GI-SPARING NSAIDs AND COMPROMISED MUCOSAL DEFENCE

GASTROINTESTINAL-SPARING EFFECTS OF NOVEL NSAIDs IN RATS WITH COMPROMISED MUCOSAL DEFENCE

By

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ABSTRACT

Nonsteroidal anti-inflammatory drugs are among the most commonly used prescription and over-the-counter medications, but they often produce significant gastrointestinal ulceration and bleeding, particularly in elderly patients and patients with certain co-morbidities. Novel anti-inflammatory drugs are seldom tested in animal models that mimic the high-risk human users, leading to an underestimate of the true toxicity of these drugs. In the present study we examined the effects of two novel NSAIDs and two commonly used NSAIDs in models in which mucosal defence was expected to be impaired. Naproxen, celecoxib, ATB-346 (a hydrogen sulfide- and naproxen-releasing compound) and NCX 429 (a nitric oxide- and naproxen-releasing compound) were evaluated in healthy, arthritic, obese, hypertensive rats, and in rats of advanced age (19 months) and rats co-administered low-dose aspirin and/or omeprazole. In all models except hypertension, greater gastric and/or intestinal damage was observed when naproxen was administered in these models than in healthy rats. Celecoxib-induced damage was significantly increased when co-administered with low-dose aspirin and/or omeprazole. In contrast, ATB-346 and NCX 429, when tested at doses that were as effective as naproxen and celecoxib in reducing inflammation and inhibiting cyclooxygenase activity, did not produce significant gastric or intestinal damage in any of the models. These results demonstrate that animal models of human co-morbidities display the same increased susceptibility to NSAID-induced gastrointestinal damage as observed in humans. Moreover, two novel NSAIDs that release mediators of mucosal defence (hydrogen sulfide and nitric oxide) do not induce significant gastrointestinal damage in these models of impaired mucosal defence.

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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
BCE	Before common era
CMC	carboxymethylcellulose
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMSO	Dimethylsulphoxide
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GI	Gastrointestinal
h	Hour(s)
H_2RA	Histamine receptor antagonist
HOX	Hydroperoxidase
H&E	Hematoxylin and eosin stain
ip	Intraperitoneal
K_m	Michaelis constant - half-maximal rate of enzyme activity
ICAM-1	Intercellular adhesion molecule-1
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

LPS	Lipopolysaccharide
mM	Millimolar
mmHg	Millimetre(s) of mercury
ng/mL	Nanograms per millilitre
NCX 429	[(S)-6-(nitrooxy)hexyl 2-(6- methoxynaphthalen-2-yl)propanoate]
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Nonsteroidal anti-inflammatory drug
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
PO	Per os
PPI	Proton pump inhibitor
ROS	Reactive oxygen species
SEM	Standard error of the mean
SHR	Spontaneous hypertensive rat
TBZ	4-hydroxythiobenzamide
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
tNSAID	Traditional nonsteroidal anti-inflammatory drug
TXA_2	Thromboxane A_2
V_{max}	Maximal rate of enzyme activity
WKR	Wistar-Kyoto rat

DECLARATION OF ACADEMIC ACHIEVEMENT

Experiments were conceived and designed by Rory Blackler and John L. Wallace. Stephanie Syer contributed to the conception and design of the polypharmacy model. Manlio Bolla and Ennio Ongini contributed to the conception and design of the pharamacokinetic experiments. Rory Blackler and Webb McKnight performed all other experiments. Stephanie Syer helped perform the polypharmacy model. Rory Blackler, Manlio Bolla, Ennio Ongini, and John L. Wallace performed data analysis. Rory Blackler wrote this dissertation with contributions from John L. Wallace.

1.0 INTRODUCTION

1.1 General Introduction

Beginning with the advent of aspirin over one century ago, nonsteroidal anti-inflammatory drugs (NSAIDs) have become one of the most widely utilized classes of drugs, due in part to their potent anti-inflammatory, analgesic, and anti-pyretic properties. 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 orally, highly bound to plasma proteins, and excreted either by glomerular filtration or by tubular secretion (Burke et al., 2006). Historically, NSAIDs are classified into 6 distinct groups based on chemical structure (Wallace, 1992)(Burke et al., 2006): 1. salicylates (e.g., aspirin), 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., phenylbutzaone) 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 into the market (Wallace, 1999a). The strong anti-inflammatory and analgesic properties of NSAIDs have made them the first-line therapy for osteoarthritis and rheumatoid arthritis (Wallace, 2007). Moreover, they are efficacious in treating mild-to-moderate pain, such as menstrual cramps, gout, and headaches. Other clinical uses are also emerging, such as cancer chemoprevention, with numerous studies indicating that frequent use of aspirin and other tNSAIDs may reduce the risk of colon cancer and possibly other gastrointestinal (GI)-related cancers (Jacobs et al., 2007).

The wide range of therapeutic uses has subsequently made the world market for NSAIDs a multi-billion dollar industry, and one that continues to expand. Most notably, the market grew considerably following the introduction of selective COX-2 inhibitors and when prescribing low-dose aspirin to attenuate the incidence of serious cardiovascular events (such as stroke and myocardial infarction) became common practice. In terms of market share, prescription costs for NSAIDs in the United States in 2001 exceeded $4.8 billion, while the estimated cost of over-the-counter oral NSAIDs that year was $3 billion (Laine, 2001). The prevalence of at least once-weekly NSAID consumption among the elderly (>65 years old) has been reported as high as 70% and half of these individuals were taking NSAIDs daily (Scarpignato and Hunt, 2010). The associated costs and prevalence of NSAID use are liable to expand as the populations of developed countries age. This is due to a concomitant increase in the prevalence of age-related diseases, such as osteoarthritis.

Despite their popular clinical use and strong efficacy in treating pain and inflammation, NSAIDs have a relatively high incidence of adverse effects. The major limitation to NSAID use is the associated GI toxicity. NSAIDs induce clinically significant ulceration and bleeding in approximately 2-4% of patients chronically taking these drugs (Silverstein et al., 2000). Moreover, NSAID use can be associated with symptoms of nausea, dyspepsia, and abdominal pain. Important risk factors for NSAID-associated upper GI clinical events include older age (≥60 years), prior history of peptic ulceration, concomitant use of anticoagulants (including low-dose aspirin) and/or corticosteroids, and the use of high-dose or multiple NSAIDs (Laine, 2006). Although selective COX-2 inhibitors, such as rofecoxib and celecoxib, cause severe GI

complications less frequently than tNSAIDs (non-selective), they are not devoid of GI-damaging effects and can adversely effect other regions of the body. For instance, "at-risk" patients 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 renal and cardiovascular adverse events (Cheng and Harris, 2004)(Kearney et al., 2006). Although modest improvements have been made in terms of NSAID-associated GI toxicity (i.e., development of selective COX-2 inhibitors), concerns still remain regarding GI and cardiovascular toxicity, which remain the major limitations to the use of these drugs. These limitations have prompted much research, both experimental and clinical, into understanding the mechanisms of NSAID-induced adverse events. A clearer understanding of these mechanisms may provide the necessary clues to develop GI- and cardiovascular-sparing NSAIDs. In this chapter, an emphasis will be made on the pathogenesis of NSAID-induced gastroduodenal and intestinal damage, along with brief summaries on the following subjects: the history of NSAIDs, biosynthesis of prostaglandins and their inhibition by NSAIDs, selective COX-2 inhibitors and the contributions of COX-1 and -2 to mucosal defence, and the therapeutic potential of novel hydrogen sulfide- and nitric oxide-releasing NSAIDs. The chapter will conclude with the objectives addressed in this thesis.

1.2 History of NSAIDs

The history of aspirin, the original NSAID, can be traced back to herbal folklore on plant extracts (e.g., willow bark and leaves) used to relieve pain and fever (Vane, 1990). As far back as 400 BCE, Hippocrates, widely regarded as the father of modern

medicine, left records indicating the practice of using willow bark concoctions for the treatment of rheumatic diseases, fever, and pain (Vane, 1990). However, this practice was most clearly documented in 1763, when the first reported "clinical trial" of willow bark administration was published. The study demonstrated that patients presenting with ague (fever) were successfully treated with a willow bark medicament (Stone, 1763). In spite of these early findings, it was not until 1829, that the active ingredient of willow bark was isolated and crystallized by French pharmacist Leroux (Burke et al., 2006). This compound was named salicin and was first synthesized by Kolbe in 1859 (Burke et al., 2006). The ensuing production of synthetic salicins (i.e., salicylic acid and sodium salicylic) began in 1874 and provided improved efficacy and solubility properties over that of isolated salicin. By the mid 1870s, synthetic salicin was a popular drug for the treatment of rheumatic fever in Europe (Vane, 1990). Although the drug was efficacious in treating pain and fever, patients complained of the strong bitter taste. This may have provided the simple impetus for a young chemist to create the first synthetic NSAID. Felix Hoffman, an employee of Bayer Corp., first synthesized acetylsalicylic acid (i.e., aspirin) from salicylic acid through an acetylation reaction in hopes of alleviating the bitter taste of salicylic acid (Vane, 1990). In 1899, the compound was named "aspirin" and formally introduced by Hermann Dreser, the chief pharmacologist at Bayer Corp. (Wallace, 1997a). This new drug was reported as an effective way of delivering salicylic acid to the body and demonstrated analgesic, antipyretic, and anti-inflammatory properties (Vane, 2000).

Despite decades of widespread aspirin use and the advent of other numerous NSAIDs (e.g., indomethacin and diclofenac), it was not until 1971 that the mechanism of

action of NSAIDs was discovered. Sir John Vane and colleagues were credited with the discovery that NSAIDs produce their anti-inflammatory effects by inhibiting the enzymatic production of prostaglandin synthesis (Vane, 1971). In addition, his studies demonstrated that aspirin itself had pharmacological properties distinct from those of salicylic acid, and does not simply act as a pro-drug that dissociated to salicylic acid in the body (Wallace, 1997a). The first evidence to emerge that NSAIDs could damage the stomach was reported by two English clinicians in 1938, based on their gastroscopic observations of patients taking aspirin (Douthwaite and Lintott, 1938). During the following decades, case reports of melena (dark, rank stools) associated with aspirin use began to compile in the literature (Wallace, 2007). However, it was not until the 1970s that larger studies documented the increasingly clear relationship between NSAID use and both gastric and duodenal ulcer formation (Levy, 1974). At the time, improved recognition of the adverse gastrointestinal effects of NSAIDs was likely prompted by the enhanced potency of NSAIDs (e.g., indomethacin and fenamates) and an increased ability to visualize the inside of the gastrointestinal tract, via flexible endoscopy (Insel, 1990).

1.3 Prostanoids, Cyclooxygenase, and NSAIDs

1.3.1 Eicosanoids

Eicosanoids are potent lipid mediators for numerous homeostatic biological functions and inflammation (Funk, 2001). The eicosanoid family consists of several arachidonate metabolite groups, including prostaglandins (PGs), prostacyclin, thromboxane A_2 (TXA_2), leukotrienes, lipoxins, and hepoxylins (Smyth et al., 2006). Eicosaoids are derived from precursor essential 20-carbon fatty acids containing multiple double bonds (Smyth et al., 2006). In mammalian systems, the most abundant precursor is

arachidonic acid (AA), which is supplied by cell membrane lipids (Smyth et al., 2006). Eicosanoids are not stored, but rather produced in response to a variety of physical, chemical, and hormonal stimuli, that activate acyl hydrolases (most notably phospholipase A_2) to make arachidonate available (Funk, 2001). The availability of this substrate is the limiting factor in the biosynthesis of eicosanoids (Smyth et al., 2006). Once liberated, AA is metabolized rapidly to oxygenated products by several distinct enzyme systems, including the cyclooxygenase (COX) isozymes. Eicosanoids operate in a hormone-like fashion, often acting in an autocrine and paracrine manner, in the local cellular milieu (Funk, 2001). Although the eicosanoid family includes several groups of lipid mediators, the following section will deal exclusively with the prostanoids.

1.3.2 Biosynthesis of Prostanoids

The biosynthesis of PGs and TXA_2, collectively known as prostanoids, occurs in a stepwise manner (Figure 1): 1. release of AA from the activated cell membrane by phospholipases 2. cyclooxygenation and hydroperoxidation of free AA by prostaglandin endoperoxide G/H synthases (colloquially known as COXs) and 3. metabolism of prostaglandin H_2 (PGH_2) by tissue specific isomerases to biologically active prostanoids. Much like the other eicosanoids, prostanoids are synthesized in response to cellular stimuli, such as mechanical stress, growth factors (e.g., epidermal growth factor), hormones (e.g., antiduretic hormone), and inflammatory stimuli. PGs both sustain homeostatic functions and mediate inflammatory processes, including the initiation and resolution of inflammation (Ricciotti and Fitzgerald, 2011).

