such as *Salmonella* and *Escherichia coli* (*E. 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 had no intestinal ulcers (Uejima *et al*., 1996). It is noteworthy that Wallace *et al*. (2011) demonstrated a similar loss of *Actinobacteria* following PPI-administration and showed that restoration of *Bifidobacteria* (a genus in the *Actinobacteria* phylum) in the small intestine during treatment with a PPI and an NSAID prevented intestinal ulceration and bleeding.

As discussed above, bile is an important factor in the pathogenesis of NSAID-enteropathy. Enteric bacteria can modify the composition of the bile and (Begley *et al*., 2005; Duboc *et al*., 2013). For instance, an association between bile dysmetabolism and intestinal dysbiosis has also been reported in patients taking inhibitors of gastric acid secretion chronically (Shindo & Fukumura, 1995; Shindo *et al*., 1998). In the present

study, PPI-induced dysbiosis was associated with an enhancement of bile cytotoxicity in NSAID- and vehicle-treated rats. The data, along with previous findings (Wallace *et al*., 2011), suggest that bile cytotoxicity may be a contributing mechanism to the exacerbation of NSAID-induced enteropathy. Similar results have also been demonstrated when rats are administered H_2RAs, another class of inhibitors of gastric acid secretion (Blackler *et al*., 2014). Although suppression of gastric acid resulted in a marked enhancement of bile cytotoxicity, this is not sufficient to produce overt intestinal damage (Wallace *et al*., 2011; Blackler *et al*., 2014). The association between bile cytotoxicity and intestinal dysbiosis may be due to the PPI-induced dysbiotic microbiota producing an increased concentration of cytotoxic, secondary bile acids in lumen of the small intestine and subsequently the bile, since bile acids are efficiently reabsorbed in the terminal ileum (Begley *et al*., 2005; Martinez-Augustin *et al*., 2008). Indeed, the addition of secondary bile acids to naïve rat bile *in vitro* is substantially more cytotoxic than the addition of primary bile acids when incubated on cultured epithelial cells (Blackler & Wallace, unpublished data). Further studies are required to establish the causal and temporal relationship between changes to the intestinal microbiota and increases in bile cytotoxicity following the suppression of gastric acid.

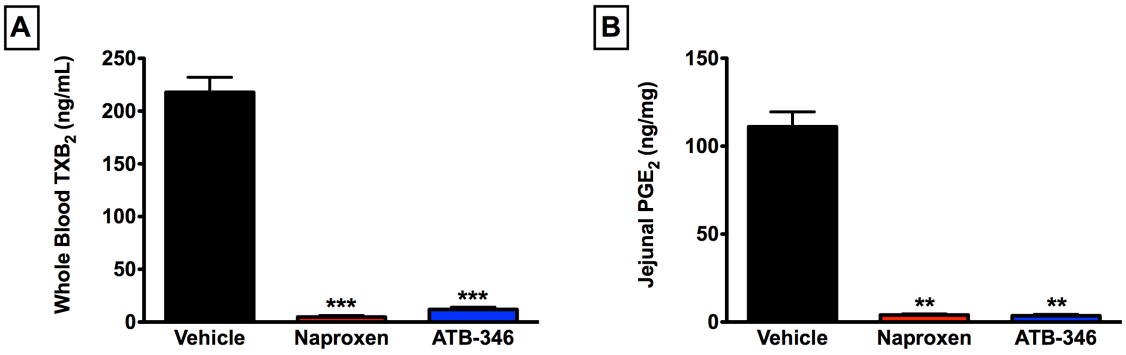
Enteric bacteria are also responsible for the enterohepatic circulation of NSAIDs. Re-absorption of NSAIDs in the small intestine requires the bacterial enzyme-catalyzed (i.e., bacterial β-glucuronidase) hydrolysis of NSAID-glucuronides (LoGuidice *et a*l., 2012). Indeed, pharmacological inhibition of bacterial β-glucuronidase, which prevents biliary delivery of the NSAID, prevents NSAID-induced intestinal damage in mice (Saitta

et al., 2014). In the present study, it is unlikely that enhancement of bile cytotoxicity observed in rats co-administered naproxen and PPIs was due to PPI-induced intestinal dysbiosis affecting the enterohepatic circulation of NSAIDs. Previously, it was demonstrated that the co-administration of omeprazole and naproxen does not increase the enterohepatic circulation of naproxen (Wallace *et al*., 2011).

The enteric microbiota can also impede the healing of GI ulcers and exacerbate the inflammatory response following intestinal injury (Elliott *et al*., 1998; Watanabe *et al*., 2008). Several studies have demonstrated that NSAID administration caused significant changes in the intestinal microbiota, often increasing the abundance of gram-negative bacteria (Kent *et al*., 1969; Uejima *et al*., 1996; Hagiwara *et al*., 2004; Dalby *et al*., 2006). As shown in the present study, microbial analysis following naproxen or PPI administration revealed an 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, which has been shown to have an important role in maintaining the integrity of the intestinal mucosa (Leonel & Alvarez-Leite, 2012). The enlarged presence of gram-negative bacteria could contribute to NSAID-induced intestinal injury by exacerbating the inflammatory response via activation of toll-like receptor 4 (TLR-4) (Watanabe *et al*., 2008). Indeed, there was a correlation between the abundance of gram-negative bacteria and intestinal damage in naproxen-administered rats in the present study.

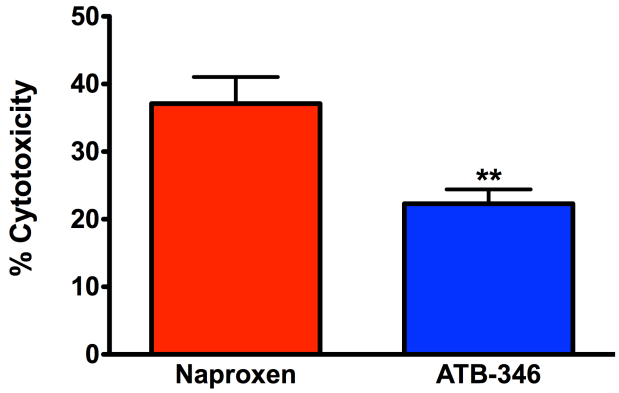
In summary, the present study provides evidence that the reduced enterohepatic circulation of ATB-346 contributes to the pre-clinical safety of this drug. We also present evidence that the enhancement of bile cytotoxicity due to PPI-administration may be a contributing factor to the exacerbation of NSAID-enteropathy. The pathogenesis of NSAID-enteropathy is complex, involving the cytotoxic effects of bile, changes in intestinal microbiota and enterohepatic circulation of the NSAID. The present study highlights that NSAID-induced small intestinal injury is dependent on the degree to which an NSAID affects these three factors. Likewise, the exacerbation of NSAID-enteropathy by the co-administration of inhibitors of gastric acid appears to be due to the suppression of gastric acid altering the nature of the bile and intestinal microbiota. These findings provide an improved understanding of the complicated pathogenesis of NSAID-enteropathy.

5.6. Supplementary Material



Supplementary Figure 5.8. ATB-346 and naproxen administration resulted in comparable suppression of cyclooxygenase (COX) 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. ATB-346, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester.



Supplementary Figure 5.9. ATB-346 administration did not enhance the cytotoxic effects of bile on human intestinal epithelial (HT-29) cells. Bile collected from rats administered naproxen (10 mg/kg) twice-daily for 4 days resulted in 37% cytotoxicity when incubated on HT-29 cells for 3 hours, whereas ATB-346 (equimolar to naproxen) administration resulted in 22% cytotoxicity (**p<0.01). Results are from the 1:6 and 1:12 dilution data and shown as mean ± SEM. The data were analyzed by an unpaired, two-tailed Student's t-test. ATB-346, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester.

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– CHAPTER 6 –

DISCUSSION

6.1. Summary

The pathogenesis of NSAID-enteropathy is mediated by complex interactions amongst bile, enteric bacteria, and the enterohepatic circulation of NSAIDs. The impact of these interactions can lead to severe small intestinal damage following NSAID use. There remains much to be understood in terms of how these mechanisms interact; however, it is clear that bile, enteric bacteria, and the enterohepatic circulation of NSAIDs are all critical to the development of small intestinal ulceration.

An improved understanding of the pathogenesis of NSAID-enteropathy is critical to the development of effective treatments and prevention strategies. *Therefore, the central aim of the research conducted during my PhD studies focused on understanding how bile, enteric bacteria, and the enterohepatic circulation of NSAIDs contribute to the pathogenesis of NSAID-enteropathy. I also evaluated whether the administration of H_2S would protect against NSAID-enteropathy and thus, whether H_2S represents a promising and effective means of treating or preventing small intestinal injury.* Collectively, the results in **Chapters 3-5** and **Appendix I** address this central aim. Each experimental approach required both an understanding of how each mechanism of injury contributed to the pathogenesis of NSAID-enteropathy and a consideration for how each mechanism could interact with and alter the nature of the other mechanisms.

The results presented as part of **Chapter 3** revealed that the administration of H_2S could protect against NSAID-enteropathy in rats, in part by reducing the cytotoxicity of bile and preventing NSAID-induced dysbiosis. The information and data presented in **Chapter 4** reviewed new findings in the pathogenesis and prevention of NSAID-

gastroenteropathy and highlighted the exacerbation of NSAID-induced intestinal injury in rats following a protocol that reflected the common clinical scenario of the co-use of an NSAID and an inhibitor of gastric acid secretion. Specifically, the results outlined in **Chapter 4** revealed that increased bile cytotoxicity as a consequence of treatment with an inhibitor of gastric acid secretion contributed to the exacerbation of NSAID-induced intestinal injury. The work outlined in **Chapter 5** addressed the roles of bile, enteric bacteria, and the enterohepatic circulation in the pathogenesis of NSAID-enteropathy by comparing the administration of a GI-safe NSAID to an NSAID with known GI toxicity. Finally, the data in **Appendix I** examined the role of enteric bacteria in the pathogenesis of NSAID-enteropathy by evaluating the effects of antibiotic co-administration on NSAID-induced intestinal injury.

The research presented in this thesis contributes to our understanding of the mechanisms of NSAID-enteropathy. Specifically, through various experimental approaches, the findings highlighted the roles of bile, enteric bacteria, and the enterohepatic circulation in the pathogenesis of NSAID-enteropathy. In addition, the results demonstrated that hydrogen sulfide represents a particularly potent therapeutic strategy for NSAID-enteropathy. Although much remains to be discovered, my work offers insight into the mechanisms through which NSAIDs cause intestinal injury and how manipulation of these mechanisms can prevent injury.

The key findings of this thesis have been discussed in detail in each manuscript (**Chapter 3-5**). Accordingly, this final chapter will discuss how each of these manuscripts relate to one another, elaborate on the overall significance and implications of my

research, identify the limitations of the thesis, and propose future directions in this field of research.