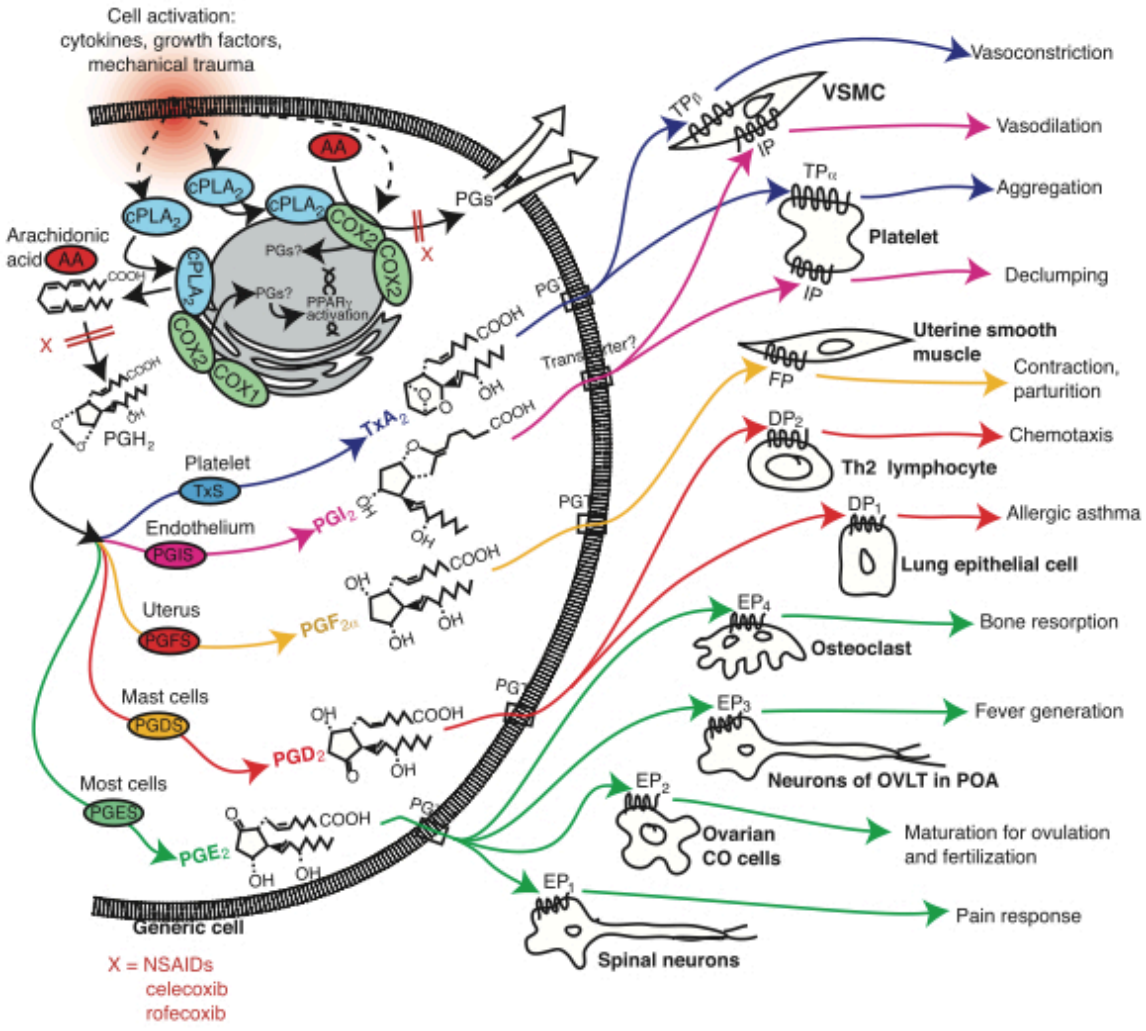

Figure 1. Prostaglandin synthesis and actions. A "generic cell" is activated by cellular stimuli, such as mechanical trauma or inflammatory stimuli, triggering the activation of phospholipases. The phospholipases release arachidonic acid (AA) from membrane lipids and COX-1 or COX-2 metabolizes AA to the intermediate PGH_2. In a cell-type restricted fashion, specific isomerases metabolize PGH_2 to biologically active prostanoids. These prostanoids may then exert paracrine or autocrine actions on a family of prostaglandin receptors to mediate a diverse number of physiological effects. "X" marks the site of inhibition by NSAIDs. (Figure credit: Funk, 2001).

In the GI tract, PGs help modulate virtually all aspects of mucosal defense, such as the secretion of luminal factors, maintenance of mucosal blood flow, and the acceleration of ulcer healing (Wallace, 2008b). Their production is ubiquitous, but generally each cell type synthesizes one or two principal PG products. For instance, COX-1-derived TXA_2 is the dominant product in platelets, whereas COX-2-derived PGE_2 and TXA_2 predominate in activated macrophages (Smyth et al., 2006). PGs are continually produced in order to help maintain homeostasis in the body, although during an inflammatory response both the level and the profile of PG production change dramatically (Ricciotti and Fitzgerald, 2011). PG production is reliant 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) to oxidize AA to PGG_2 and hydroperoxidase (HOX) to reduce PGG_2 to PGH_2 (Smyth et al., 2006). The PGH_2 produced is the chemically unstable precursor for the formation of all prostanoids (Funk, 2001). The final step in the formation of prostanoids is reliant on the coupling of PGH_2 synthesis to downstream isomerases or synthases that are intricately orchestrated in a cell-specific fashion (Funk, 2001). In vivo, there are four main bioactive PGs and one thromboxane group generated (Funk, 2001). Prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) are formed 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 formed. Both PGI_2 and TXA_2 are unstable, active intermediates, which are broken down

non-enzymatically to the biologically inactive compounds 6-keto-PGF_{α} and TXB_2, correspondingly (Smyth et al., 2006).

Once generated, prostanoids may undergo facilitated transport from the cell through a known prostaglandin transporter (PGT) or other carriers to exert their actions locally on a variety of specific membrane receptors (Schuster, 2002). Prostanoid receptors can be classified into 5 different groups; designated by the same letter as the natural prostanoid with the greatest affinity (Smyth et al., 2009). One receptor has been identified for each of TXA_2, PGI_2, and PGF_2 (TP, IP, and FP, respectively), while four distinct PGE_2 receptors ($EP_{1\text{-}4}$) and two PGD_2 receptors (DP_1 and DP_2) have been identified (Smyth et al., 2009). All eicosanoid receptors are G protein-coupled receptors that interact with G_s, G_i, and G_q to modulate the activities of adenylyl cyclase and phospholipase C (Smyth et al., 2009). G-protein activation results in the generation of secondary messengers that help amplify the original receptor signal and mediate cellular effects (Smyth et al., 2009). To conclude, the complex and widespread biosynthesis of prostanoids underscores their important physiological and pathophysiological actions. In the following sections many prostanoid functions will be discussed in a GI context.

1.3.3 Cyclooxygenase Isozymes

As previously stated, two isoforms of cyclooxygenase have been identified, COX-1 and -2. COX-1 was originally identified in the mid 1970s and cloned in 1988 (Hemler et al., 1976)(Vane et al., 1998). It was postulated as early as 1972 that a second COX isoform existed, but not until 1991 was its existence confirmed by two separate groups (Kujubu et al., 1991)(Xie et al., 1991). The COX isozymes are inserted predominately in the endoplasmic reticulum (ER) and nuclear membrane with their binding pocket exposed

to pick up free AA nearby (Crofford, 1997). Biochemically, COX-1 and -2 display comparable enzymatic function when AA is used as a substrate (i.e., similar V_{max} and K_m)(Crofford, 1997). In addition, the structure of both enzymes is extraordinarily analogous, with only one significant amino acid difference leading to a larger "side pocket" for substrate access in COX-2 (Smith et al, 2000). In spite of the structural similarities, COX-2 will accept a wider range of fatty acid substrates (e.g., eicosapentaenoic acid and linoleic acid) and bind these substrates more efficiently than COX-1 (Vane et al., 1998). In terms of COX tissue localization, COX-1 is expressed constitutively in most cells, whereas COX-2 is upregulated by cytokines, shear stress, and growth factors. Thus, in simplistic terms COX-1 is generally regarded as the housekeeping, basal enzyme responsible for homeostatic PG levels such as maintaining mucosal blood flow in the GI tract (Funk, 2001). On the other hand, COX-2 is important in various inflammatory and "induced" settings, such as cancer (Funk, 2001). There are notable exceptions to this over-simplification, and it is important to remember that both isozymes contribute to the physiological and pathophysiological prostanoid production.

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 elucidated this mechanism of action in 1971; when they demonstrated that low concentrations of aspirin and indomethacin inhibited the enzymatic production of PGs. NSAIDs inhibit PG production by acting as reversible (excluding aspirin), competitive inhibitors of cyclooxygenase activity. They do not inhibit the lipooxygenase pathways of AA metabolism and hence do not suppress leukotriene formation (Burke et al., 2006). All NSAIDs inhibit COX by interacting with

the bis-oxygenase subunit, as a result preventing the introduction of molecular oxygen and cyclization of AA. Although they compete directly with AA for binding to the COX site (inhibiting cyclooxygenase activity), they have little effect on the peroxidase activity of the enzyme (Smith et al., 2000).

Two general points can be made on the mechanism by which NSAIDs inhibit cyclooxygenase activity. First, there are two classes of NSAIDs: (1) tNSAIDs (non-selective for COX isoforms) and (2) selective COX-2 inhibitors. All tNSAIDs can inhibit COX-1 and -2 but in general bind more tightly with COX-1 (Smith et al., 2000). As their name suggests, selective COX-2 inhibitors exhibit selectivity toward COX-2. Second, while all NSAIDs compete with AA for the cyclooxygenase active site, they can exhibit one of three modes of inhibition: (a) rapid, simple, reversible competitive inhibition (e.g., ibuprofen and naproxen); (b) rapid, lower affinity, reversible binding followed by time-dependent, higher affinity, slowly reversible binding (e.g., indomethacin and flurbiprofen); (c) rapid, reversible binding followed by irreversible, covalent modification (acetylation) (e.g., aspirin) (Smith et al., 2000). NSAIDs that exhibit the first mode (a) of inhibition do not modify the conformation of COX (i.e., non-covalent modification) and increasing the availability of AA can restore the enzymatic activity (Burke et al., 2006). The second mode (b) of inhibition results in an enzyme-inhibitor complex and a resulting conformational change in the COX protein over time. It is important to note that this conformational change is not a covalent interaction and thus, allows the COX protein to slowly (time-dependent) revert back to its original state and re-establish its PG synthesis abilities (Smith et al., 1996). The third mode (c) of inhibition, exclusive to aspirin, involves the covalent modification (an irreversible conformation change) of COX-1 and -

2 by the acetylation of Ser530 at position 530 and 516 on each isozyme, respectively (Smith et al., 2000)(Burke et al., 2006). The resulting acetyl group prevents AA from accessing the active site by protruding into the binding space, permanently inactivating the enzyme. The modified COX enzyme cannot therefore synthesize prostanoids even after the drug is removed. However, the effect of aspirin and salicylates on COX-2 differs from that of COX-1. Acetylated COX-2 will still oxidize AA but to 15-hydroxyeicosatetraenoic acid (15-HETE) instead of PGH_2, whereas acetylated COX-1 will not oxidize AA at all (Lecomte et al., 1994). The 15-HETE may still undergo metabolism by 5-lipoxygenase to yield 15-epilipoxin A4, which has potent anti-inflammatory properties (Serhan and Oliw, 2001). Indeed, the ability to acetylate COX-1 is the basis for the unique, long-lived cardioprotective effects of aspirin on platelet aggregation because circulating platelets, unlike most cells, do not synthesize new COX-1 enzymes to replace the deactivated, acetylated enzymes (Smith et al., 1996). Of note, all selective COX-2 inhibitors cause a time-dependent inhibition of COX-2 but not COX-1. They exhibit COX-2 selectivity because of their mixed mode of inhibition; inhibiting COX-2 in a time-dependent, reversible conformational change manner, whereas they inhibit COX-1 by a rapid, competitive, reversible mechanism (Smith et al., 2000).

1.5 Selective COX-2 Inhibitors

After the initial discovery COX-2 in 1991, it was subsequently shown that the COX-2 isoform was expressed at markedly high levels at sites of inflammation while only low levels of expression could be found in healthy tissues (Vane et al., 1994). An enticing theory quickly emerged and captured the imagination of the pharmaceutical world. Subsequently, a vast amount of resources were dedicated to the development of

novel NSAIDs that exhibit COX-2 selectivity. The theory was simple: inhibit the inducible COX-2 isoform only (sparing COX-1) and these novel NSAIDs would reduce fever, pain, and inflammation, while sparing the gastrointestinal tract of injury. However, this theory was reliant on two central suppositions: 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 gastrointestinal homeostasis (Wallace, 1999a). Using the framework of this theory, it was perceived that NSAID-induced GI toxicity was due to a lack of selectivity of tNSAIDs for COX-1 and -2 at clinically effective doses (Wallace, 1999a). Numerous selective COX-2 inhibitors were created based on this elegant theory, including celecoxib, rofecoxib, and valdecoxib. Initially, it was anticipated that these selective COX-2 inhibitors would abolish NSAID-related GI toxicity. However, in clinical use, it soon became apparent that selective COX-2 inhibitors only reduce, but do not eliminate, gastroduodenal damage (Laine et al., 2003a)(Lanas et al., 2007). The failure to abolish GI toxicity is partly explained by the fact that at clinically effective doses in humans, selective COX-2 inhibitors were inhibiting the synthesis of COX-2 derived PGs as well as suppressing COX-1 derived PGs (Wallace, 1999a).

Other major concerns are associated with the use of selective COX-2 inhibitors and tNSAIDs, including significant cardiovascular and renal toxicities (Wallace, 2008). In fact, the heightened cardiovascular 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 incidence of cardiovascular events (i.e., myocardial infarction and stroke), patients taking selective COX-2 inhibitors

are often co-prescribed low-dose aspirin (Kearney et al., 2006). Somewhat ironically, this abolishes any beneficial effects the patient would have gained by using selective COX-2 inhibitors over tNSAIDs in terms of GI toxicity (Laine et al., 2003b).

1.6 Contributions of COX-1 and -2 to Mucosal Defence

Although the 'selective COX-2 inhibitor' theory proved incorrect, it provided an impetus or greater understanding of the pathogenesis of NSAID-induced gastroduodenal damage. For instance, the advent of selective COX-2 inhibitors helped unearth evidence that COX-1 and COX-2 have overlapping roles in the maintenance of the GI tract. COX-1 contributes the majority of PGs produced by the healthy stomach, but plenty of evidence indicates that the production of COX-2-derived PGs substantially increases following mucosa damage (Gretzer et al., 2001), periods of ischemia (Maricic et al., 1999), or when COX-1 is inhibited (Davies et al., 1997). The up-regulation of COX-2-derived PGs after these insults appears important in fortifying mucosal defense mechanisms (e.g., increasing blood flow) and enhancing injury repair (e.g., ulcer healing) (Smith and Langenbach, 2001)(Ma et al., 2002). Thus, due to the overlapping roles of COX-1 and -2 in mucosal defence, selective inhibition of COX-1 or COX-2 is unlikely to produce significant gastroduodenal damage. Indeed, it has been demonstrated in rats that NSAID-induced gastroduodenal damage requires the inhibition of both COX-1 and -2 (Wallace et al., 2000a). The concept that mucosal defense is dually mediated by both COX-1 and -2 is further exemplified by studies in mice where one of the isozymes has been genetically altered. For example, COX-1-deficient mice have low endogenous levels of gastric mucosal PG synthesis, but surprisingly do not spontaneously develop gastric ulcers (Langenbach et al., 1995). Also, COX-2-deficient mice demonstrate an impaired ability to

resolve inflammation, suggesting that COX-2 is not only a source of inflammatory PGs but also an important contributor to the production of anti-inflammatory mediators (Wallace, 2008). Although COX-1 and -2 play important roles in the gastrointestinal tract, the inhibition of these isozymes by NSAIDs cannot fully explain NSAID-induced GI toxicity. The following two sections address other important mechanisms of NSAID-induced gastroduodenal injury and enteropathy, and how they differ.

1.7 Pathogenesis of NSAID-induced Gastroduodenal Injury

To date, the most important adverse effects of NSAID use have been the ulceration and bleeding of the upper GI tract following chronic administration of these drugs. NSAID administration can often lead to superficial erosions primarily in the corpus region of the stomach and ulcerations (i.e., penetration through the muscularis mucosa) in the antral region (Sostres et al., 2010). Undoubtedly, the latter is more clinically relevant, given that ulcers are more likely to perforate and bleed (McCarthy, 1990). The bleeding is partly attributable to the inability of platelets to aggregate in acidic environments (i.e., pH <4) (Green et al., 1978). The mechanisms by which NSAIDs produce gastroduodenal ulceration and bleeding can be divided into two broad categories: (1) the local, topical damaging actions on the epithelium and (2) the systemic actions. The following figure depicts the primary contributing mechanisms to NSAID-induced gastroduodenal injury (Figure 2):

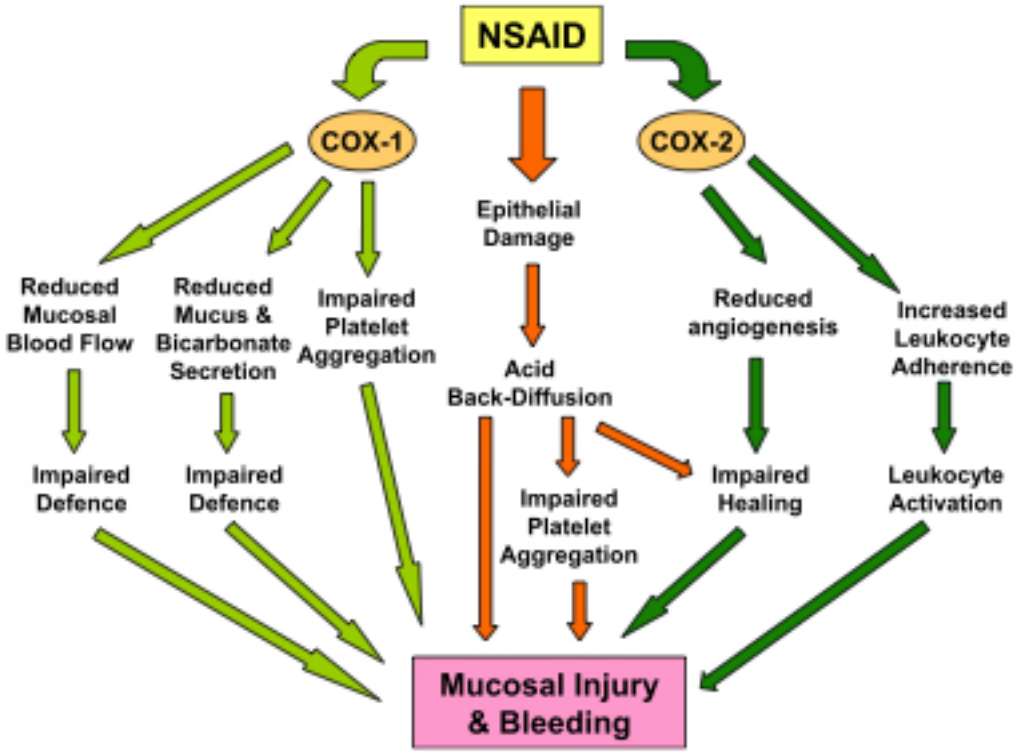

Figure 2. Pathogenesis of NSAID-induced gastric injury and bleeding. NSAIDs induce injury/bleeding via their direct, cytotoxic effects on the local epithelium and the systemic inhibition of cyclooxygenase (COX) activity. It is important to note that the effects elicited via only one of these damaging pathways (e.g., selective inhibition of COX-1 or COX-2) are unlikely to produce clinically significant damage. (Figure credit: Wallace, 2008).

The topical actions of NSAIDs on the epithelium involve several mechanisms. As previously mentioned, many NSAIDs are organic acids, and thus can theoretically kill epithelial cells if they come in direct contact (Tarnawski et al., 1988). For instance, it has been suggested that charged NSAIDs (due to stomach acidity) can become trapped within epithelial cells and induce osmotic lysis, subsequently leading to the uncoupling of

oxidative phosphorylation and cell death (Somasundaram et al., 1995). NSAIDs can also directly render the mucosa susceptible to luminal acid damage by disrupting the layer of surface-active phospholipids on the mucosal surface (Giraud et al., 1999)(Lichtenberger et al., 2006). Lastly, NSAIDs can directly inhibit epithelial repair by interfering with epithelial growth factor (EGF) signaling pathways, which are important in epithelial cell proliferation (Kajanne et al., 2007)(Pai et al., 2001). While the topical damaging effects of NSAIDs likely contribute to NSAID-induced gastroduodenal damage, they are unlikely to produce significant damage on their own. In fact, evidence from various studies demonstrates that topical exposure of the gastroduodenal mucosa to NSAIDs is not necessary for ulcer formation. For example, parenteral administration of NSAIDs can elicit gastric ulcers (Estes et al., 1993)(Wallace and McKnight, 1993b). Further downplaying the role of NSAID-induced topical damage to the gastric mucosa are the observations that enteric-coated and/or prodrug NSAID formulations exhibit comparable incidences of gastric ulceration and bleeding to that of orally administered NSAIDs (Graham et al., 1985)(Wallace, 2008). Conversely, considerable evidence exists that the systemic effects (i.e., suppression of mucosal PG synthesis) is the primary mechanism of action by which NSAIDs damage the gastroduodenal mucosa. Indeed, the extent to which various NSAIDs inhibit mucosal PG synthesis correlates very well with their ability to induce gastroduodenal damage (Whittle, 1981)(Rainsford and Willis, 1982). A strong temporal correlation is also evident between the first signs of gastroduodenal damage and the suppression of mucosal PG synthesis (Whittle, 1981) (Wallace, 2008). However, the suppression of gastric PG synthesis does not guarantee ulceration but rather leads to mucosal susceptibility (Ligumsky et al., 1983)(Wallace et al, 2000a). As previously

stated, PGs are important modulators of mucosal defense and as such, their suppression by NSAIDs leads to a weakened mucosal defense. For example, NSAIDs impair protective gastric mucus and bicarbonate secretions, mucosal blood flow, and inhibit epithelial repair (Wallace, 2008). Without mucosal PGs, the mucosa is rendered vulnerable to the damaging effects of luminal agents, such as gastric acid, pepsin, ethanol, and even NSAIDs themselves (Wallace, 2008). The damaging effects of gastric acid secretion in the pathogenesis of NSAID-induced gastroduodenal injury is highlighted by the clinical effectiveness of histamine receptor antagonists (H_2RAs) and proton pump inhibitors (PPIs) in reducing upper GI tract bleeding and ulcerations (Wallace, 2008). Another critical event involved in NSAID-induced gastroduodenal injury is the increase in leukocyte adherence (primarily neutrophils) to the vascular endothelium shortly after NSAID administration and the potentiating role of the cytokine tumor necrosis factor-α (TNF-α) in this pathway. It has been observed that rats made neutropenic through treatment with an anti-neutrophil antibody do not develop hemorrhagic lesions upon NSAID administration (Wallace et al., 1990). Furthermore, pre-treating rats with specific monoclonal antibodies that prevent leukocyte adherence to vascular endothelium significantly attenuates NSAID-induced gastric damage (Wallace et al., 1993c). Increased leukocyte adherence to the gastric endothelium could contribute to gastric mucosal injury in two major ways (Wallace and Granger, 1999b). Firstly, adhered neutrophils are likely activated and thus capable of inducing cellular injury via the release of reactive oxygen metabolites and proteases (Vaananen et al., 1991). Secondly, neutrophil adherence in the microcirculation could obstruct mucosal blood flow, thereby furthering mucosal susceptibility (Wallace and Granger, 1999b). The release of TNF-α potentiates leukocyte

adherence due to its ability to potently stimulate intercellular adhesion molecule-1 (ICAM-1) expression on the gastric vascular endothelium. While TNF-α helps mediate leukocyte adherence, other mediators may be just as important in this process. The suppression of PGI_2, which is an important inhibitor of neutrophil activation and adherence, may partly contribute to the increased neutrophil adherence witnessed after NSAID administration (Wallace, 1992). In addition, two endogenously produced gaseous mediators (i.e., nitric oxide (NO) and hydrogen sulfide (H_2S)) have been shown to reduce leukocyte-endothelial cell adhesion in the gastric vasculature (Wallace et al., 1997b)(Zanardo et al., 2006). The ability to reduce leukocyte-endothelial cell adhesion may partially explain why both NO and H_2S are able to prevent or reduce NSAID-induced gastroduodenal injury.

1.8 Pathogenesis of NSAID-induced Enteropathy

The ability of NSAIDs to cause significant bleeding and ulceration in the stomach and duodenum is well recognized (Wallace, 2008). Likewise, the mechanisms responsible for these events are well characterized and numerous therapies have been developed to help curtail the incidence of gastroduodenal damage (e.g., PPIs). On the other hand, the ability of NSAIDs to cause intestinal damage remains less appreciated (Wallace, 2012). It was not until 1993 that it became clear NSAID use was also associated with significant damage to the distal regions of the small intestine (Bjarnason et al., 1993). It remains a challenge to examine and document NSAID-induced intestinal damage in patients, despite the improved ability to explore the entirety of the intestine (e.g., use of video capsule endoscopy). Complicating intestinal evaluation is the time it takes for the NSAID enteropathy to manifest. Unlike gastroduodenal damage, NSAID enteropathy occurs over

a much longer period of time and the analgesic properties of NSAIDs themselves often mask the patient's symptoms (Wallace, 2012). Because of these difficulties, NSAID enteropathy remains largely overlooked in clinical studies. This is a troubling trend since the small bowel may be a more common site for NSAID-induced damage than the stomach (Scarpignato and Hunt, 2010). As demonstrated by numerous studies using video capsule endoscopy, the incidence of small intestinal damage in healthy volunteers taking NSAIDs plus PPI over a 2-week period was between 55-75% (Goldstein et al., 2005)(Fujimora et al., 2010). Moreover, the past decade has witnessed a decrease in NSAID-related upper GI complications versus an increasing trend in both the number of lower GI complications and their clinical severity (Lanas et al., 2009). The majority of insight into the mechanisms of NSAID-induced enteropathy has been obtained from animal studies. From these studies, it appears that NSAID enteropathy is multifactorial and that a clear, unifying hypothesis (akin to NSAID gastropathy) may not adequately explain the damage process. Indeed, it is unmistakable that the mechanisms responsible for NSAID-induced enteropathy are distinct from that of NSAID gastropathy (Wallace, 2012). The following figure highlights key events in the development of NSAID enteropathy (Figure 3):