6.2. Bile, Enteric Bacteria, and the Enterohepatic Circulation of NSAIDS are Paramount to Intestinal Injury

For decades it has been recognized that bile, enteric bacteria, and the enterohepatic circulation of NSAIDS are necessary for the development of significant NSAID-enteropathy. For instance, ligation of the bile duct in rats prevents NSAID-induced intestinal damage and NSAIDs that do not undergo enterohepatic circulation do not elicit small intestinal damage (Kent *et al*., 1969; Wax *et al*., 1970; Beck *et al*., 1990; Reuter *et al*., 1997; Somasundaram *et al*., 1997). A key observation for the role of enteric bacteria is that germ-free rodents develop little to no damage in the small intestine following NSAID administration (Robert & Asano, 1977; Konaka *et al*., 1999). However, it is a challenge to separate the specific roles of bile, enteric bacteria, and the enterohepatic circulation of NSAIDs in the pathogenesis of NSAID-enteropathy. The reason for this being there is strong interplay amongst each of these mechanisms of injury. An understanding of the relative contributions of each of these mechanisms, as well as how they interact and feed back on each other, is vital to providing insight into the pathogenesis of NSAID-enteropathy.

6.2.1. Bile and enterohepatic circulation: critical factors in NSAID-enteropathy.

Drug secretion into the bile is a typical clearance route for many pharmaceuticals. However, the biliary delivery of NSAIDs and their metabolites evokes intestinal injury due to direct toxic actions and an indirect disruption of intestinal homeostasis (Reuter *et al*., 1997; Petruzzelli *et al*., 2007; Zhou *et al*., 2010). Indeed, it has been reported that differences in the extent of NSAID secretion into the bile amongst various species (i.e., human, rat, dog, etc) corresponds to species differences in vulnerability to NSAID-enteropathy (Duggan *et al*., 1975). The reason for this being, the enterohepatic circulation of NSAIDs repeatedly exposes the intestinal epithelial cells to the topical damaging effects of the NSAID, the metabolites of the NSAID, and to bile itself (Wallace, 2013a).

To examine the critical role of enterohepatic circulation in the pathogenesis of NSAID-enteropathy, I administered NSAIDs and/or various interventions to rats and then collected and analyzed the bile. The collected bile was then used in an *in vitro* model of rat epithelial cell injury that I established to examine the cytotoxic effects of the bile. In addition, the amount of NSAID was analyzed within the collected bile and explored whether this influenced *in vivo* ulcerogenicity and *in vitro* bile cytotoxicity. The results in **Chapter 5** are especially important in demonstrating the influence of the enterohepatic circulation on NSAID-induced intestinal injury. I compared the enterohepatic circulation and bile cytotoxicity of naproxen, a GI-toxic NSAID, to ATB-346, a GI-safe and H_2S-releasing naproxen derivative. Previously, I demonstrated that, unlike naproxen, the administration of ATB-346 did not produce any ulceration in the small intestine of rats, even when administered to rats with comorbidities and to rats that were repeatedly co-administered with other drugs (i.e., PPIs, low-dose ASA) (Blackler *et al*., 2012).

Interestingly, the administration of ATB-346 in rats resulted in lower levels of biliary naproxen and naproxen glucuronides compared to rats that received naproxen (Blackler *et al*., 2012). This observation is indicative of reduced enterohepatic circulation of ATB-346 or of the naproxen that is liberated from this drug. Since the combination of an NSAID and bile is particularly damaging to intestinal epithelial cells, I anticipated that the bile collected from naproxen-treated rats would be more cytotoxic than bile collected from rats treated with ATB-346 (Uchida *et al*., 1997; Dial *et al*., 2007; Zhou *et al*., 2010). Indeed, the bile collected from naproxen-treated rats was more cytotoxic to rat epithelial cells *in vitro* than bile collected from ATB-346-treated rats (**Chapter 5**). The *in vitro* cytotoxicity of the bile correlated well with the *in vivo* ulcerogenicity observed in the naproxen-treated and the ATB-346-treated rats. In addition, at each dose that was tested, naproxen administration resulted in greater levels of naproxen in the bile as compared to ATB-346 treatment. The altered pharmacokinetic behaviour observed with ATB-346 versus the parent NSAID, naproxen, is likely a critical reason that ATB-346 administration does not cause intestinal ulceration or an enhancement of bile cytotoxicity. These novel findings indicate that the reduced enterohepatic circulation of ATB-346 or of the naproxen liberated from this drug contributes to the GI-safety of this drug. On the other hand, results in **Chapter 3** and **Appendix I** suggest that the inability of an H_2S-releasing agent (i.e., DADS) and of broad-spectrum antibiotics to entirely prevent NSAID-induced small intestinal injury may be due to the inability to limit the enterohepatic circulation of naproxen. While the co-administration of DADS with naproxen prevented naproxen-induced dysbiosis and bile cytotoxicity, biliary levels of

naproxen in this group did not differ from rats administered naproxen alone. Likewise, the co-administration of broad-spectrum antibiotics with naproxen prevented naproxen-enhanced bile cytotoxicity, but the enterohepatic circulation of naproxen remained intact.

While biliary levels of an NSAID contribute to enhancement of bile cytotoxicity, the results in **Chapter 3-5** and **Appendix II** suggest that the manipulation of bile composition can also affect the cytotoxicity of bile, and subsequently, the extent of intestinal injury following NSAID-administration. For instance, co-treatment with antisecretory drugs (i.e., PPIs or H_2RAs) enhanced naproxen-induced enteropathy and bile cytotoxicity in rats. However, it was previously demonstrated that PPIs do not increase the enterohepatic circulation of naproxen (Wallace *et al*., 2011). In addition, the co-treatment of rats with an H_2S-releasing agent (i.e., DADS) reduced naproxen-induced enteropathy and bile cytotoxicity, while not altering the biliary levels of naproxen. It is noteworthy that the cytotoxicity of collected, naïve rat bile with added naproxen (300 μM) was less cytotoxic than bile collected from rats that were administered naproxen (20 mg/kg for 4.5 days) (58% vs. 50%, respectively), despite having a lower (~100-fold) concentration of naproxen. This enhancement of bile cytotoxicity, independent of the naproxen concentration, may be the result of numerous alterations *in vivo* to bile composition. For instance, the dysbiotic enteric microbiota that resulted from naproxen treatment may have an increased ability to deconjugate bile acids and/or convert primary bile acids to secondary bile acids. Indeed, it has been demonstrated that indomethacin administration can increase the abundance of secondary bile acids in the bile (Yamada *et al*., 1996). Furthermore, results in **Appendix II** demonstrated that secondary bile acids

are particularly damaging to rat intestinal epithelial (IEC-6) cell cultures as compared to primary bile acids. On the other hand, it was previously demonstrated that bile collected from naproxen-administered rats also contain naproxen-glucuronides, which may contribute to bile cytotoxicity (Seitz & Boelsterli, 1998; Blackler *et al*., 2012). Lastly, naproxen administration could alter the abundance of other constituents found in the bile, such as GSH and phospholipids, which may influence bile cytotoxicity. Both GSH and phospholipids can protect intestinal epithelial cells from injury (Lash *et al*., 1986; Aw *et al*., 1994; Zhou *et al*., 2010).

As a whole, the data presented in this thesis provide evidence that the biliary delivery of NSAIDs is essential for the development of small intestinal damage and contributes to the increased bile cytotoxicity and dysbiotic microbiota that is observed following NSAID administration. In addition, the results in **Chapter 5** are a novel demonstration that the GI-safety of the H_2S-releasing NSAID, ATB-346, is partly related to the reduced enterohepatic circulation of ATB-346 and of the naproxen that is liberated from this drug. It will be important for future studies to determine how the bile composition is altered by NSAID administration, as this is a limitation of the current thesis results. Furthermore, there is currently a lack of well-designed clinical trials that address the cytotoxic effects of the combination of bile and NSAIDs.

6.2.2. Enteric bacteria: a necessary, multifactorial contributor to NSAID-induced intestinal injury.

There is an abundance of evidence that the presence or absence of specific intestinal bacteria contributes to the pathogenesis of NSAID-enteropathy. For instance, numerous studies have reported that germ-free (no microbiota) rodents monocolonized with gram-positive bacteria, or wild-type rodents treated with antibiotics are resistant to NSAID-induced small intestinal injury (Kent *et al*., 1969: Robert & Asano, 1977; Uejima *et al*., 1996; Watanabe *et al*., 2008). There are several, potential explanations for the causal role of enteric bacteria in the modulation of the extent of NSAID-induced small intestinal damage; however, which of these roles predominates remains unclear.

Bacteria exacerbate the inflammatory response following mucosal injury by releasing LPS and other cell wall components that bind to and activate TLRs (Watanabe *et al*., 2008). Bacteria can also rapidly colonize mucosal ulceration sites and interfere with ulcer healing (Elliott *et al*., 1998). These observations, along with several studies that reported an enrichment of gram-negative bacteria following NSAID administration in rats, likely explain why antibiotics that effectively reduce the abundance of enteric bacteria (particularly gram-negative) can diminish small intestinal damage (Kent *et al*., 1969; Koga *et al*., 1999; Hagiwara *et al*., 2004; Dalby *et al*., 2006). However, much remains to be understood in terms of targeting bacteria with antibiotics in order to limit NSAID-enteropathy. For instance, I observed that the administration of the gram-negative targeting antibiotic, kanamycin, exacerbated naproxen-enteropathy (**Appendix I**). On the other hand, when I co-treated rats with a mixture of antibiotics that provided broad-spectrum targeting of enteric bacteria, the extent of naproxen-induced ulceration and intestinal inflammation was significantly reduced (**Appendix I**).

Despite a significant reduction in intestinal injury, considerable intestinal damage remained in naproxen-treated rats that were co-treated with the broad-spectrum antibiotic mixture. Several studies have demonstrated similar results following the administration of a broad-spectrum antibiotic, including a paper by Kent *et al*. (1969), which remarked "since the antibiotics do not prevent completely the ulceration, I think that these agents reduce the severity of the lesion by allowing healing to start sooner" (Yamada *et al*., 1993). Nevertheless, the causal roles of enteric bacteria in NSAID-enteropathy extend beyond the exacerbation of inflammation and impedance of mucosal injury. For instance, enteric bacteria also participate the enterohepatic circulation of NSAIDs via the microbial enzyme-catalyzed (i.e., β–glucuronidase) hydrolysis of NSAID-glucuronides in the small intestine, and as demonstrated in **Chapter 5**, the biliary delivery of NSAIDs directly influenced NSAID-enteropathy (Treinen-Moslen & Kanz, 2006; LoGuidice *et al*., 2012). Indeed, it has recently been demonstrated that an inhibitor of bacterial β-glucuronidase prevented NSAID-induced intestinal damage in mice (Saitta *et al*., 2014). It is, therefore, possible that the inability of the broad-spectrum antibiotic mixture to prevent the enterohepatic circulation of naproxen likely contributed to the intestinal injury following naproxen administration (**Appendix I**).

The composition of the intestinal microbiota can greatly influence the pathogenesis of NSAID-enteropathy. NSAIDs themselves alter the composition of the microbiota, as evidenced by the unbalanced overgrowth of specific bacteria following NSAID administration to rodents (Reuter *et al*., 1997; Hagiwara *et al*., 2004; Dalby *et al*., 2006). In **Chapter 3 and 5**, I demonstrated that naproxen administration resulted in a

dysbiotic GI-microbial community, and that there was an increased relative abundance of gram-negative bacterial families in rats with intestinal damage. NSAID-induced dysbiosis may antagonize (e.g., activate the LPS-TLR axis) intestinal inflammation following injury, but could also modify the overall β–glucuronidase activity of the microbiota. For instance, it has been demonstrated that NSAID administration to rodents enhanced the abundance of *E. coli,* which substantially increased (30-fold) β–glucuronidase activity compared to other bacterial taxonomic groups (Qian *et al*., 1993; Hagiwara *et al*., 2004; Dabek *et al*., 2008). Consequently, an increase in the overall β–glucuronidase activity of the microbiota would likely enhance the release of NSAIDs or NSAID-metabolites in the small intestine and thus, further damage enterocytes and promote enterohepatic recirculation.