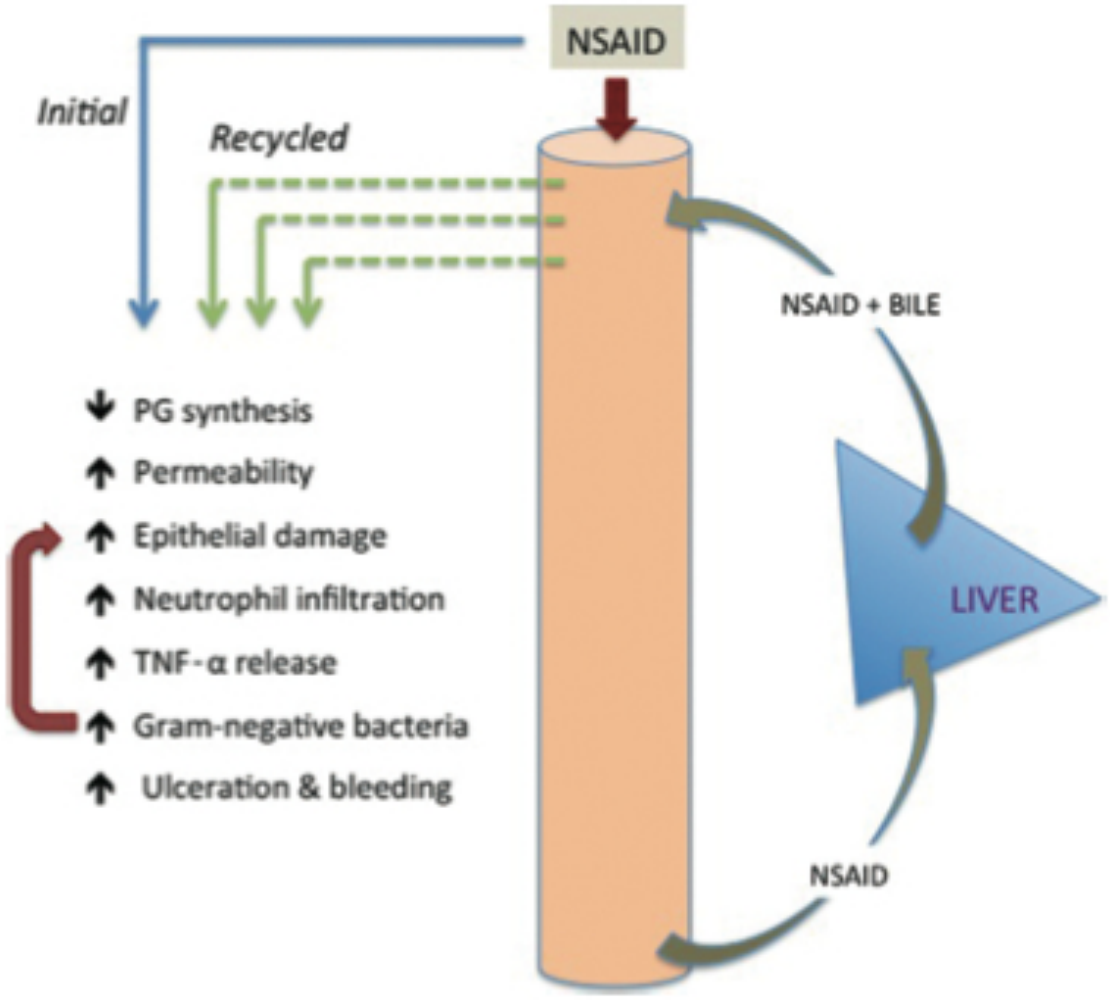

Figure 3. Pathogenesis of NSAID enteropathy. Inhibition of prostaglandin (PG) synthesis occurs with all NSAIDs and renders the intestinal mucosa susceptible to damage. However, COX inhibition does not appear to be the primary mechanism of damage. Initially, NSAIDs can increase intestinal permeability and cause direct epithelial damage. Once the injury process has commenced, infiltrating neutrophils and TNF-α release likely contribute to tissue injury. However, only NSAIDs that undergo enterohepatic recirculation will cause significant ulceration. It is likely that NSAIDs combined with bile exhibit a much greater capacity to damage the tissue and exacerbate the above-mentioned mechanisms. Furthermore, the increase in gram-negative bacteria is

a result of NSAID enterohepatic circulation and particularly important for the generation of ulcers. (Figure credit: Wallace, 2012).

Similar to gastroduodenal injury, the inhibition of mucosa PG synthesis renders the small intestine more susceptible to injury and less able to undergo repair after topical irritant damage (Reuter et al., 1997)(Tanaka et al., 2002). However, it does not appear that COX inhibition plays a primary role in NSAID-induced enteropathy (Reuter et al., 1997). This is evident by the lack of correlation between the extent of intestinal PG synthesis inhibition and subsequent degree of intestinal ulceration and bleeding. Furthermore, the appearance of NSAID-induced intestinal damage is not temporally synchronized with the suppression of intestinal PG synthesis (Whittle, 1981)(Reuter et al., 1997). The following are the main contributing factors in NSAID-induced enteropathy: altered intestinal permeability (i.e., barrier disruption), epithelial cell damage, neutrophil infiltration, TNF-α release, an increase in luminal gram-negative bacteria, and the enterohepatic recirculation of NSAIDs (Wallace, 2012).

An increase in intestinal epithelial permeability can be detected both in humans and rats within 12 hours of NSAID administration (Reuter et al., 1997)(Bjarnason et al., 1986). This may be due to the ability of some NSAIDs to uncouple oxidative phosphorylation in epithelial mitochondria causing ATP deficiencies and an ensuing disruption of tight junctions (Somasundaram et al., 1995). This could cause barrier disruption and facilitate the entry of damaging agents (e.g., bacteria and bile acids) into the lamina propria (Somasundaram et al., 1995). On the other hand, it has been hypothesized that the suppression of PG synthesis leads to increases in intestinal

permeability. For instance, some studies have demonstrated that exogenous PG administration has the ability to prevent NSAID-induced increases in intestinal permeability and ulceration in humans (Bjarnason, 1990). As a result, it is not clear whether the increase in permeability is due to the topical irritant properties of NSAIDs or the suppression of PG synthesis (Wallace, 2012). The possibility therefore exists that either hypothesis may be correct depending on the NSAID being evaluated. Moreover, many NSAIDs have widely variable effects on small intestinal permeability while still producing significant intestinal injury; thus this mechanism may not play a critical role in enteropathy (Choi et al., 1995).

Similar to NSAID-induced gastropathy, NSAIDs themselves can cause intestinal epithelial cell damage. Consequently, this topical damage may initiate a cascade of events leading to inflammation and ulcer formation (Somasundaram et al., 2000)(Zhou et al., 2010). The cell damage may be the result of oxidative phosphorylation uncoupling and/or a consequence of epithelial cell lipid bilayer disruption (Somasundaram et al., 2000)(Zhou et al., 2010). As a result of the inflammatory signals arising from both increased intestinal permeability and epithelial cell damage, neutrophils infiltrate the inflamed mucosa and can contribute to NSAID enteropathy. Activated neutrophils in the mucosa generate damaging levels of reactive oxygen species (ROS) and release proteases that cause collateral damage to surrounding cells (Antoon and Perry, 1997). However, unlike NSAID gastropathy, leukocyte adherence to the vascular endothelium does not appear critical in the pathogenesis of NSAID enteropathy (Wallace, 2012). Likewise, evidence exists that TNF-α contributes to NSAID enteropathy, but it plays a limited role in the process (Reuter and Wallace, 1999). For instance, instead of actively potentiating

leukocyte adherence to the vascular endothelium (as occurs in the early stages of NSAID-induced gastric injury), TNF-α may simply be produced as a consequence of damage and participate in driving acute phase inflammation (Appleyard et al., 1996)(Watanabe et al., 2008a). Nonetheless, attenuating 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 antibodies against TNF-α prevented the damage by 67% (Watanabe et al., 2008a).

The role bacteria play in NSAID-enteropathy is supported by evidence that treatment with broad-spectrum antibiotics can prevent experimental NSAID-enteropathy and that germ-free rodents do not develop intestinal ulcers when administered NSAIDs (Konaka et al., 1999)(Robert and Asano, 1977). In particular, results from rodent studies strongly signify that the generation of small intestinal ulcers after NSAID administration is dependent on the increased presence of gram-negative bacteria (Reuter et al., 1997)(Hagiwara et al., 2004). This is further supported by a study in which germ-free mice colonized with *Escherichia coli or Eubacterium limosum* (both gram-negative bacteria) were rendered susceptible to NSAID enteropathy, but when colonized with *Bifidobacter adolescentis* or *Lactobacillus acidophilus* (both gram-positive bacteria) the mice retained their resistance to NSAID enteropathy (Uejima et al., 1996). In addition, genetically altered mice lacking TLR-4, a receptor stimulated by bacterial endotoxin (i.e., lipopolysaccharide (LPS), an outer cell membrane component of gram-negative bacteria), do not develop small intestinal ulcers when administered indomethacin (Watanabe et al., 2008a).

Although the above-mentioned mechanisms of NSAID enteropathy are important, the most critical mechanism for inducing significant NSAID ulceration is the enterohepatic recirculation of the drug (Wallace, 2012). NSAIDs that undergo extensive enterohepatic recirculation exhibit a much greater propensity to cause small intestinal ulceration (Kent et al., 1969)(Reuter et al., 1997). Indeed, ligation of the bile duct to prevent the enterohepatic circulation of an NSAID prevents intestinal damage (Kent et al., 1969). When NSAIDs are re-absorbed in the ileum and subsequently secreted back into the duodenum (i.e., enterohepatic recirculation) the intestinal epithelial cells are repeatedly exposed to the topical damaging effects of the drug. Furthermore, it is hypothesized that the combination of NSAIDs and bile results in toxic micelles that are more damaging than bile salts or NSAIDs on their own (Yamada et al., 1993)(Petruzzelli et al., 2007). NSAID enterohepatic circulation not only perpetuates epithelial damage, it appears necessary to significantly alter intestinal bacteria and promote the growth of damaging, gram-negative bacteria (Reuter et al., 1997). Although a wealth of information exists implicating the roles of enterohepatic circulation and endogenous bacteria, much still remains to be clarified in NSAID enteropathy.

1.9 Hydrogen Sulfide- and Nitric Oxide-releasing NSAIDs: Rationale, Efficacy, and GI Tolerability

Although effective co-therapies exist to prevent NSAID gastropathy, such as misoprostol or PPI administration, both therapies are unable to prevent NSAID enteropathy and can have significant drawbacks (Wallace, 2012). For instance, despite the gastroprotective benefits of misoprostol its use is limited by a high incidence of diarrhea. As for PPIs, recent animal studies suggest that the co-administration of PPIs and NSAIDs

may induce small intestinal bacterial alterations that exacerbate NSAID enteropathy (Wallace et al., 2011). Therefore, not only are there no proven therapies for preventing NSAID-induced enteropathy, the main gastroprotective therapy for NSAIDs (i.e., PPIs) may perpetuate enteropathy (Wallace, 2012). The failure to develop preventative therapies for NSAID enteropathy has made the development of novel NSAIDs that elicit significantly less GI toxicity a very attractive objective. A particularly promising class of NSAIDs, gaseous mediator-releasing NSAIDs, has demonstrated reduced GI toxicity and is currently receiving considerable attention. The development of these novel drugs was fueled by the discovery that two endogenous mediators, hydrogen sulfide (H_2S) and nitric oxide (NO), are capable of eliciting many PG-like effects in terms of GI mucosal defence (Wallace and Vong, 2008). For example, in the stomach both H_2S and NO induce vasodilation, inhibit leukocyte adherence to the vascular endothelium, increase mucus and bicarbonate secretions, and promote the healing of ulcers (Wallace, 2007)(Wallace and Vong, 2008). The importance of these mediators in GI mucosal defence is highlighted by studies that demonstrated inhibition of gastric mucosal H_2S or NO synthesis led to an increased susceptibility to NSAID-induced gastric damage (Whittle, 1993)(Fiorucci et al., 2005)(Wallace and Vong, 2008). The converse is also true; administration of H_2S or NO donors increased the resistance of the gastric mucosa to NSAID-induced injury (MacNaughton et al., 1989)(Fiorucci et al., 2005). In addition, the administration of these donors accelerated the healing of pre-existing gastric ulcers (Elliot et al., 1995)(Wallace et al., 2007). Not only are H_2S and NO capable of enhancing mucosal defense, they also exhibit potent anti-inflammatory effects (Wallace and Miller, 2000)(Zanardo et al., 2006).

The following figure illustrates some of the key actions that hydrogen sulfide exerts to dampen inflammation (Figure 4):

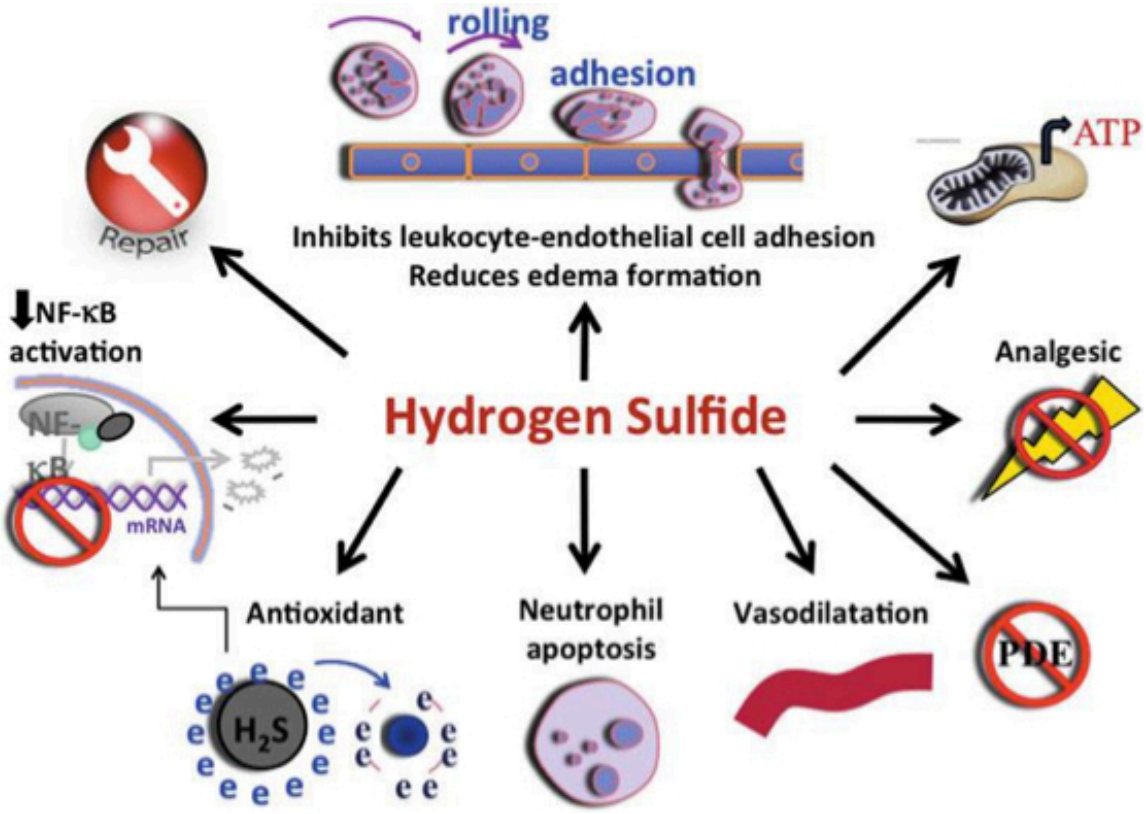

Figure 4. Anti-inflammatory effects of hydrogen sulfide (H_2S). H_2S suppresses leukocyte adherence to the vascular endothelium and infiltration into the inflamed tissue. The ability of H_2S to promote mucosal injury repair is likely due to a combination of up-regulation of COX-2 expression, vasodilation, and promotion of angiogenesis. In addition, it can reduce the expression and release of pro-inflammatory cytokines and chemokines, most likely through suppression of NF-κB activity. H_2S is also an antioxidant and can induce neutrophil apoptosis. 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 credit: Wallace et al., 2012).

Although figure 4 focuses on H_2S, it is important to note that NO shares many similar anti-inflammatory actions. For instance, in the stomach both gases can reduce pro-inflammatory cytokine expression and release through inhibition of the NF-κB pathway (Wallace et al., 2004)(Li et al., 2007). They are also potent anti-oxidants and capable of dampening inflammation by inducing neutrophil apoptosis (Wallace and Miller, 2000)(Wallace and Vong, 2008). It is these actions that make H_2S and NO promising candidates for coupling with NSAIDs. The theory of this approach is that the novel NSAIDs would slowly release protective gaseous mediators to compensate, in terms of mucosal defense, for the reduction of PG synthesis inhibition, thus maintaining mucosal integrity (Wallace and Vong, 2008). Furthermore, these compounds might exhibit enhanced anti-inflammatory activity compared to their parent derivatives due to the potent anti-inflammatory actions of H_2S and NO. In recent years, the theory of improved GI toxicity has been convincingly demonstrated. H_2S- and NO-releasing NSAIDs produce considerably less GI damage than their parent NSAIDs in animal studies (Wallace et al., 2010). In humans, much remains to be evaluated with the H_2S-releasing drugs, although NO-releasing NSAIDs have performed well in clinical trials (Wilder-Smith et al., 2006). It is important to note that these studies confirmed that the novel NSAIDs still have the ability to suppress PG synthesis, and thus, retain the key effect through which NSAIDs exert anti-inflammatory, anti-pyretic and analgesic effects (Wallace et al., 2004)(Wallace et al., 2010). Two novel, gaseous mediator-releasing

NSAIDs were evaluated in this thesis; ATB-346 [2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester] and NCX 429 [(S)-6-(nitrooxy)hexyl 2-(6-methoxynaphthalen-2-yl)propanoate]. ATB-346 consists of a molecule of naproxen linked to an H_2S-releasing moiety (i.e., 4-hydroxythiobenzamide (TBZ); via an ester bond). Likewise, NCX 429 consists of a molecule of naproxen linked to an NO-releasing moiety via an ester bond. During our studies, these novel NSAIDs were evaluated in healthy rats and in rats with clinically significant co-morbidities. The following subsection offers a specific introduction to the work conducted with these drugs and the significance evaluating their GI safety using animals with co-morbidities.

1.10 Thesis Introduction and Relevance

Therapies aimed at preventing NSAID-induced GI injury have largely focused on gastroduodenal damage. The most common approach used clinically to minimize gastroduodenal injury is to co-administer a proton pump inhibitor (PPI) with the NSAID. This has been shown to significantly reduce the incidence of gastroduodenal damage (Scheiman et al., 2006), but recent animal studies suggest that suppression of acid secretion can lead to exacerbation of NSAID-induced small intestinal injury and bleeding (Wallace et al., 2011). There are several clinical studies that report high levels of intestinal damage in healthy volunteers taking NSAIDs plus a PPI, and one study showing significant elevation of a marker of intestinal inflammation (i.e., calprotectin) in patients taking PPIs (Goldstein et al., 2005)(Maiden et al., 2005)(Poullis et al., 2003). Selective inhibitors of COX-2 entered the marketplace at the turn of the last century with great promise for GI safety. This promise has largely been unfulfilled (Graham et al., 2011). However, even the small upper GI benefit gained through use of a selective COX-2

inhibitor versus a non-selective COX inhibitor is lost when low-dose aspirin is co-administered (Laine et al., 2003). This co-therapy is aimed at reducing the incidence of cardiovascular events associated with the use of selective and most non-selective NSAIDs (Kearney et al., 2006). Low-dose aspirin, alone, can also cause significant small intestinal injury (Watanabe et al., 2008). Studies to evaluate the effects on the GI tract of the combined use of an NSAID, a PPI and low-dose aspirin, which is now a common combination in clinical practice, have not been reported.

One of the problems encountered in attempts to develop GI-sparing NSAIDs is that preclinical studies have largely focused on the stomach (ignoring the small intestine) and are usually performed using healthy animals. The latter may give false security about the safety of the drug, which in humans will be used by individuals with significant co-morbidities and compromised mucosal defence. It is therefore important to evaluate the safety and efficacy of novel NSAIDs in models that more closely resemble the patients who will be the major users of these drugs. NSAID-induced gastroduodenal injury has been reported to be elevated in elderly patients, and in patients with co-morbidities such as obesity, hypertension and rheumatoid arthritis (Solomon and Gurwitz, 1997)(Hernández-Díaz and Rodríguez, 2002)(Aro et al., 2006). Novel NSAIDs should also be evaluated in combination with the drugs that are often co-prescribed with NSAIDs (e.g., PPIs and low-dose aspirin), given that these drugs may exacerbate NSAID-induced GI damage. This approach will make the data more predictive of the human response, therefore providing more insight on the potential GI safety of drugs intended for use as treatments of inflammatory conditions.

In the present study, we examined the effects of a number of NSAIDs in models that attempt to mimic relevant clinical scenarios of NSAID use. Two of the most commonly used NSAIDs (naproxen and celecoxib) were compared to each other and to two novel, putative GI-sparing NSAIDs (both chemically related to naproxen; one nitric oxide-releasing and the other hydrogen sulfide-releasing). As previously stated, both NO and H_2S have been shown to exert protective effects in the GI tract, and NSAID compounds that release one of these gaseous mediators produce significantly less GI damage than their respective parent drugs in healthy animals (Davies et al., 1997)(Wallace et al., 2010). In addition to examining the GI safety of these compounds when administered together with low-dose aspirin and/or a PPI, we evaluated them in models in which mucosal defence may be compromised (i.e., obese rats, arthritic rats, hypertensive rats and aged rats). In all studies we compared the test drugs at doses that produced comparable anti-inflammatory effects in rats with adjuvant arthritis.

1.11 Objectives

The following primary objectives were addressed in this thesis:

1. To evaluate the extent of NSAID-induced GI damage in rat co-morbidity models that closely resemble relevant clinical scenarios of NSAID use, and in models where mucosal defence may be compromised.

2. To determine whether ATB-346 and NCX 429 exhibit superior GI safety compared to naproxen in rat co-morbidity models.

2.0 METHODS AND MATERIALS

2.1 Animals

Male, Wistar rats weighing 180–220 g and male, Zucker rats (both lean and obese, weighing ~360 and ~560 g, respectively), spontaneously hypertensive rats (SHR) and normotensive rats (Wistar-Kyoto; WKR) (180–220 g) were obtained from Charles Rivers (Montreal, QC, Canada). 19-month old, male, Sprague Dawley rats (mean weight of 525 ± 30 g) were obtained from Harlan Laboratories (Indianapolis, IN, USA). 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). All experimental procedures described herein were approved by the Animal Care Committee of the Faculty of Health Sciences at McMaster University. The studies were carried out in accordance with the guidelines of the Canadian Council of Animal Care. The health of the animals was assessed at least twice-daily, and any animals in distress or having lost >15% of their original body weight were euthanized by an overdose of sodium pentobarbital.

2.2 Test Drugs

Naproxen and ATB-346 (2-(6-methoxy-napthalen-2-yl)-propio-nic acid 4-thiocarbamoyl-phenyl ester) were tested in all models, and in some models the effects of celecoxib and NCX 429 [(S)-6-(nitrooxy)hexyl 2-(6-methoxynaphthalen-2-yl)propanoate] were also examined. Naproxen and celecoxib were administered at a dose of 10 mg/kg. This dose was selected because it produced significant and comparable activity in reducing paw swelling in rats with adjuvant arthritis (see 3.1)(Cicala et al., 2000). To test

the enteric-sparing ability of TBZ as a separate entity, naproxen (20 mg/kg) was co-administered with TBZ at a dose of 13 mg/kg (equimolar to TBZ quantity in a 32 mg/kg dose of ATB-346). In all studies described below, ATB-346 and NCX 429 were given at doses equimolar to the dose of naproxen. All test drugs were suspended in vehicle (dimethylsulfoxide/1% carboxymethylcellulose; 5:95 ratio).

2.3 Adjuvant Arthritis Model

Polyarthritis was induced in Wistar rats via an injection into the base of the tail of 100 μL of Freund's Complete Adjuvant containing 0.75 mg of heat-killed *Mycobacterium butirricum* (Cicala et al., 2000). To evaluate the ensuing inflammatory process, the volume of the hind paws of each rat was blindly measured using a hydroplethysmometer (Ugo Basile, Comerio, Italy) prior to the injection of the adjuvant, and on days 7, 10, 14 and 18 after adjuvant administration. Groups of rats (n = 8 each) were treated twice-daily beginning on day 7 with celecoxib (10 mg/kg), naproxen (10 mg/kg), or equimolar doses of ATB-346 (14.5 mg/kg) or NCX 429 (15 mg/kg). Two control groups (one with adjuvant arthritis and one naive) were treated with an equal volume of vehicle. At the end of the study the stomach and small intestine were excised and blindly evaluated for hemorrhagic damage, as described below (see 2.4).

2.4 NSAID-induced Gastroenteropathy

Unless otherwise noted, studies of NSAID-induced gastroenteropathy were performed in healthy (2-month old) Wistar rats. Rats were given one of the test drugs or vehicle orally, twice each day for 4.5 days (9 administrations in total). Three hours after the final administration of drug or vehicle, the rats were anesthetized with sodium pentobarbital (ip) and blood was drawn from the aorta for ELISA measurement of whole

blood thromboxane B_2 (TXB_2)-synthesis, as an index of systemic COX-1 activity (see 2.11)(Wallace et al., 1998). The stomach and small intestine were then excised and blindly evaluated for hemorrhagic damage. This involved measuring the lengths, in mm, of all hemorrhagic lesions. Separate gastric and intestinal damage scores were then calculated by summing the lengths of all lesions for each rat (Wallace et al., 2011). After scoring, samples of the corpus region of the stomach were collected for the measurement of prostaglandin (PG)E_2 synthesis, as described below (see 2.11). Finally, specimens of gastric and jejunal tissues were fixed and processed for histological examination (H&E staining).

2.5 Polypharmacy Model

Groups of Wistar rats (n > 6/group) were treated for a total of 9 days with one or more drugs. The rats received omeprazole (10 mg/kg) or vehicle twice-daily (ip) throughout the 9 days. Beginning on day 2, the rats received vehicle or low-dose aspirin (10 mg/kg) orally once daily. Beginning on day 5, the rats received an NSAID or vehicle orally twice-daily. The rats were euthanized 3 hours after the final administration of the NSAID or vehicle for blind evaluation of the extent of damage to the stomach and small intestine, as described above. Samples were taken for measurement of prostaglandin and thromboxane synthesis, as described above. Previously we demonstrated that the dose of omeprazole used in this study produced a 99% inhibition of gastric acid secretion by the 5th day of administration (when NSAID treatment was initiated) (Wallace et al., 2011). The dose of aspirin was chosen based on the 81 mg per day dose in patients prescribed low-dose aspirin for ‘cardio-protection’. It was adjusted to a 10 mg/kg daily administration since this dose produced a 95% inhibition of whole blood thromboxane

synthesis by the 3rd day of administration (when NSAID treatment was initiated) in rats (Wallace et al., 2011).

2.6 Advancing Age Model

Studies were performed, as described above (see 2.4), using Sprague Dawley rats that were 19 months of age (n = 6 per group).

2.7 Obesity Model

Male, Zucker rats of the fa/fa phenotype spontaneously develop to an obese state due to a mutation of the leptin receptor, whereas their Fa/fa littermates exhibit normal weight gain (Zucker and Antoniades, 1972). Obese and lean Zucker rats (n = 6/group) were treated orally twice-daily with naproxen (10 mg/kg), celecoxib (10 mg/kg), ATB-346 (14.5 mg/kg), or vehicle (1% CMC, DMSO (95:5)) for a total of 4.5 days. Three hours after the final dose, the rats were euthanized, the stomach and small intestine were blindly evaluated for damage and sample collection was conducted as described above (see 2.4). In order to determine if any of the rats were diabetic, as has been reported, blood glucose levels were determined prior to and after NSAID dosing using a Freestyle Freedom Lite unit (Augstein and Salzsieder, 2009)(Abbott Diabetes Care, Saint-Laurent, QC, Canada). A sample of non-fasting blood was used for blood glucose determination and collected via a tail snip performed at the same time of day both before and after NSAID administration.