Results in **Chapters 3-5** demonstrate that drugs given concomitantly with NSAIDs could also alter the composition of the microbiota and enhance NSAID-enteropathy. Previously, it was demonstrated that PPI-induced exacerbation of NSAID-enteropathy in rats was attributable to a dysbiosis that occurred following PPI administration (Wallace *et al*., 2011). The novel data presented in **Chapter 5** has helped to elucidate how PPI-induced dysbiosis triggers the exacerbation of NSAID-enteropathy via the enhancement of bile toxicity. Various clinical reports have associated the chronic use of PPIs or H_2RAs with small intestinal bacterial overgrowth (SIBO) and bile acid dysmetabolism (Shindo & Fukumura, 1995; Shindo *et al*., 1998; Williams & McColl, 2006; Lombardo *et al*., 2010). The ensuing bile acid dysmetabolism in patients with SIBO is likely the result of the disproportionate increase in number of microbes capable of

deconjugating bile acids and/or of converting primary bile acids to secondary bile acids (Shindo & Fukumura, 1995; Shindo *et al*., 1998; Begley *et al*., 2005). This may explain why the administration of PPI or H_2RA alone to rats increased the cytotoxic effects of bile (**Chapter 4 and 5**). The connection between dysbiosis and bile dysmetabolism may also explain why the co-administration of DADS or broad-spectrum antibiotics prevented naproxen-induced bile cytotoxicity and reduced small intestinal damage (**Chapter 3 and Appendix I**). Interestingly, not only can a dysbiotic microbiota influence the composition of the bile, but bile can also modulate the rat intestinal microbiota, as bile acids have varying degrees of anti-microbial effects (Begley *et al*., 2005; Islam *et al*., 2011).

In summary, the enteric microbiota contributes to the pathogenesis of NSAID-enteropathy via numerous mechanisms. Antibiotic administration likely reduces NSAID-induced intestinal damage by preventing the growth of bacteria in the small intestine, and thus dampening the inflammatory response and preventing the impedance of mucosal healing; however, this incompletely protects the small intestine. The dysbiotic bacterial community following antisecretory drug or NSAID administration alters bile toxicity and results in enhanced intestinal damage. However, the findings in this thesis only begin to describe patterns of microbial colonization associated with NSAID-enteropathy. Therefore, it will be important to begin determining the functional characteristics of the altered microbial community. For example, targeting bacterial enzymes responsible for the enterohepatic circulation of NSAIDs and the modification of bile acids may be a more useful target for novel therapies of NSAID-related enteropathy.

6.3. Hydrogen Sulfide: a Potent Therapeutic for NSAID-enteropathy

There is substantial evidence for the importance of H_2S in enhancing GI-mucosal resistance to damage and in modulating inflammation and repair (Wallace *et al*., 2014). The administration of H_2S-donating agents to rodents elevates mucosal blood flow, prevents leukocyte-endothelial adhesion, reduces oxidative stress, and stimulates angiogenesis (Wallace, 2010). Conversely, the administration of inhibitors of the key enzymes that contribute to H_2S synthesis exacerbates inflammation in the GI tract, decreases COX-2 expression, enhances leukocyte infiltration, and delays the healing of ulcers in the stomach and colon of rodents (Wallace *et al*., 2014).

The GI-protective actions of H_2S have been exploited in the design of novel, H_2S-releasing NSAIDs, which produce negligible damage in the GI tract. In particular, the H_2S-releasing NSAID, ATB-346, has reduced toxicity in the small intestine in preclinical studies (Wallace *et al*., 2010; Blackler *et al*., 2012). However, it was not known whether other H_2S-releasing agents protect the small intestine from NSAID-enteropathy. In **Chapter 3**, I demonstrated that co-administration of diallyl disulfide, an H_2S-releasing agent, protected against NSAID-induced small intestinal injury in rats, in part by limiting two important factors in the pathogenesis of NSAID-enteropathy: the cytotoxicity of bile and NSAID-induced dysbiosis. Interestingly, these two effects may be related, since changes in enteric bacteria can lead to altered bile metabolism, and an alteration of bile cytotoxicity (Duboc *et al*., 2013). This is similar to the association I observed in **Chapter 4 and 5** with the administration of inhibitors of gastric acid secretion and may explain why the co-administration of DADS to naproxen-treated rats resulted in a reduction of

bile cytotoxicity, but the biliary levels of naproxen were unaltered by DADS treatment. Further studies are required to better understand how DADS administration resulted in an altered microbiota and reduced bile cytotoxicity.

The ability of an H_2S-releasing agent to protect against NSAID-induced intestinal damage may depend on the nature of the agent. In **Appendix II**, our results demonstrate that substantial release of H_2S from DADS is obtained when a reducing agent is incubated with DADS. This suggests that H_2S is not spontaneously released from DADS and thus, may allow for the DADS to reach mucosal injury sites before expending the H_2S-releasing capability. Interestingly, bile is a major source of GSH and it has been demonstrated that the delivery of GSH via bile to the small intestine protected intestinal epithelial cells from oxidative injury in rats (Lash *et al*., 1986; Aw *et al*., 1994). It is possible that GSH in the lumen of the small intestine created an ideal (i.e., reducing) environment for DADS to release H_2S *in vivo*, which may explain why DADS co-administration is so effective at protecting naproxen-treated rats from small intestinal injury. It would be interesting to explore whether other H_2S-releasing agents are as effective as DADS at preventing NSAID-induced small intestinal damage.

It is noteworthy that DADS co-administration was considerably more effective than antibiotic co-administration at reducing NSAID-induced small intestinal damage. For instance, the administration of a broad-spectrum antibiotic mixture significantly reduced the naproxen-induced intestinal damage score by 54%, whereas the two protective doses of DADS (30 and 60 $mmol \cdot kg^{-1}$) resulted in an 83% and 85% reduction in naproxen-induced intestinal damage score, respectively. In addition, the co-

administration of the broad-spectrum antibiotic mixture was unable to prevent the severe blood loss caused be naproxen treatment, whereas DADS co-administration with the two protective doses prevented significant bleeding. The use of antibiotics as a therapeutic approach does not appear promising, considering the lack of effectiveness of antibiotics in preventing NSAID-enteropathy. Furthermore, there is the potential for adverse effects and drug resistance associated with long-term antibiotic use in humans.

While DADS is an effective therapeutic, it could not achieve complete protection against naproxen-induced enteropathy. In contrast, ATB-346 treatment spares the small intestine and gastric mucosa of damage in rats, even when dosed in co-morbidity and polypharmacy models (Wallace *et al*., 2010; Blackler *et al*., 2012; Elsheikh *et al*., 2014). It is unlikely that the differences in intestinal protection between ATB-346 and DADS are simply related to the different amounts of H_2S released.

In an effort to examine what other mechanisms contribute to the intestinal safety of ATB-346, I dosed naproxen or ATB-346 to rats and explored the effects of each drug had on the bile and on the intestinal microbiota (**Chapter 5**). Naproxen caused a dose-dependent increase in intestinal damage, inflammation, and bile cytotoxicity. On the other hand, ATB-346 administration, at doses equimolar to each naproxen dose, resulted in negligible intestinal damage and did not increase either intestinal inflammation or bile cytotoxicity. As discussed in **Section 6.1**, the reduced enterohepatic recirculation of ATB-346 or of naproxen liberated from this drug is likely a significant contributor to the intestine-sparing effect of ATB-346. For instance, the considerably lower levels (67-82%) of biliary naproxen observed with ATB-346 administration were associated with an

insignificant increase in bile cytotoxicity. In addition, it has been reported that the enterohepatic circulation of NSAIDs is necessary to significantly alter the intestinal microbiota and to promote the growth of damaging, gram-negative bacteria (Reuter *et al*., 1997). Indeed, I demonstrated that the administration of naproxen, which underwent significant enterohepatic circulation, resulted in a dysbiotic microbiota that contained a higher abundance of gram-negative bacterial families (**Chapter 3 and 5**). Conversely, I observed that ATB-346 underwent significantly less enterohepatic recirculation and did not increase the abundance of gram-negative bacterial families.

In summary, H_2S is an endogenous gaseous mediator that promotes mucosal integrity, repair, and resolution of inflammation. Our results demonstrate that the co-administration of an H_2S-releasing agent protected against naproxen enteropathy, in part by reducing the cytotoxicity of bile and preventing NSAID-induced dysbiosis. In addition, the intestine-sparing effects of the H_2S-releasing NSAID, ATB-346, are partly related to the reduced enterohepatic circulation of ATB-346 or the naproxen liberated from this drug. The preclinical safety of H_2S-releasing NSAIDs, such as ATB-346, is encouraging and may supersede the requirement of therapies to prevent the GI-toxicity of NSAIDs in the future.

6.4. Future Perspectives

Substantial progress has been made in understanding the mechanisms that contribute to the pathogenesis of NSAID-enteropathy, however, much still remains to be understood. In particular, future studies should explore to what extent the critical

mechanisms (i.e., enteric bacteria, bile, and the enterohepatic recirculation of NSAIDs) of NSAID-enteropathy affect one another. For instance, there are many interactions between the enteric microbiota and bile (Begley *et al*., 2005). An understanding of these interactions may help to explain the phenomenon of unbalanced overgrowth of specific bacteria (e.g., gram-negative) following NSAID administration (Kent *et al*., 1969; Reuter *et al*., 1998; Hagiwara *et al*., 2004). This phenomenon has been recognized for decades, although it is not entirely known *why* this dysbiosis occurs. Interestingly, many bacterial species exhibit varying degrees of sensitivity to bile acids (Floch *et al*., 1972). For example, gram-negative bacteria are quite insensitive to the anti-microbial properties of bile acids (Floch *et al*., 1972). It is therefore possible that changes in bile composition following NSAID administration could favour the unbalanced overgrowth of specific bacteria.

Further studies are also required to understand how H_2S alters the enteric microbiota and bile, and whether these alterations could protect the GI tract from other detrimental clinical conditions, such as inflammatory bowel disease and hepatobiliary diseases. I demonstrated that the administration of an H_2S-releasing agent (i.e., DADS) altered the enteric microbiota. It would be interesting to explore whether the altered microbiota from H_2S-administered rodents could be transferred to germ-free rodents and impact the susceptibility of those rodents to NSAID-enteropathy or to another model of gastrointestinal damage, such as colitis.

Lastly, it will be important to further explore PPI-induced alterations to the enteric microbiota and bile. As demonstrated in this thesis, these alterations contribute to the

enhancement of NSAID-induced intestinal damage, but it is possible that these alterations promote other detrimental effects clinically. It will also be imperative to evaluate the pathogenesis of NSAID-enteropathy in the context of common co-morbidities as many of these co-morbidities affect the critical mechanisms of damage, such as the enteric microbiota, and thus the extent of intestinal damage.