2.8 Hypertension Model

SHR rats develop hypertension spontaneously without exception at the age of 7-15 weeks (Yamori et al., 1984). The systolic blood pressure of mature males is ~200 mmHg (Roba et al., 1976). To confirm that the spontaneously hypertensive rats were

indeed hypertensive, blood pressure was measured in SHR and normotensive (WKR) controls using a CODA Non-Invasive (tail-cuff) Blood Pressure System (Kent Scientific Corporation, Torrington, CT, USA). The CODA system utilizes a volumetric pressure recording method to simultaneously measure systolic blood pressure, diastolic blood pressure, mean blood pressure, heart pulse rate, tail blood volume and tail blood flow. In order to minimize stress-induced alterations in blood pressure, each rat underwent a daily 15-minute training session in the restraining device for 3 days prior to blood pressure determination. The rats were acclimated for 10 minutes in advance of blood pressure readings and placed on a heating blanket (36°C) to promote thermo-regulation and maintain tail blood flow. Blood pressure measurements were performed two days prior to beginning NSAID administration. The rats received naproxen (10 mg/kg), an equimolar dose of ATB-346 (14.5 mg/kg) or vehicle orally twice-daily for 4.5 days. 3 hours after the final administration of the test drugs the rats were euthanized and the extent of gastric and small intestinal damage was blindly evaluated, as described above. Samples were taken for measurement of gastric PGE_2 and whole blood TXB_2 synthesis, as described below (see 2.11).

2.9 Pharmacokinetics

Rats were treated with naproxen (10 mg/ kg), ATB-346 (14.5 mg/kg) or NCX 429 (15 mg/kg). Subgroups (n = 4 each) of rats received either a single, oral dose or 4 doses at 12 h intervals. Four hours after the final administration, all rats were anesthetized with sodium pentobarbital. The bile duct was cannulated with polyethylene cannula (PE-10; Clay Adams, Parsipany, NJ, USA) and bile was collected for 30 minutes, after which a blood sample was drawn from the descending aorta (Reuter et al., 1997). Blood samples

were allowed to clot at room temperature for 45 minutes, after which they were centrifuged at 1000 *g* for 10 minutes. After being centrifuged, serum was collected and frozen in liquid nitrogen. Samples were stored at -80°C for subsequent analysis using LC-MS/MS. 50 μL of bile or serum samples were de-proteinated by adding three volume of acetonitrile and 10 μL of DMSO. Samples were then centrifuged for 10 min at 4°C (3200 *g*) and the supernatants were transferred to a 96-well plate for analysis using a LC-MS/MS system for quantification of naproxen (Wallace et al., 2011). A calibration curve of naproxen in both serum and bile was prepared in the concentration range 0.1–300 μM.

2.10 Pharmacodynamics

Rats were treated orally with a single dose of naproxen (10 mg/kg), ATB-346 (14.5 mg/kg), or vehicle (1% CMC, DMSO (95:5)). Subgroups (n = 4 per group) of rats were then anesthetized with sodium pentobarbital at time-points 15 min, 1 h, 3 h, 6 h, and 12 h. A blood sample was drawn from the descending aorta for ELISA measurement of whole blood thromboxane B_2 (TXB_2)-synthesis and samples of the corpus region of the stomach were collected for the measurement of prostaglandin (PG)E_2 synthesis.

2.11 Measurement of Cyclooxygenase Enzyme Activity

Whole blood thromboxane B_2 (TXB_2) synthesis was used as an index of the inhibitory effects of the test drugs on COX-1 activity and measured using a previously verified method (Patrono et al., 1980). The production of thromboxane under the conditions of this method occurs almost exclusively via COX-1 in platelets (Brideau et al., 1996). Rats were anesthetized with sodium pentobarbital (65 mg/kg ip) and a midline laparotomy was performed to expose the aorta. Approximately 3 mL of blood was drawn from the descending aorta using a 3 cc syringe with an 18-gauge needle. The blood was

then transferred to a glass tube and allowed to stand at 37°C for 45 minutes, after which 100 μL of indomethacin (100 μg/mL in 1.25% sodium bicarbonate solution) was added. The samples were then transferred to wet ice for 5 minutes before being centrifuged (1000 *g* for 10 min). The resulting serum was collected and frozen at -80°C for subsequent determination of TXB_2 levels using an ELISA (Caymen Chemical Company, Ann Arbor, MI, USA). As a measurement of the PG biosynthetic capacity (i.e., COX activity) of the stomach, a sample of the corpus region was excised, weighed, and added to a tube containing 1 mL of sodium phosphate buffer (10 mmol/L; pH 7.4)(Wallace et al., 2000a). Using scissors, the tissue sample was minced for 30 seconds, and then placed in a shaking water bath (37°C) for 20 min. The samples were then removed and centrifuged (14,000 *g*) for 30 seconds. The supernatant was collected and stored at -80°C until the concentration of PGE_2 could be determined by ELISA (Caymen Chemical Company, Ann Arbor, MI, USA).

2.12 Materials

ATB-346 (2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thio-carbamoyl-phenyl ester) was provided by Antibe Therapeutics Inc. (Toronto, ON, Canada) and NCX 429 [(S)-6-(nitrooxy)hexyl 2-(6-methoxynaphthalen-2-yl)propanoate] was provided by NicOx S.A. (Sophia Antipolis, France). Celecoxib was purchased from American Custom Chemical Corp. (San Diego, CA, USA). Sodium naproxen was purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA kits for measuring TXB_2 and PGE_2 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Freund's Complete Adjuvant and *Mycobacterium butirricum* were purchased from Difco Laboratories (Detroit, MI, USA).

2.13 Statistical Analysis

Data are expressed as the mean ± SEM. Comparisons among groups of data were performed by one-way analysis of variance followed by a post hoc test (Dunnett's Multiple Comparison Test for parametric data and Mann Whitney Test for non-parametric data). An associated probability (p value) of less than 5% was considered significant.

3.0 RESULTS

3.1 Gastrointestinal Damage in Healthy, Young Rats

In healthy, 2-month old Wistar rats, administration of naproxen (10 mg/kg) twice-daily for 4.5 days resulted in very little damage in the stomach or small intestine. The mean damage scores were 1.3 ± 0.5 for the stomach and 1.0 ± 0.5 for the small intestine (n = 8; see Figure 7 for intestinal damage). Similarly, celecoxib, ATB-346, NCX 429 and low-dose aspirin produced negligible gastric and small intestinal damage in healthy young rats. The mean gastric PGE_2 levels and whole blood TXB_2 levels were 134.7 ± 22.2 (ng/mg) and 211.9 ± 16.7 (ng/mL), respectively, in vehicle-treated rats.

It was previously demonstrated that the gastric-sparing property of ATB-346 was not evident when the two components of this drug (naproxen and TBZ) were administered as separate entities (Wallace et al., 2010). However, it was unknown as to whether TBZ administered as a separate entity with naproxen could prevent intestinal damage. Healthy rats (n = 5) co-administered the molar equivalents of naproxen (20 mg/kg) and TBZ (13 mg/kg) twice-daily for 4.5 days developed significant small intestinal damage that did not differ significantly in its severity from that induced by naproxen alone (Figure 5). An equivalent dose of ATB-346 did not produce significant intestinal damage (Figure 5). TBZ administration had no effect on gastric PGE_2 synthesis or whole blood TXB_2 synthesis.

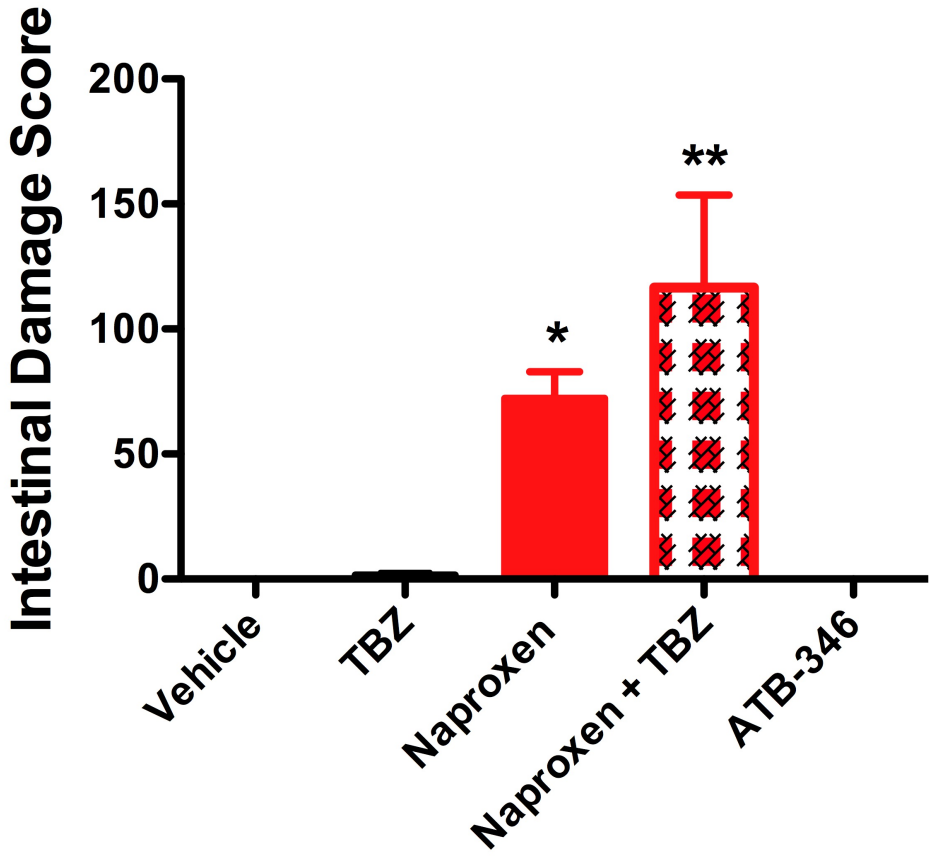

Figure 5. Intestinal damage in rats administered naproxen and TBZ as separate entities. The intestine sparing property of ATB-346 was not evident when the two components of this drug (naproxen and TBZ) were administered as separate entities to healthy, young rats. Both the naproxen and naproxen + TBZ groups had significantly greater intestinal damage scores than the vehicle-rats. *$p < 0.05$, **$p < 0.01$ versus the vehicle-treated group. n = 5 per group.

3.2 Efficacy Studies in Adjuvant Arthritis

Each of the drugs, at the doses tested, produced comparable reductions in paw edema, reducing paw volumes to levels not different from those of healthy rats (Figure 5A). Consistent with the comparable anti-inflammatory effects, the test drugs produced comparable suppression of gastric prostaglandin E_2 synthesis (Figure 5B, top) and systemic COX-1 activity (whole blood thromboxane synthesis) (Figure 5B, bottom). Despite this, the extent of gastric and small intestinal damage differed among the treatment groups. Naproxen caused significant gastric and intestinal damage, while no significant damage was observed in rats treated with celecoxib, ATB-346 or NCX 429 (not significantly different from that in vehicle-treated rats) (Figure 5D). Furthermore, the propensity of naproxen to cause gastric and intestinal damaged was higher in arthritic rats compared to young, healthy rats (~5-fold increase).

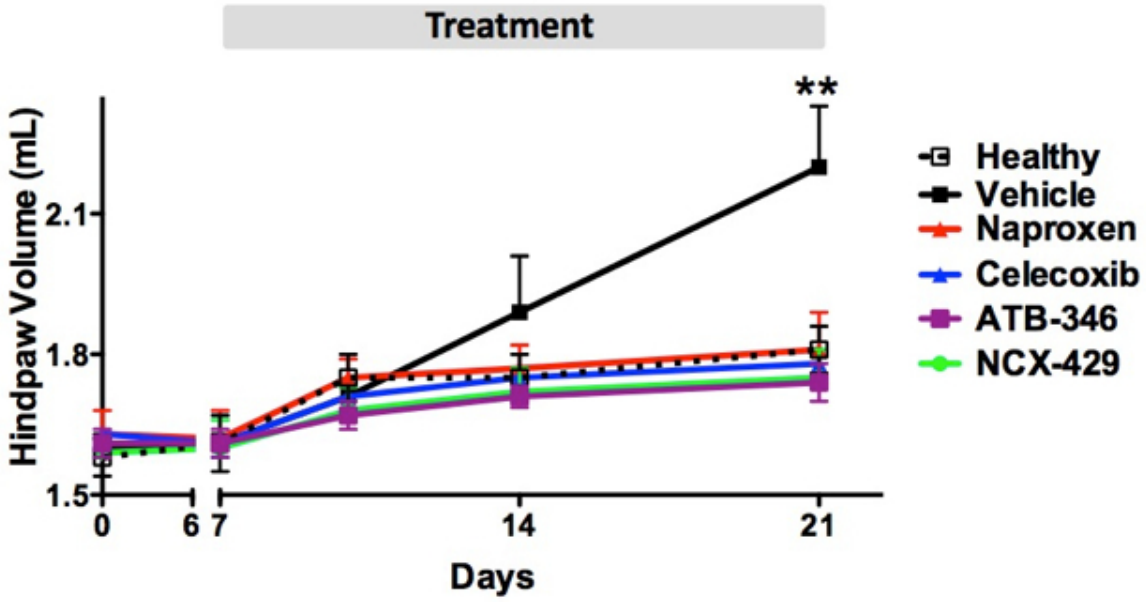

Figure 6A. Anti-inflammatory effects of test drugs. Polyarthritis was evident in rat hind paws within 7 days of administration of Freund's Complete Adjuvant. Each of the test drugs (administered twice-daily from day 7 to 21) produced significant and comparable reductions of paw edema. **$p < 0.01$ versus the naproxen-treated group. $n = 8$ per group.

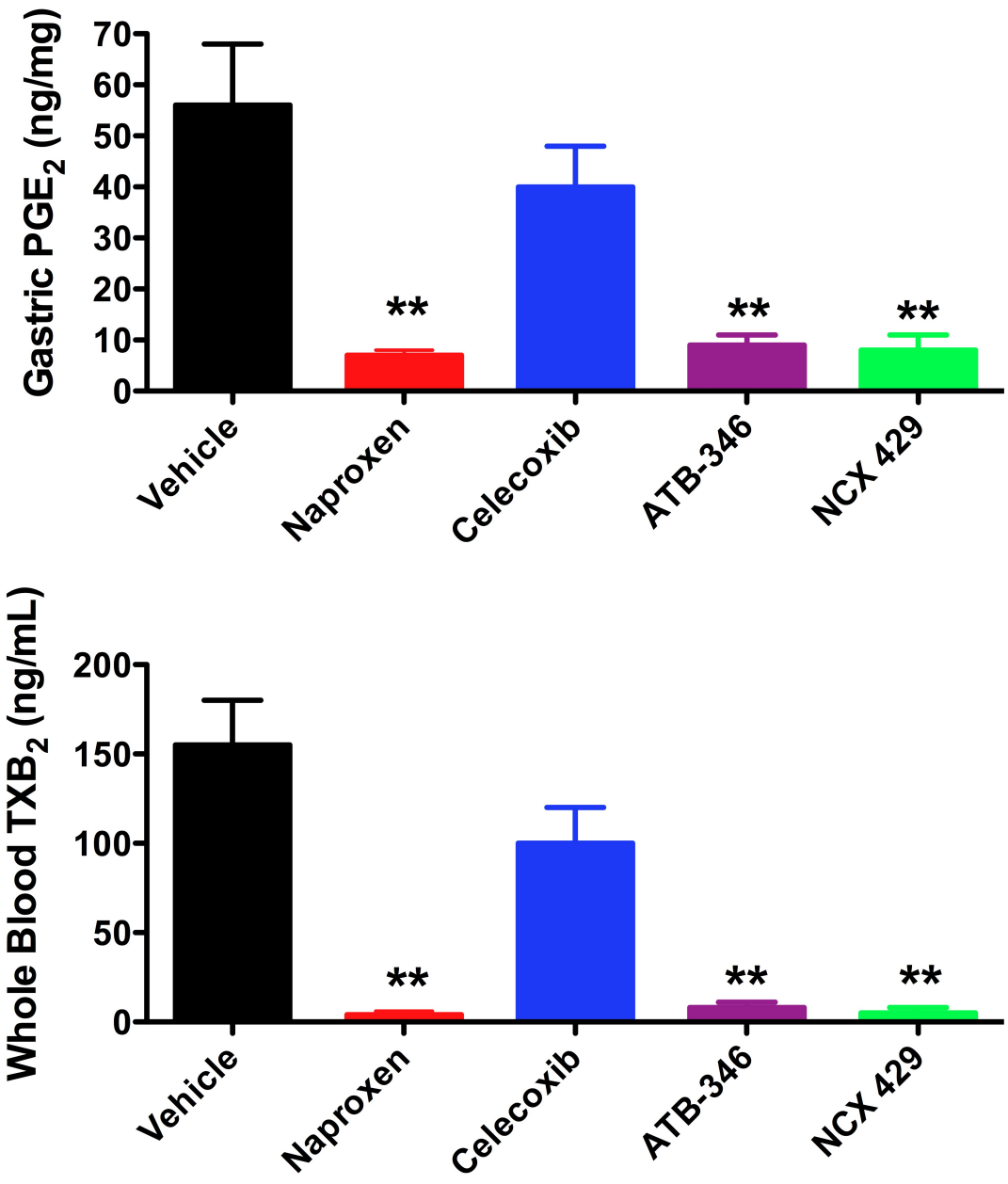

Figure 6B. Inhibition of cyclooxygenase activity. The test drugs produced comparable suppression of gastric prostaglandin E_2 synthesis (top panel) and systemic cyclooxygenase (COX)-1 activity (whole blood thromboxane synthesis) (bottom panel) in the adjuvant arthritis model. **$p < 0.01$ versus the vehicle-treated group. $n = 8$ per group.

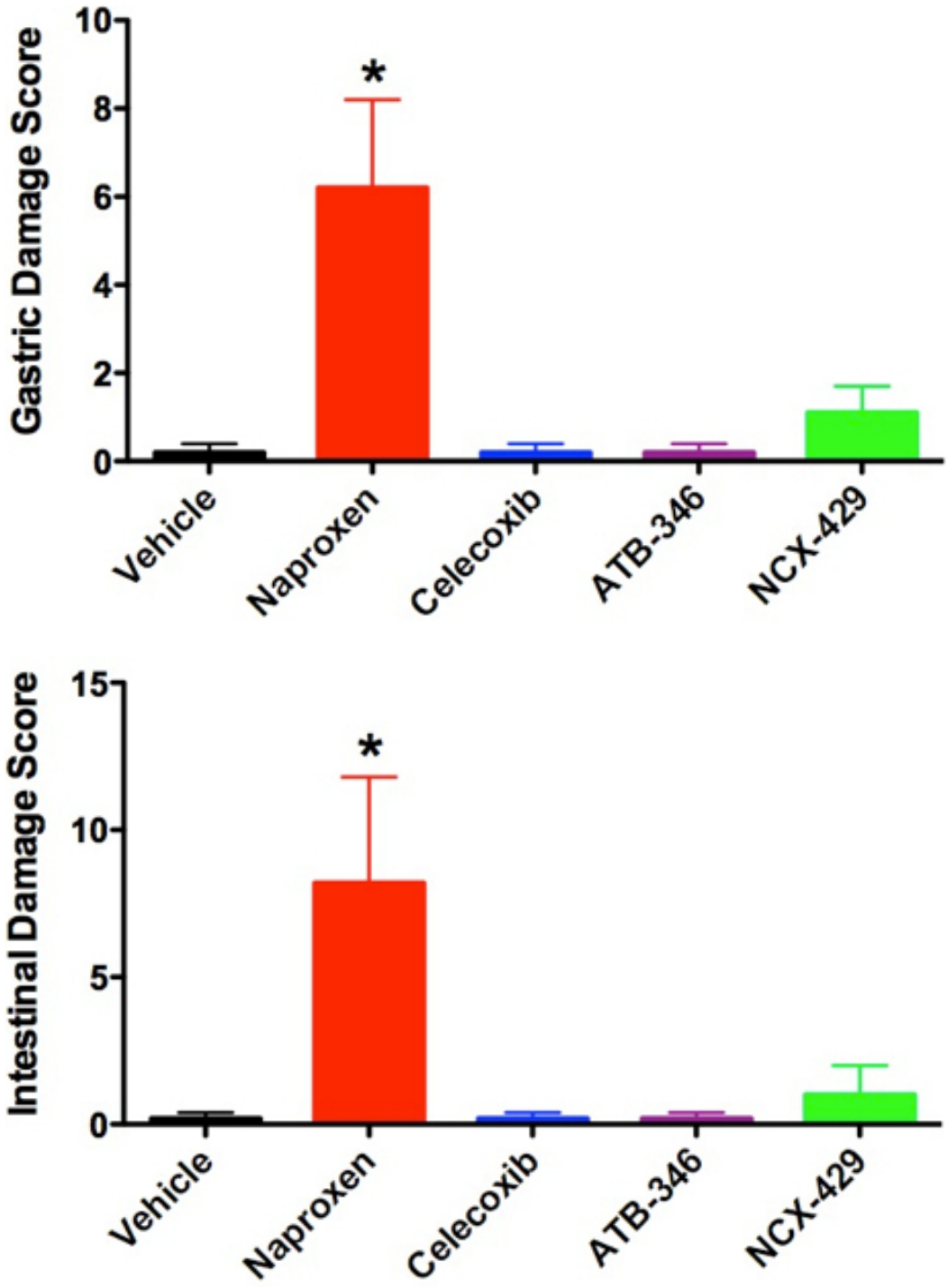

Figure 6C. Gastrointestinal damage in rats with adjuvant arthritis. Naproxen was the only test drug that caused significant gastric (top panel) and intestinal (bottom panel)

damage compared to the vehicle-treated group. *p < 0.05, versus the vehicle-treated group. n = 8 per group.

3.3 Polypharmacy Model

The test drugs were also assessed for their ability to induce gastrointestinal damage in circumstances mimicking the clinical scenario in which patients receive co-treatment with a 'gastro-protective' drug and/or low-dose aspirin (to provide 'cardio-protection'). The combination of either naproxen or celecoxib with both low-dose aspirin and omeprazole resulted in the highest intestinal damage scores (Figure 7). In sharp contrast to the effects observed with naproxen and celecoxib, administration of ATB-346 or NCX 429 together with low-dose aspirin and/or omeprazole did not result in significant intestinal damage (Figure 7).

When naproxen or celecoxib were administered together with low-dose aspirin, the extent of hemorrhagic injury in the small intestine increased markedly ($p < 0.05$) over that observed with either drug alone (Figure 7). Similarly, co-administration of the proton pump inhibitor (i.e., omeprazole) with naproxen or celecoxib resulted in a dramatic increase in the extent of small intestinal damage. Gastric damage was negligible in rats co-treated with one of the NSAIDs and omeprazole and/or low-dose aspirin (mean damage scores of <3).

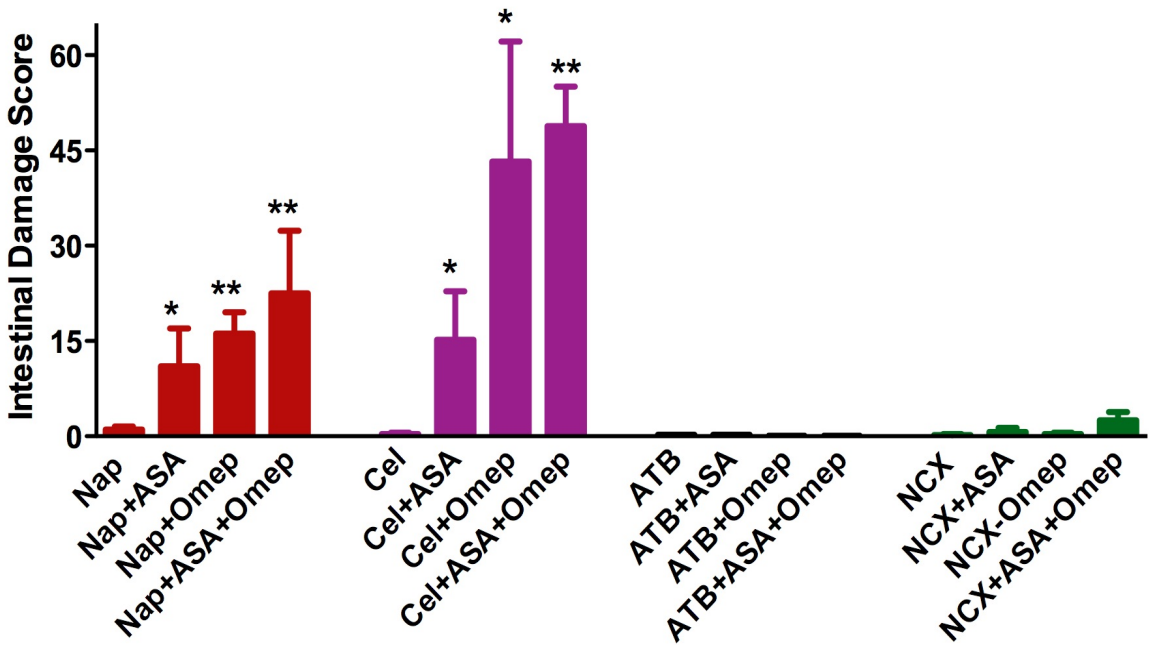

Figure 7. Co-administration of naproxen or celecoxib with omeprazole and/or low-dose aspirin results in marked exacerbation of small intestinal damage. In contrast, rats given a naproxen derivative (ATB-346 or NCX 429) did not develop significant intestinal injury when given alone or in combination with omeprazole, low-dose aspirin, or both. *$p < 0.05$, **$p < 0.01$ versus the corresponding group treated with NSAID alone ($n \geq 6$ per group). Aspirin and omeprazole, alone or given together, did not elicit significant intestinal damage.

3.4 Studies in Aged Rats

In rats that were 19 months of age, twice-daily administration of naproxen for 4.5 days resulted in the development of extensive gastric damage that consisted of both of erosions and penetrating ulcers (Figure 8A). Histological evaluation confirmed that damage in the older rats penetrated through the muscularis mucosae into the submucosa. Similar to the studies in younger rats, the older rats did not develop detectable intestinal

damage when treated with naproxen. Older rats treated with celecoxib, ATB-346 or NCX 429 did not develop significant gastric or intestinal damage. However, ATB-346 and NCX 429 suppressed (<95%) gastric PGE_2 and whole blood thromboxane synthesis to the same extent as naproxen (Figure 8B).