6.5. Conclusions

The work presented in this thesis provids an improved understanding of the complicated pathogenesis of NSAID-enteropathy by confirming that the nature of the bile, the enterohepatic circulation of NSAIDs, and the nature of the intestinal microbiota are each of importance. The results in this thesis also demonstrate that H_2S represents an effective preventative therapy for NSAID-enteropathy and that H_2S-releasing NSAIDs, such as ATB-346, have preclinical safety. Further studies will aid in the development of effective preventative or curative therapies for NSAID-enteropathy.

– APPENDIX I –

UNPUBLISHED RESULTS: THE EFFECTS OF TARGETING ENTERIC BACTERIA WITH ANTIBIOTICS IN NSAID-ENTEROPATHY

7.1. Objectives

The **central aim** of the following studies was *to clarify the role of enteric bacteria in the pathogenesis of NSAID-enteropathy.*

Objective 1: *To explore whether the administration of a specific antibiotic or combination of antibiotics prevent NSAID-enteropathy in a chronic administration model.*

Objective 2: *To investigate whether antibiotic administration can alter the cytotoxic effects of bile and the enterohepatic circulation of NSAIDs.*

7.2. Introduction

There is a wealth of evidence that enteric bacteria play a critical role in the pathogenesis of NSAID-enteropathy. It remains unclear whether bacteria play a primary role by initiating the tissue injury, or a secondary role by exacerbating NSAID-induced tissue injury and hindering mucosal repair. Nevertheless, the contribution of enteric bacteria to the NSAID-induced intestinal injury and inflammation, to the enterohepatic circulation of NSAIDs, and to the impedance of mucosal repair, suggests that the therapeutic targeting of enteric bacteria may prevent NSAID-enteropathy (Scarpignato, 2008).

Several studies have documented dramatic shifts in the small intestinal microbiota following NSAID administration. Generally, these studies reported an increase in gram-negative bacteria, and a concomitant reduction in gram-positive bacteria, which has promoted the idea that gram-negative bacteria in particular contribute to NSAID-induced

intestinal injury (Hagiwara *et al*., 2004; Dalby *et al*., 2006; Kato *et al*., 2009). This hypothesis has been highlighted by numerous findings. For instance, germ-free rodents developed little to no damage when administered an NSAID, but when colonized with gram-negative bacteria, these rodents became susceptible to NSAID-enteropathy (Robert & Asano, 1977; Uejima *et al*., 1996). In addition, mice that lacked the endotoxin receptor, TLR-4, which binds LPS from gram-negative bacteria, developed significantly less intestinal injury following NSAID administration compared to the wild type controls (Watanabe *et al*., 2008).

It has also been reported that the co-administration of antibiotics, particularly ones that target gram-negative bacteria, with NSAIDs can limit the development of intestinal injury and bleeding (Koga *et al*., 1999; Watanabe *et al*., 2008). For instance, Koga *et al* demonstrated that the occurrence of intrarectally dosed indomethacin induced intestinal ulcerations could be dose-dependently reduced by prior oral kanamycin administration once daily for seven days. Kanamycin is an aminoglycoside antibiotic, often used to treat serious gram-negative bacterial infections. Orally dosed kanamycin is not absorbed, thus limiting its effect to killing luminal bacteria (Koga *et al*., 1999). The authors further confirmed that this protocol of kanamycin decreased the number of intestinal gram-negative bacteria and intestinal inflammation (i.e., MPO activity) in a dose-dependent manner. Therefore, the authors suggested that the favourable effects of kanamycin were due to its strong antibacterial action on gram-negative bacteria (Koga *et al*., 1999). On the other hand, antibiotics that specifically target gram-positive bacteria do not appear to protect rodents from NSAID-enteropathy. Vancomycin is a very large, hydrophilic,

glycopeptide antibiotic that is poorly absorbed orally, thus its antimicrobial effects are limited to killing luminal bacteria (Watanabe et al., 2008). It is often used in the treatment of gram-positive bacterial infections and is largely inactive against gram-negative bacteria. Two oral doses of vancomycin within 24 hrs of a single dose of indomethacin did not alter the severity of indomethacin-induced intestinal damage (Watanabe *et al*., 2008).

While animal studies have demonstrated that co-treatment with an antibiotic may be a viable therapeutic strategy for reducing NSAID-enteropathy, there are limitations to these findings. For instance, NSAID-enteropathy is often modeled by administering a single, large dose of an exceptionally GI-toxic NSAID, such as indomethacin, which is not reflective of a clinical setting. These studies have also not addressed whether the beneficial effects of antibiotic treatment could be partly due to antibiotic-induced intestinal dysbiosis. Intestinal dysbiosis could affect two important mechanisms in NSAID-enteropathy by altering the bile or the enterohepatic circulation of NSAIDs (Wallace, 2013a).

The following experiments were undertaken to clarify the role of enteric bacteria in the pathogenesis of NSAID-enteropathy. First, I compared the severity of NSAID-enteropathy between rats administered kanamycin and rats administered vancomycin. Rats received multiple doses of naproxen, a commonly prescribed NSAID, which is more reflective of a clinical scenario. I also evaluated the influence of kanamycin administration and vancomycin administration on the cytotoxic effects of bile. Our second approach involved administering a combination of antibiotics with broad-

spectrum coverage in order to greatly decrease the enteric bacterial load (Rakoff-Nahoum *et al*., 2004; Croswell et al., 2009). The mixture regimen consisted of four antibiotics: ampicillin, vancomycin, neomycin, and metronidazole. Both ampicillin and metronidazole demonstrate activity against gram-positive and -negative bacteria (only anaerobes for metronidazole) and are well absorbed from the GI tract, but rapidly cleared from systemic circulation (Rakoff-Nahoum *et al*., 2004; Croswell et al., 2009). On the other hand, vancomycin and neomycin are not well absorbed in the GI tract. Neomycin has broad-spectrum activity but is particularly efficacious against gram-negative bacteria, whereas vancomycin targets gram-positive bacteria. This experiment was particularly important to evaluate whether antibiotics could indeed protect against chronic naproxen-induced enteropathy, and whether antibiotic administration could affect bile toxicity and the enterohepatic circulation of NSAIDs.

7.3. Methods

Single-antibiotic Administration. Rats ($n \geq 6$ per group) were treated orally, twice daily, with kanamycin (100 mg/kg), vancomycin (50 mg/kg), or vehicle orally, daily for 9 days. On the evening of the 7th day, the rats received 4 oral doses (at 12 hour intervals) of naproxen (20 mg/kg) or vehicle (dimethylsulfoxide and 1% carboxymethylcellulose; 5:95 ratio) for 2 days.

Antibiotic Mixture Administration. Rats ($n \geq 6$ per group) received either regular drinking water or drinking water supplemented with a mixture of four antibiotics for 9 days. The

antibiotic drinking water was prepared as follows: ampicillin (1 g/L), metronidazole (1 g/L), neomycin (1 g/L), and vancomycin (500 mg/mL). On the evening of the 7th day, the rats received 4 doses (at 12 hour intervals) of naproxen (20 mg/kg) or vehicle (dimethylsulfoxide and 1% carboxymethylcellulose; 5:95 ratio) orally over 2 days.

Naproxen-Induced Enteropathy. One hour after the final administration of drug or vehicle, bile was collected. The small intestine was then excised and blindly evaluated for hemorrhagic damage. Refer to the methods section in Chapter 3.3 for further details. 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).

Collection of Bile. One hour after the final administration of naproxen or vehicle, the 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 and then stored at -80°C until use in the cytotoxicity assay.

Intestinal Epithelial Cell Culture. Rat intestinal epithelial (IEC-6) cells were obtained from the American Type Culture Centre (Manassas, VA, USA). For detailed culturing procedures, refer to the methods section in Chapter 3.3. Cells between passages 17-20 were used for bile cytotoxicity assays.

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 to 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. Solutions of bile were added to IEC-6 cells for 3 h. Following the incubation period, the cells were centrifuged and the supernatants collected for lactate dehydrogenase measurement, using a Cytoscan-LDH Cytotoxicity Assay Kit. Refer to the methods section in Chapter 3.3 for further details.

Biliary Naproxen Levels. Concentrations of naproxen in bile were blindly 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 the jejunum (~200 mg; with the luminal contents preserved) from rats treated with vehicle or antibiotics, and co-treated with vehicle or naproxen, were collected under sterile conditions and homogenized in PBS. Homogenates were serially diluted and plated onto Columbia (with 5% sheep blood) (CBA) agar and incubated for 48 h under aerobic conditions. Plates containing between 20 and 200 colony-forming units (CFU) were analyzed to determine bacterial numbers, and the results expressed as CFU per gram of tissue. Refer to the methods section in Chapter 3.3 for further details.

DNA Extraction and Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis. Bacterial DNA was extracted from cecal content samples as previously described (Park *et al.*, 2013). The hypervariable V3 region of the bacterial 16s ribosomal DNA gene was amplified using a polymerase chain reaction with universal bacterial primers (HDA-1 and HDA-2) (Mobixlab, McMaster University core facility, Hamilton, Ontario, Canada). Denaturing gradient gel electrophoresis (DGGE) was then performed and the gels were stained with SYBR Green and viewed by ultraviolet transillumination. Similarities between bacterial profiles were determined using the Dice coefficient, and the Ward algorithm. Refer to the methods section in Chapter 3.3 for further details.

Materials. Naproxen sodium, kanamycin sulfate, neomycin trisulfate salt hydrate, and metronidazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ampicillin sodium salt and vancomycin hydrochloride were purchased from MP Biomedicals (Santa Ana, CA, USA). Columbia agar media plates were purchased from Becton-Dickinson (Mississauga, ON, CA).

7.4. Results

Dosing with a single antibiotic did not protect against naproxen-induced ulceration. The co-administration of naproxen at 20 mg/kg for 2 days to vehicle-treated rats resulted in severe intestinal damage (Figure 7.1 A and B). The co-administration of naproxen to kanamycin-treated rats significantly ($p<0.05$) increased small intestinal damage as compared to rats co-treated with naproxen and vehicle (Figure 7.1 A). The extent of small

intestinal damage was similar in vancomycin-treated rats that were co-administered naproxen to vehicle-treated rats that received naproxen (Figure 7.1 B). Kanamycin or vancomycin administration alone did not result in any observable intestinal damage.

The co-administration of naproxen at 20 mg/kg for 2 days to vehicle-treated rats resulted in increased jejunal granulocyte infiltration (MPO activity) as compared to rats treated with vehicle only (Figure 7.1 C and D). Administration of kanamycin or vancomycin was unable to prevent the increase in MPO activity caused by naproxen treatment. Kanamycin or vancomycin administration alone did not significantly alter intestinal MPO activity.

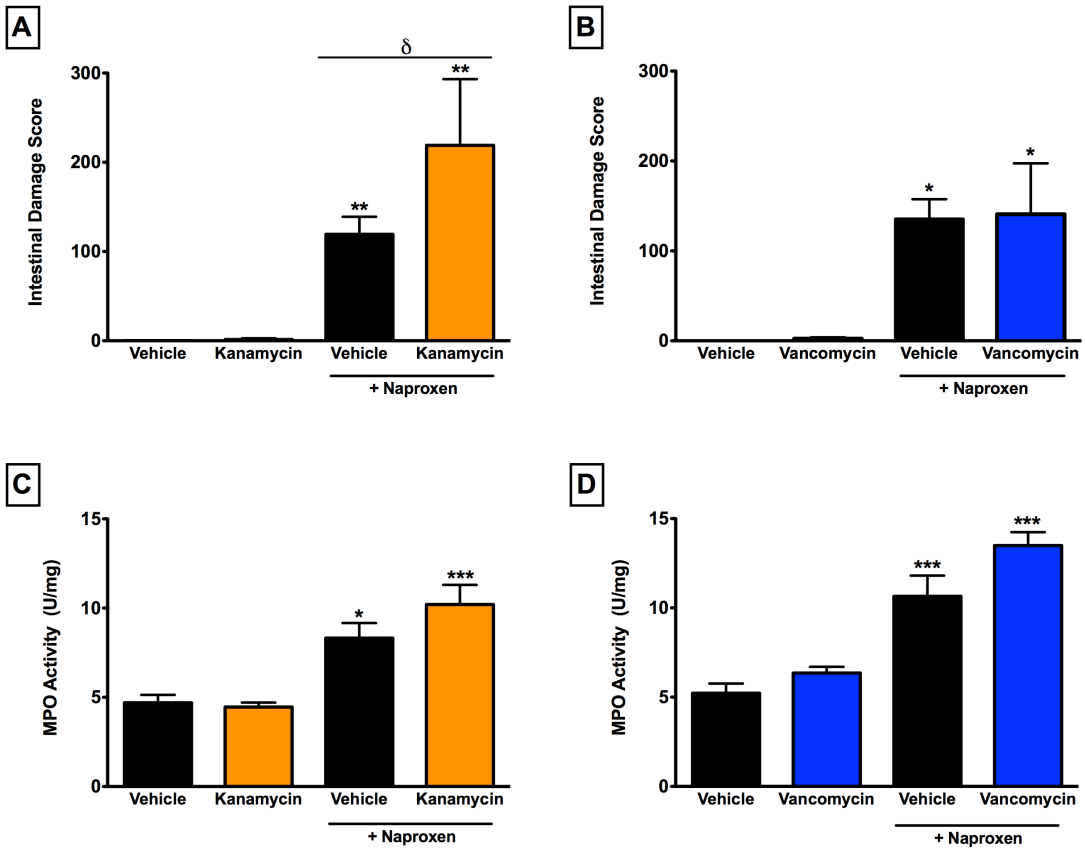


Figure 7.1. Kanamycin co-treatment exacerbated naproxen-induced ulceration. Panel A and B: The administration of naproxen (20 mg/kg) twice daily for 2 days resulted in significant intestinal damage (**$p<0.01$, *$p<0.05$; Panel A and B, respectively) compared to vehicle-only treated rats. Co-treatment with kanamycin significantly ($^{\delta}p<0.05$; Student's t-test) worsened naproxen-induced intestinal erosions, whereas co-treatment with vancomycin did not alter intestinal erosions. Panel C and D: Co-treatment with kanamycin or vancomycin did not significantly alter the increase in intestinal myeloperoxidase (MPO) activity caused by naproxen administration. Results are shown as mean ± SEM ($n\geq5$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

Dosing with a single antibiotic did not reduce naproxen-enhanced bile cytotoxicity. When tested at dilutions of 1:6 or 1:12, bile collected from rats that were administered naproxen at 20 mg/kg over 2 days exhibited significantly increased cytotoxic effects on IEC-6 intestinal epithelial cells as compared to bile from rats that were administered vehicle (Figure 7.2 A and B). The cytotoxicity of the bile collected from rats that were co-administered either kanamycin or vancomycin with naproxen was not significantly different from the cytotoxicity of the bile collected from rats treated with naproxen and vehicle. Kanamycin or vancomycin administration alone did not significantly alter the cytotoxic effects of bile.

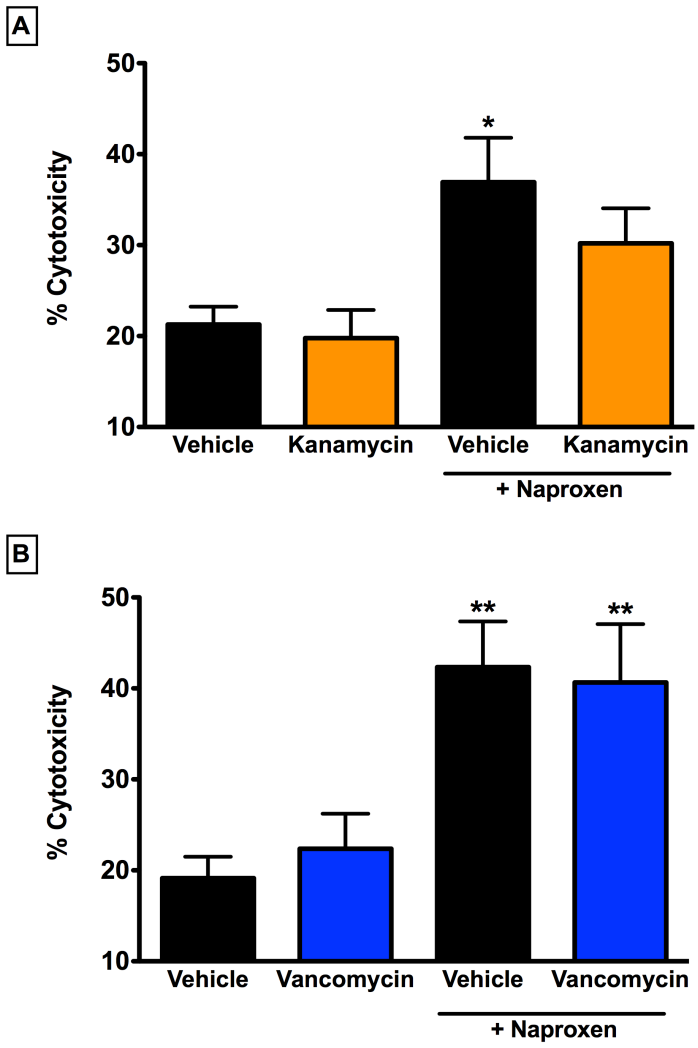
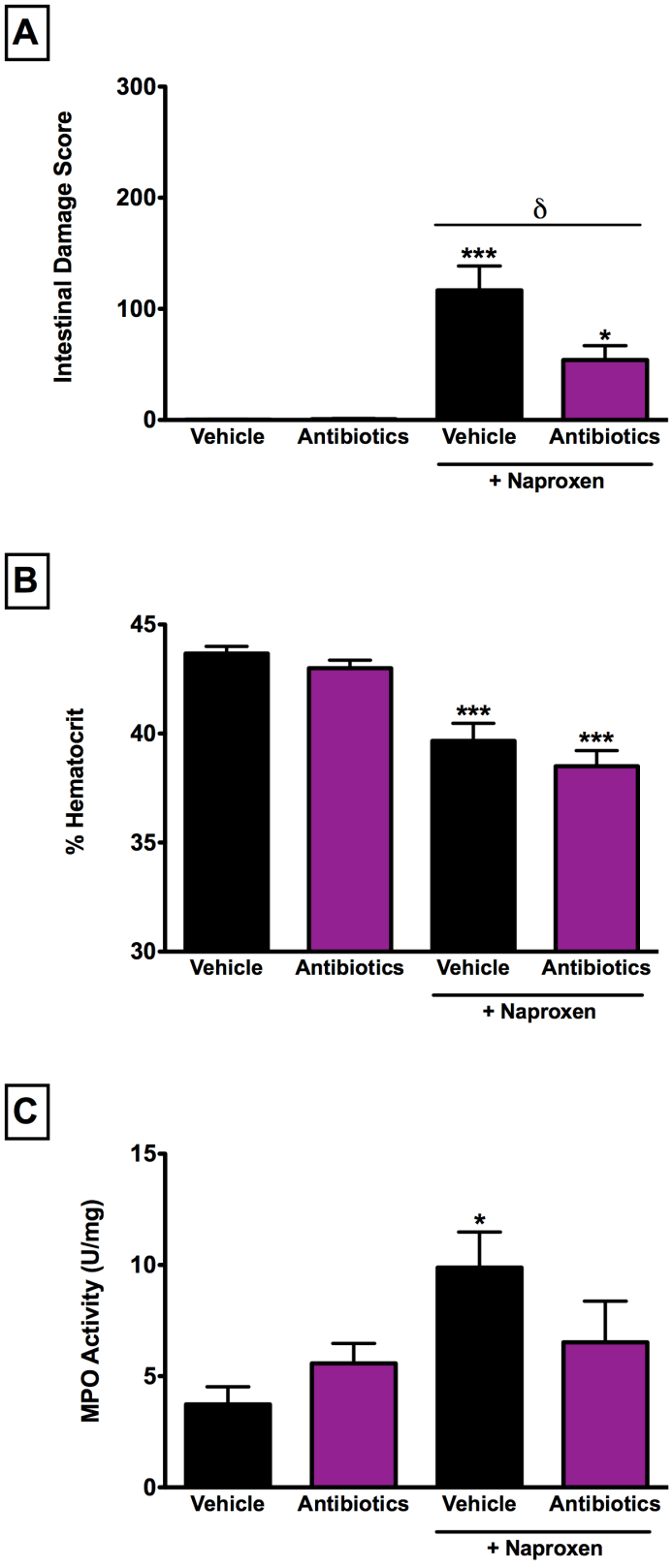


Figure 7.2. Naproxen-enhanced bile cytotoxicity is unaltered by kanamycin or vancomycin co-treatment. Bile collected from rats treated with naproxen at 20 mg/kg twice daily for 2 days was significantly more cytotoxic to cultured rat intestinal epithelial (IEC-6) cells than bile collected from vehicle-treated rats (*$p<0.05$, **$p<0.01$; Panel A and B, respectively). Naproxen-induced bile cytotoxicity was not significantly different between the bile collected from rats co-treated with kanamycin or vancomycin compared to rats that were co-treated with vehicle. The data shown are combined from the 1:6 and 1:12 dilutions of bile samples, and are expressed as mean ± SEM ($n\geq5$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

Administration of the antibiotic mixture reduced naproxen-enteropathy. Rats administered naproxen (20 mg/kg) twice daily for 2 days had extensive intestinal damage and blood loss (Figure 7.3 A and B). On the other hand, the administration of naproxen to rats receiving the antibiotic mixture resulted in a significant ($p<0.05$) reduction in the extent of intestinal damage (Figure 7.3 A). However, treatment with the antibiotic mixture did not prevent naproxen-induced bleeding, as rats that received either antibiotics or vehicle had similar reductions in hematocrit after naproxen treatment (Figure 7.3 B). The administration of naproxen significantly ($p<0.05$) increased jejunal granulocyte infiltration (MPO activity) as compared to rats treated with vehicle only (Figure 7.3 C). Conversely, naproxen-induced granulocyte infiltration was prevented in rats that received antibiotics.


A
Intestinal Damage Score
300
200
100
0
δ
***
*
Vehicle
Antibiotics
Vehicle
Antibiotics
+ Naproxen
B
% Hematocrit
45
40
35
30
***
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Vehicle
Antibiotics
Vehicle
Antibiotics
+ Naproxen
C
MPO Activity (U/mg)
15
10
5
0
*
Vehicle
Antibiotics
Vehicle
Antibiotics
+ Naproxen

Figure 7.3. A broad-spectrum antibiotic mixture reduced intestinal ulceration and inflammation. Panel A: Naproxen (20 mg/kg) administration twice daily for 2 days resulted in severe small intestinal ulceration (***$p<0.001$; versus vehicle-treated rats). The co-treatment of antibiotics with naproxen significantly reduced intestinal ulceration in naproxen-treated rats ($^{\delta}p<0.05$; Student's t-test). Panel B: Naproxen administration to vehicle- or antibiotic-treated rats caused significant bleeding (decrease in hematocrit) compared to rats treated with vehicle only (***$p<0.001$). Panel C: Naproxen co-administration to vehicle-treated rats significantly increased intestinal myeloperoxidase (MPO) activity (*$p<0.05$; versus vehicle-treated rats), whereas MPO activity in naproxen plus antibiotic co-treated rats was not significantly different from that of vehicle-treated rats. The antibiotic mixture contained ampicillin, metronidazole, neomycin, and vancomycin. Results are shown as mean ± SEM ($n\geq5$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

The antibiotic mixture reduced naproxen-enhanced bile toxicity. The naproxen-induced enhancement of bile cytotoxicity was reduced in rats that were co-treated with antibiotics (Figure 7.4 A). Bile collected from rats treated with naproxen was significantly ($p<0.01$) more cytotoxic to IEC-6 intestinal epithelial cells as compared to bile from rats that were administered vehicle. On the other hand, bile collected from rats co-treated with naproxen and antibiotics was not significantly different, in terms of cytotoxicity, from the bile collected from rats treated with vehicle only. The administration of antibiotics alone did not significantly alter the cytotoxic effects of bile.

To explore whether the co-administration of the antibiotic mixture reduced biliary concentrations of naproxen, I measured the concentrations of naproxen in bile from rats treated with naproxen (20 mg/kg) alone and from rats co-treated with antibiotics. The

concentration of naproxen in the bile of naproxen-treated rats did not differ significantly when the rats were co-treated with antibiotics (Figure 7.4 B).

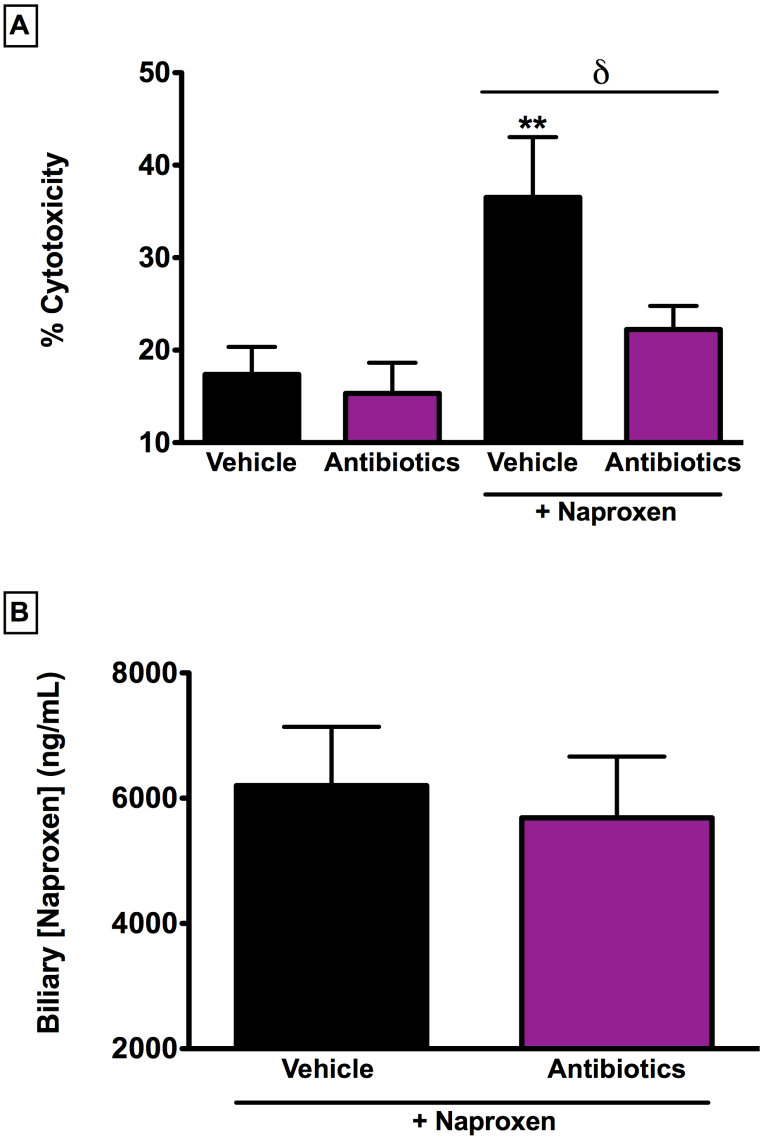

Figure 7.4. A broad-spectrum antibiotic mixture reduced naproxen-enhanced bile toxicity, but biliary naproxen concentration remained unaltered. Panel A: Bile collected from rats treated with naproxen at 20 mg/kg twice daily for 2 days was significantly more cytotoxic to cultured rat intestinal epithelial (IEC-6) cells than the bile collected from vehicle-treated rats (**$p<0.01$). Bile cytotoxicity was significantly reduced in rats that

were co-treated with naproxen and antibiotics ($^{\delta}p<0.05$; versus naproxen plus vehicle group; Student's t-test). The data shown are combined from the 1:6 and 1:12 dilutions of bile samples. Panel B: Biliary concentrations of naproxen were unaltered by the co-treatment with the mixture of antibiotics. Bile was collected 1 h after the final drug administration. Biliary naproxen concentrations were measured by liquid chromatography/mass spectrometry. The antibiotic mixture contained ampicillin, metronidazole, neomycin, and vancomycin. Results are shown as mean ± SEM ($n\geq5$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

The antibiotic mixture caused profound dysbiosis and reduced intestinal bacterial growth. The total number of aerobes in the jejunum of rats treated with antibiotics was significantly ($p<0.05$) reduced compared to vehicle-treated rats, regardless of whether naproxen was co-administered (Figure 7.5 A). DGGE analysis of cecal contents demonstrated that the treatment with antibiotics caused a marked shift in the composition of the microbiota. DGGE analysis was performed to compare the microbial composition in rats treated with vehicle, antibiotics, naproxen (20 mg/kg) plus vehicle, or naproxen plus antibiotics. Antibiotic administration caused cecal dysbiosis in rats co-treated with either vehicle or naproxen, as analyzed by the Dice coefficient and Ward algorithm to determine similarities (Figure 7.6 B). Naproxen administration alone resulted in cecal samples clustering separately from vehicle-treated rats, indicating that naproxen treatment can also alter the composition of the microbiota. The clustering observed in Figure 7.5 B was confirmed via a second DGGE analysis with another 3 samples for each group.

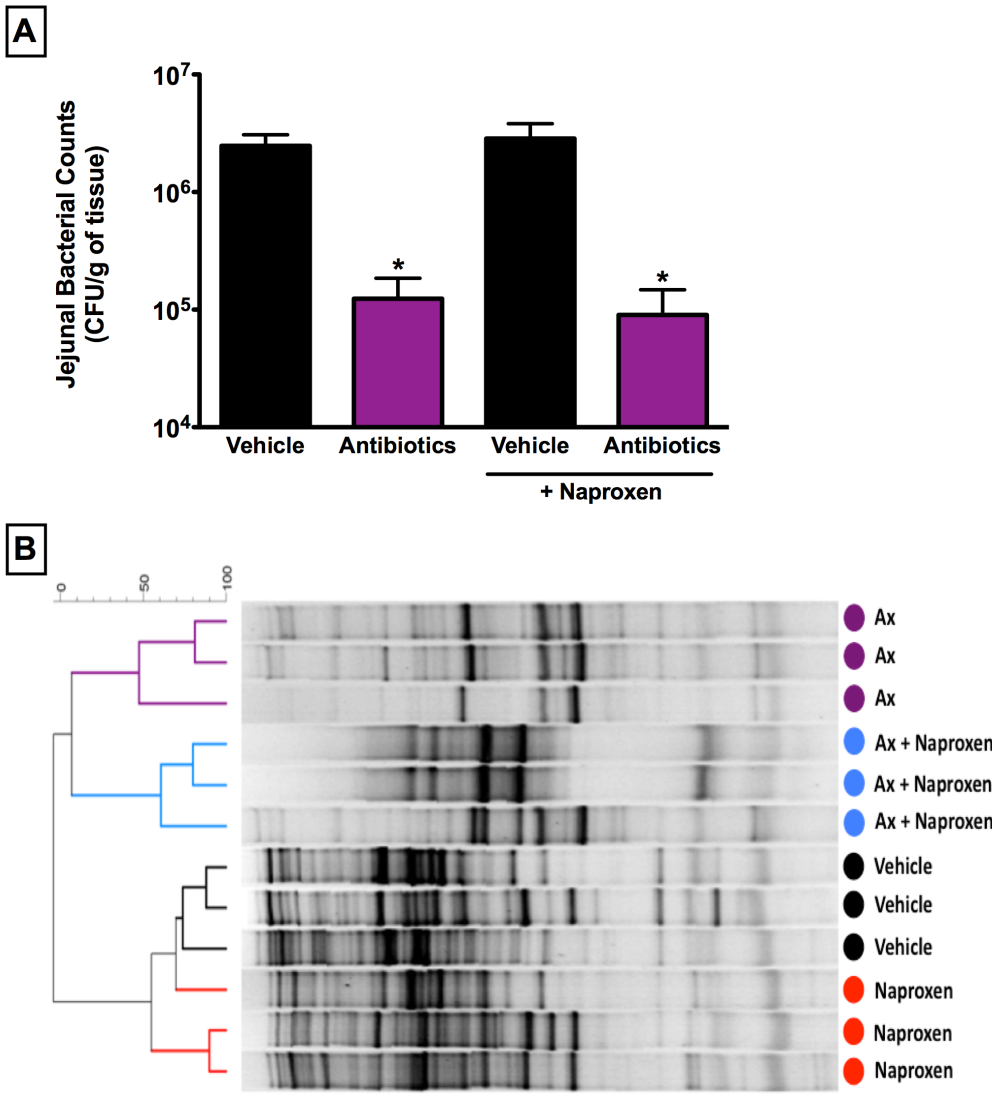

Figure 7.5. A broad-spectrum antibiotic mixture altered the intestinal microbiota. Panel A: Co-treatment with antibiotics (ampicillin, metronidazole, neomycin, and vancomycin) for 9 days significantly decreased the number of total aerobes in the jejunum of rats (*$p<0.05$; versus the vehicle- or naproxen-treated group). Panel B: Denaturing gradient gel electrophoresis analysis revealed that the administration of the antibiotic mixture caused cecal dysbiosis in rats. The clustering observed in the dendrogram was constructed using the Dice coefficient and Ward algorithm. Results in panel A are shown as mean ± SEM ($n\geq5$ per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

7.5. Discussion

NSAID-enteropathy is a major clinical concern because of the widespread use of NSAIDs and the lack of effective preventative and therapeutic agents to protect users from small intestinal damage (Wallace, 2013a). It has long been understood that enteric bacteria are critical in the pathogenesis of NSAID-enteropathy, since germ-free rodents do not develop intestinal damage (Kent *et al*., 1969; Robert & Asano, 1977). Moreover, NSAID administration results in changes to the type of bacteria present within the intestine (Hagiwara *et al*., 2004; Dalby *et al*., 2006). Additionally, blocking bacterial-host interaction (e.g., preventing TLR-4 activation) can reduce the NSAID-induced mucosal injury (Watanabe *et al*., 2008). These findings have prompted the idea of manipulating the bacterial flora or specifically targeting enteric bacteria to reduce and possibly prevent the incidence and severity of NSAID-enteropathy. Specifically, the use of antibiotics as a therapeutic option has been explored.

Several studies have demonstrated that specifically targeting gram-negative bacteria can protect rodents from NSAID-enteropathy (Koga *et al*., 1999; Watanabe *et al*., 2008). Koga *et al.* reported that kanamycin co-administration could dose-dependently reduce the intestinal damage and inflammation caused by indomethacin administration in rats. In our model of naproxen-induced enteropathy, kanamycin co-administration offered no intestinal protection and unexpectedly exacerbated naproxen-induced mucosal injury. Furthermore, co-treatment with kanamycin also significantly increased the extent of jejunal MPO activity in naproxen-administered rats. These results contradict the prevailing notion of the importance of gram-negative bacteria in the pathogenesis of

NSAID-enteropathy. I then targeted gram-positive bacteria via vancomycin administration to investigate how it would affect naproxen-enteropathy. Wantanabe *et al* (2008) reported that vancomycin administration conferred no protection to indomethacin-induced enteropathy in rodents. Indeed, I demonstrated similar results, as vancomycin co-treatment did not reduce naproxen-enteropathy. On the other hand, treatment with a mixture of antibiotics that provided broad-spectrum targeting of enteric bacteria significantly reduced the extent of naproxen-induced ulceration and intestinal inflammation in rats.

The vast majority of studies that indicated that antibiotic administration could protect against NSAID-enteropathy have administered a single dose, or limited doses, of indomethacin, a highly GI toxic NSAID (Kent *et al*., 1969; Koga *et al*., 1999; Watanabe *et al*., 2008). This modeling of NSAID-enteropathy may not adequately represent the repeated injury to the intestinal epithelium that occurs in chronic NSAID administration protocols and thus, may overestimate the beneficial effects of antibiotic co-administration. Indeed, our results with the kanamycin administration and repeated naproxen dosing protocol highlight the discrepant ability of antibiotics to protect against NSAID-enteropathy. Furthermore, it is possible that not all NSAID-induced (i.e., naproxen) intestinal damage is promoted by the presence of gram-negative bacteria.

The reduction in mucosal injury that I observed in rats that were co-administered a broad-spectrum antibiotic mixture and naproxen may be the result of numerous beneficial effects. Treatment with the broad-spectrum antibiotic mixture reduced the bacterial load in the small intestine, which likely limited the extent of interaction between

enteric bacteria and the epithelium. This may have limited bacterial colonization of NSAID-induced mucosal lesions, which would have impeded the healing of injured tissue (Elliott *et al*., 1998). Furthermore, once the mucosal barrier has been disrupted by NSAIDs, bacterial components are able to enter the tissue and activate pattern recognition receptors (e.g., TLRs) that trigger an inflammatory cascade and subsequent neutrophil recruitment, which can cause further mucosal injury (Scarpignato, 2008; Watanabe *et al*., 2008). This could account for the lack of granulocyte infiltration that was observed in naproxen-treated rats co-administered the broad-spectrum antibiotic mixture. On the other hand, treatment with the broad-spectrum antibiotic mixture caused a profound dysbiosis in rats and altered bile toxicity. It is possible that the antibiotic-induced changes in the microbiota prevented the growth of specific bacterial species that are associated with NSAID-induced intestinal injury (Hagiwara *et al*., 2004; Dalby *et al*., 2006; Kato *et al*., 2009). In chapters 3, 4, and 5, I demonstrated that the increased bile toxicity in NSAID-administered rats was associated with increased intestinal injury. Therefore, the reduction in mucosal injury observed in naproxen-treated rats co-administered the broad-spectrum antibiotic mixture may be partly explained by the lack of bile toxicity in this group. The levels of naproxen in the bile were similar between rats that were co-administered either vehicle or the antibiotic mixture, but bile toxicity may also affected by the type of bile acids that are present within the bile (Zhou et *al*., 2010). Enteric bacteria are responsible for the production of secondary bile acids, which are particularly damaging to epithelial cells due in part to the increased hydrophobicity of these bile acids (Hofmann, 1999; Martinez-Augustin *et al*., 2008). Therefore, the reduction in enteric flora caused by the

broad-spectrum antibiotic mixture may have reduced the concentration of secondary bile acids in the bile and in the lumen of the intestine.

In chapter 5, I demonstrated that the severity of naproxen-induced intestinal damage correlated well with the concentrations of naproxen in the bile after administration of naproxen to rats. The bacterial deconjugation of NSAID-glucuronides, by the enzyme bacterial β-glucuronidase, is necessary for the reabsorption of NSAIDs via enterocytes (Gadelle *et al*., 1985; LoGuidice *et al*., 2012). The co-administration of the broad-spectrum antibiotic mixture was unable to prevent the enterohepatic circulation of naproxen (i.e., the levels of naproxen in bile were unaltered by antibiotic treatment), which may explain why a substantial amount of intestinal injury still remained in this group. Likewise, the failure of kanamycin or vancomycin to protect against NSAID-enteropathy may also be due to the inability of these antibiotics to limit the enterohepatic circulation of naproxen.

In summary, the present data of the investigation of the ability of antibiotics to prevent NSAID-enteropathy highlights the difficulty of targeting enteric bacteria in order to limit intestinal injury. The enteric bacteria contribute to the pathogenesis of NSAID-enteropathy in multiple ways. Their critical role of bacteria in enterohepatic circulation, as well as in the conversion of primary to secondary bile acids, is important in promoting the acute injury and inflammation (i.e., topical damage of NSAIDs and bile to the epithelium). Once mucosal injury has occurred, enteric bacteria play an important role in exacerbating and/or perpetuating injury and inflammation. The reduction in naproxen-induced intestinal injury that was seen in rats following the co-administration of the

broad-spectrum antibiotic mixture was likely due to the diminished levels of bacteria in the intestine, which rendered a dampened inflammatory response upon injury. On the other hand, the inability of the broad-spectrum antibiotic mixture to prevent the enterohepatic circulation of naproxen likely contributed to the substantial intestinal injury that remained. Considering the lack of effectiveness of antibiotics in preventing NSAID-enteropathy and the potential for adverse effects and drug resistance associated with long-term antibiotic use, this therapeutic approach does not appear to be particularly attractive. Future studies may want to explore different ways of manipulating the bacterial flora, such as by using probiotics, or by specifically targeting the bacterial enzymes responsible for the enterohepatic circulation of NSAIDs.

– APPENDIX II –

UNPUBLISHED RESULTS: SUPPLEMENTARY MATERIAL

8.1. Introduction

It has been previously demonstrated that diallyl disulfide (DADS), along with various other garlic-derived compounds, are hydrogen sulfide (H_2S)-releasing agents (Benavides *et al*., 2007). Orally administrated DADS may encounter various oxidizing or reducing environments in the gastrointestinal (GI) tract following absorption. The release of H_2S from DADS occurs via a reduction reaction (Benavides *et al*., 2007). I investigated to what extent various reducing agents would promote the release of H_2S from DADS *in vitro*.

I also explored if the addition of naproxen or commercially purchased bile acids to bile collected from naïve rats would increase the cytotoxic effects of bile when exposed to rat epithelial cells *in vitro*. These experiments were conducted to explore if the direct, cytotoxic effects of naproxen and/or specific bile acids can affect bile cytotoxicity.

8.2. Methods

Hydrogen sulfide-release from DADS. The ability of DADS to release H_2S was measured using a modified version (Qu *et al*., 2006) of an assay first described by Stipanuk and Beck (1982). This method used zinc acetate to trap H_2S which was subsequently acidified with N,N'-dimethyl-p-phenylenediamine (NNDP) and ferric (III) chloride ($FeCl_3$) to produce methylene blue. The colour intensity of the methylene blue that formed was indicative of the concentration of trapped H_2S and was detected by spectrophotometry to represent relative levels of H_2S. This assay was performed using various concentrations of DADS (0.5 mL), buffer (0.4 mL) and various reducing agents

(L-cysteine, L-glutathione, or dithiothreitol (DTT)) at 1 mM (0.1 mL) added to 20 mL capacity vials. A smaller 2 mL tube containing a piece of filter paper soaked with zinc acetate (1%; 0.3 mL) was placed inside the larger vial. The larger vials were then flushed with nitrogen gas for 20 sec and capped with an airtight serum cap. The vials were transferred to a 37°C shaking water bath for 30 min. Samples were transferred to wet ice for 10 min, after which trichloroacetic acid (TCA; 50%; 0.4 mL) was injected into the reaction mixture through the serum cap. The mixture was then transferred to a 50°C shaking water bath for 60 min to allow for the trapping of evolved H_2S by the zinc acetate. The zinc acetate used to trap H_2S did not come into contact with the DADS solution at any point during the reaction. The serum cap was then removed and NNDP (20 mM; 50 μL) in 7.2 M HCl and $FeCl_3$ (30 mM; 50 μL) in 1.2 M HCl were added to the inner tube containing zinc acetate. Samples were left to stand in the dark for 20 min. 200 μL of each sample was plated and absorbance at 670 nm was measured with a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). A calibration curve of absorbance versus H_2S concentration was generated using sodium hydrosulfide (NaHS) of varying concentrations.

Addition of naproxen or bile acids to naïve rat bile. A bile duct cannulation was performed to collect naïve bile from healthy, untreated Wistar rats. The bile was then diluted 1:3, 1:6, 1:12, 1:24, and 1:48 immediately prior to incubation on IEC-6 cell cultures. To each dilution, naproxen (10-1000 μM) or commercially purchased bile acids (10-1000 μM) were added. The three bile acids added were either a conjugated, primary

bile acid [i.e., taurocholic acid (TCA)], a primary bile acid [i.e., cholic acid (CA)] or a secondary bile acid [i.e., deoxycholic acid (DCA)]. The bile solutions were then incubated on IEC-6 cells for 3 h. Following the incubation period, the supernatant was assayed for lactate dehydrogenase (LDH), as a measure of cell lysis. Refer to the methods section in Chapter 3.3 for further details.

Materials. Diallyl disulfide, dithiothreitol, L-cysteine, L-glutathione, sodium naproxen, sodium hydrosulfide, cholic acid, deoxycholic acid, and taurocholic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Colourmetric assay kits for LDH determination were purchased from G-Biosciences (St. Louis, MO, USA).

8.3. Results

Reducing agent required for hydrogen sulfide release from DADS. In the absence of a reducing agent, the release of H_2S from DADS *in vitro* was negligible at the concentrations tested (Figure 8.1). However, in the presence of reducing agents (L-cysteine, GSH, or DTT) the amount of H_2S released from DADS was substantially increased compared to DADS incubated in buffer alone.

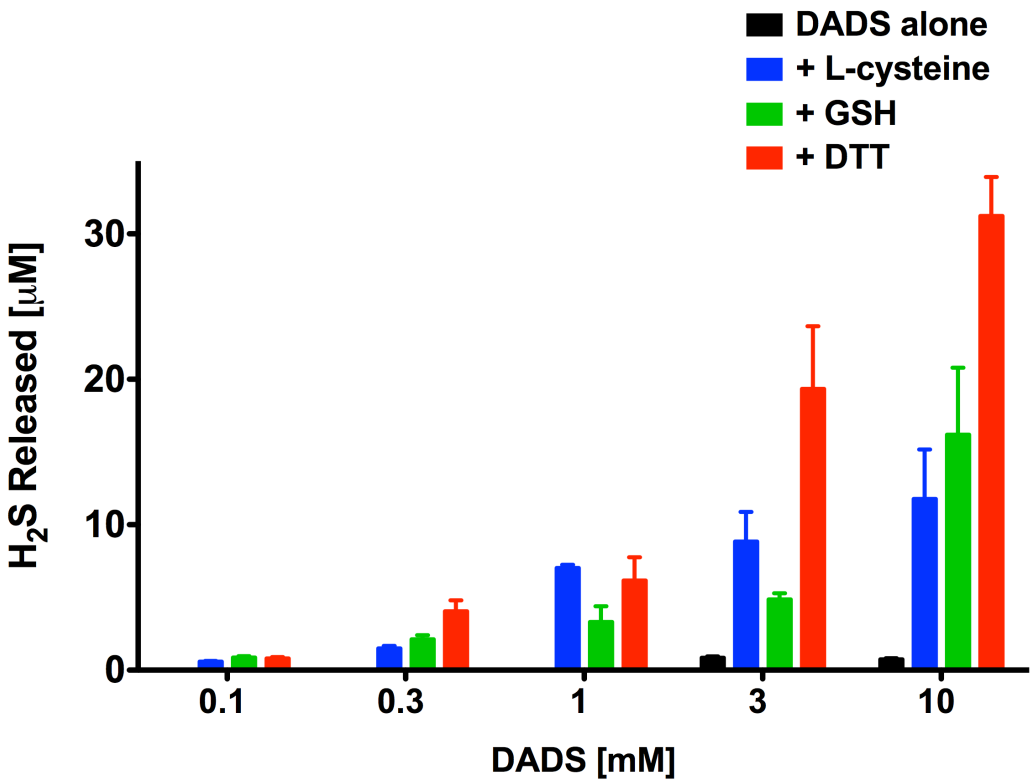

Figure 8.1. In vitro release of hydrogen sulfide (H_2S) from diallyl disulfide (DADS). At all concentrations tested, H_2S release from DADS in the absence of a reducing agent was negligible. However, when incubated with reducing agents (at 1 mM) [i.e., L-cysteine, glutathione (GSH), or dithiothreitol (DTT)], the release of H_2S from DADS was substantially greater than when DADS was incubated in buffer alone. Each bar represents the mean ± SEM (n=4 per group).

The addition of naproxen and bile acids enhanced the cytotoxic effects of bile. When tested at dilutions of 1:6 or 1:12, the addition of naproxen to bile collected from healthy, untreated rats enhanced the cytotoxic effects of the bile on rat intestinal epithelial (IEC-6) cells in a concentration-dependent manner (***$p<0.001$ vs. rat bile with no naproxen

added). Bile solutions were incubated on IEC-6 cells for 3 h and results for the 1:6 dilutions are shown in Figure 8.2.

The addition of DCA, a secondary bile acid, to naïve rat bile significantly enhanced the cytotoxic effects of bile at all concentrations of DCA tested (***$p<0.001$ vs. rat bile with no bile acids added) (Figure 8.3). On the other hand, the addition of CA, a primary bile acid, to naïve rat bile significantly enhanced the cytotoxic effects of bile in a concentration-dependent manner. At the two lower concentrations tested, the addition of DCA to bile resulted in significantly ($^{\delta\delta\delta}p<0.001$) enhanced bile cytotoxicity as compared to bile with CA added. Addition of the conjugated, primary bile acid (TCA) to naïve rat bile significantly enhanced bile cytotoxicity only at the highest concentration (1.5 mM) tested (**$p<0.01$ vs. rat bile with no bile acids added).

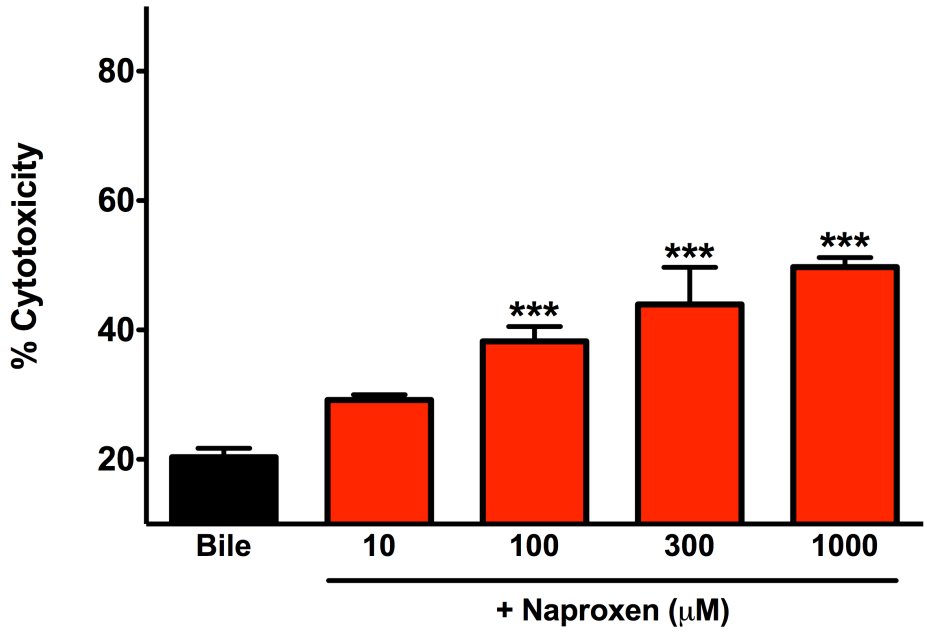

Figure 8.2. In vitro addition of naproxen to naïve rat bile enhanced bile cytotoxicity. The cytotoxic effects of bile collected from untreated (naïve) rats were concentration-

dependently enhanced by the addition of naproxen when incubated on rat intestinal epithelial (IEC-6) cells for 3 hours (***p<0.001 vs. rat bile with no naproxen added). The data shown are from the 1:6 dilutions and each bar represents the mean ± SEM (*n*=4 per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

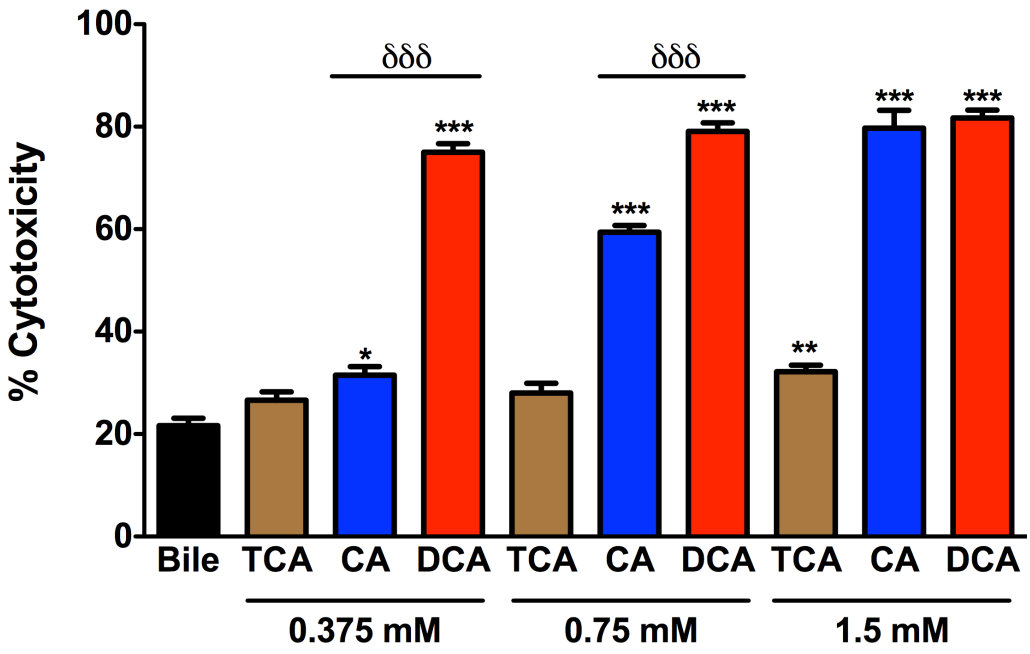

Figure 8.3. Secondary bile acids are cytotoxic to cultured rat epithelial cells in vitro. The addition of deoxycholic acid (DCA) to naïve rat bile significantly enhanced the cytotoxic effects of bile when incubated on rat intestinal epithelial (IEC-6) cells for 3 hours at all concentrations tested (***p<0.001 vs. rat bile with no bile acids added). The addition of DCA to bile resulted in significantly ($^{\delta\delta}$p<0.01; Student's t-test) enhanced bile cytotoxicity as compared to bile with CA added at the two lowest concentrations tested. The data shown are from the 1:6 dilutions and each bar represents the mean ± SEM (*n*=4

per group). The data were analyzed by a one-way analysis of variance followed by Dunnett's post-hoc test.

8.4. Discussion

The *in vitro* measurement of H_2S release from DADS suggests that DADS requires a reducing agent in order to release appreciable levels of H_2S. This may be particularly important for the ability of DADS to protect the small intestine from injury, as it appears that H_2S is not spontaneously released from DADS and thus, may allow for the compound to reach the sites of injury before expending its H_2S-releasing capacity. In fact, bile is a major source of GSH and it has been demonstrated that the delivery of GSH via bile to the small intestine protected intestinal epithelial cells from oxidative injury in rats (Lash *et al*., 1986; Aw *et al*., 1994). It is possible that the presence of GSH in the small intestine creates an ideal (i.e., reducing) environment for DADS to release H_2S *in vivo*, which may explain why DADS co-administration was particularly effective at reducing naproxen-inducing small intestinal injury.

Bile is a digestive secretion that plays a crucial role in the emulsification and solubilization of lipids, but it can also disrupt epithelial cellular membranes and cause cell death (Hofmann, 1999). Likewise, NSAIDs can damage intestinal epithelial cells and the combination of bile and NSAIDs is particularly damaging to epithelial cells (Dial *et al*., 2006; Zhou *et al*., 2010). Therefore, it is unsurprising that the addition of naproxen to rat bile significantly enhanced bile cytotoxicity *in vitro*. Interestingly, our *in vitro* results also suggest that the type of bile acids present within the bile can affect bile cytotoxicity.

When bile enters the small intestine, bacterial reactions can deconjugate, dehydroxylate, and dehydrogenate bile acids, all of which increase the hydrophobicity of bile acids (Begley *et al*., 2005). Deconjugated bile acids are more hydrophobic than conjugated ones, and secondary bile acids are more hydrophobic than primary bile acids. Hydrophobic bile acids are proficient at disrupting cellular membranes and causing cell death, which likely explains why I observed that DCA is particularly damaging to cultured IEC-6 cells (Hofmann, 1999).

In summary, naproxen and specific bile acids are able to significantly enhance the cytotoxic effects of bile *in vitro*. NSAID administration to rats may enhance bile cytotoxicity *in vivo* due to a number of factors; such as the drug itself within the bile or due to altered levels of specific bile acids, or a combination of both.

– APPENDIX III –

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– APPENDIX IV –

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