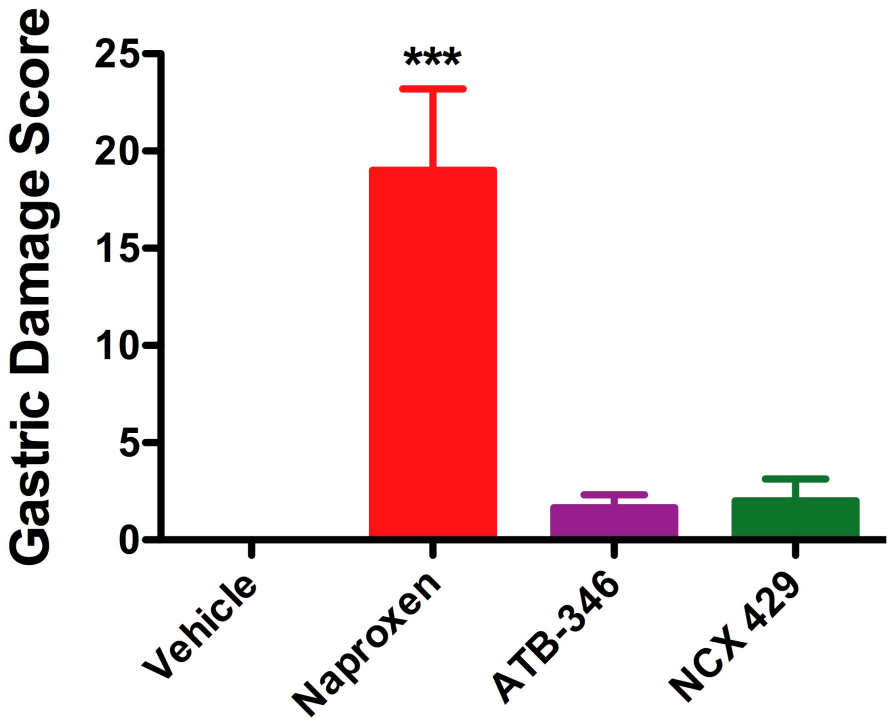

Figure 8A. Extensive gastric damage in aged rats treated with naproxen. Older (19 months of age) rats develop extensive gastric damage when given naproxen, but not when given equimolar doses of a hydrogen sulfide-releasing naproxen derivative (ATB-346) or a nitric oxide-releasing naproxen derivative (NCX 429). ***$p < 0.001$ versus the vehicle-treated group. n = 6 per group.

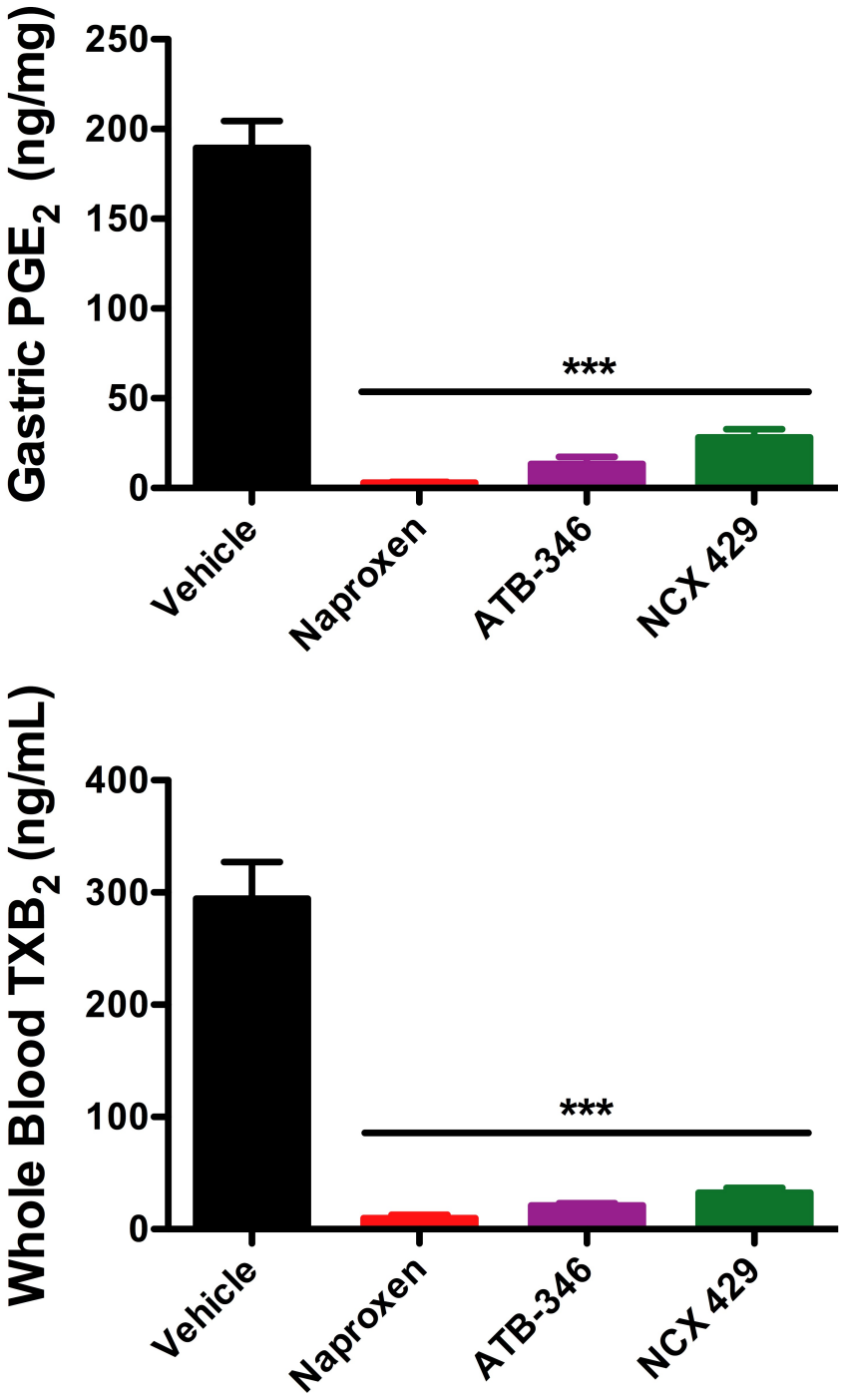

Figure 8B. COX inhibition in aged rats treated with test drugs. ATB-346 and NCX 429 suppressed gastric PGE_2 (top panel) and whole blood thromboxane synthesis (bottom panel) to the same extent (<95%) as naproxen. ***$p < 0.001$ versus the vehicle-treated group. n = 6 per group.

3.5 Studies in Obese Rats

Treatment of lean Zucker rats (mean weight of ~360 g) with naproxen twice-daily for 4.5 days resulted in a small amount of damage in the stomach and intestine, similar to that seen in healthy, Wistar rats (Figure 9A). Obese Zucker rats (mean weight of ~560 g) did not exhibit gastric damage, but they developed much more severe small intestinal damage when treated with naproxen ($p < 0.01$ versus the lean counterparts) (Figure 9A). Furthermore, the small intestine of naproxen-treated obese rats was hyperemic and friable. ATB-346 did not produce detectable gastric or intestinal damage in either lean or obese Zucker rats. Whole blood thromboxane synthesis was markedly higher in the obese rats than in the lean littermates (632 ± 110 vs. 105 ± 36 ng/mL, respectively; $p < 0.01$). However, in both groups of rats naproxen and ATB-346 caused near-complete suppression (<95%) of gastric PGE_2 synthesis and whole blood thromboxane synthesis (Figure 9B). Diabetes was not observed in either lean or obese Zucker rats. Blood glucose levels were all within the normal, non-fasting range (5.5 ± 1.1 mM in lean vs. 5.5 ± 1.2 mM in obese).

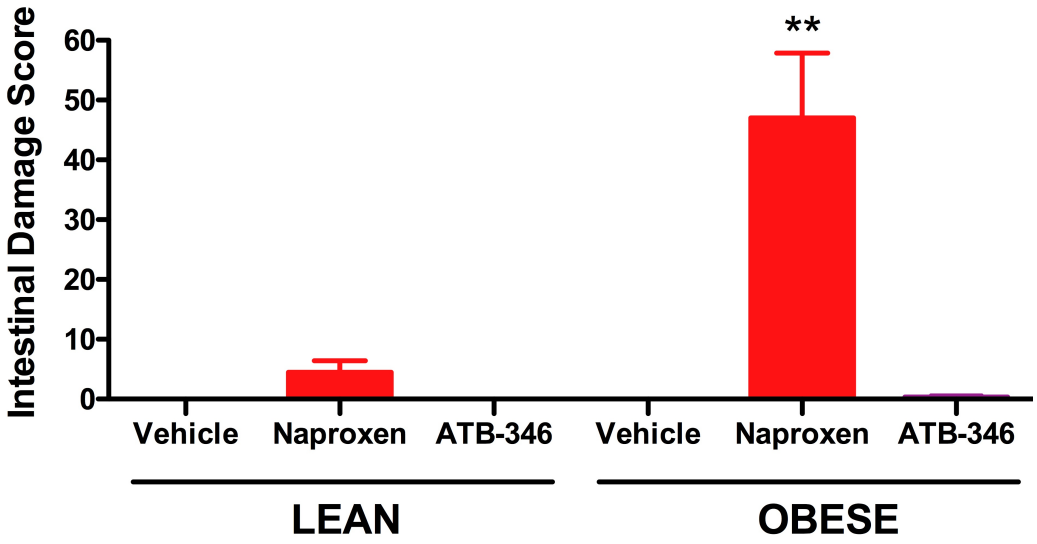

Figure 9A. Increased naproxen-induced small intestinal damage in obese versus lean rats. Neither lean nor obese rats developed intestinal damage when administered ATB-346. **$p < 0.01$ versus the corresponding vehicle- and ATB-346-treated rats. n = 6 per group.

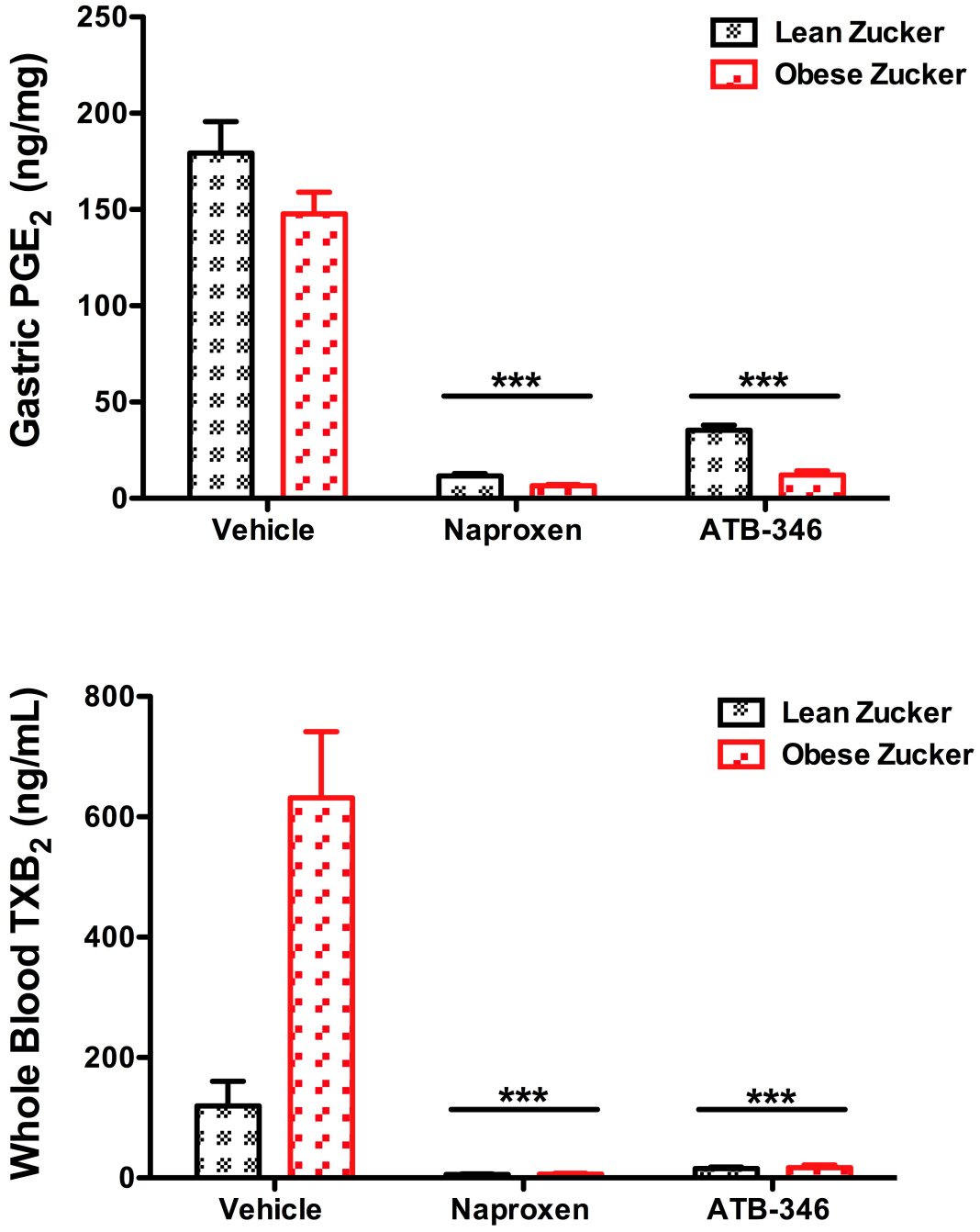

Figure 9B. COX inhibition in obese and lean rats treated with test drugs. In both lean and obese rats, naproxen and ATB-346 caused near-complete suppression (<95%) of gastric PGE_2 synthesis (top panel) and whole blood thromboxane synthesis (bottom panel). ***$p < 0.001$ versus the vehicle-treated group. n = 6 per group.

3.6 Studies in Hypertensive Rats

SHR rats had markedly elevated systemic blood pressure as compared to the normotensive (Wistar-Kyoto) rats (Figure 10A, top). When treated with naproxen or ATB-346 twice-daily for 4.5 days, no significant gastric damage was observed in either group of rats (Figure 10A, bottom). Intestinal damage was not observed in either group of rats with either test drug. The test drugs produced comparable suppression of gastric PGE_2 and whole blood thromboxane synthesis in both hypertensive and normotensive rats (Figure 10B). However, the control hypertensive rats did exhibit marked elevations of whole blood thromboxane synthesis (~3.5-fold) compared to control normotensive rats, as has been reported previously (Purkerson et al., 1986).

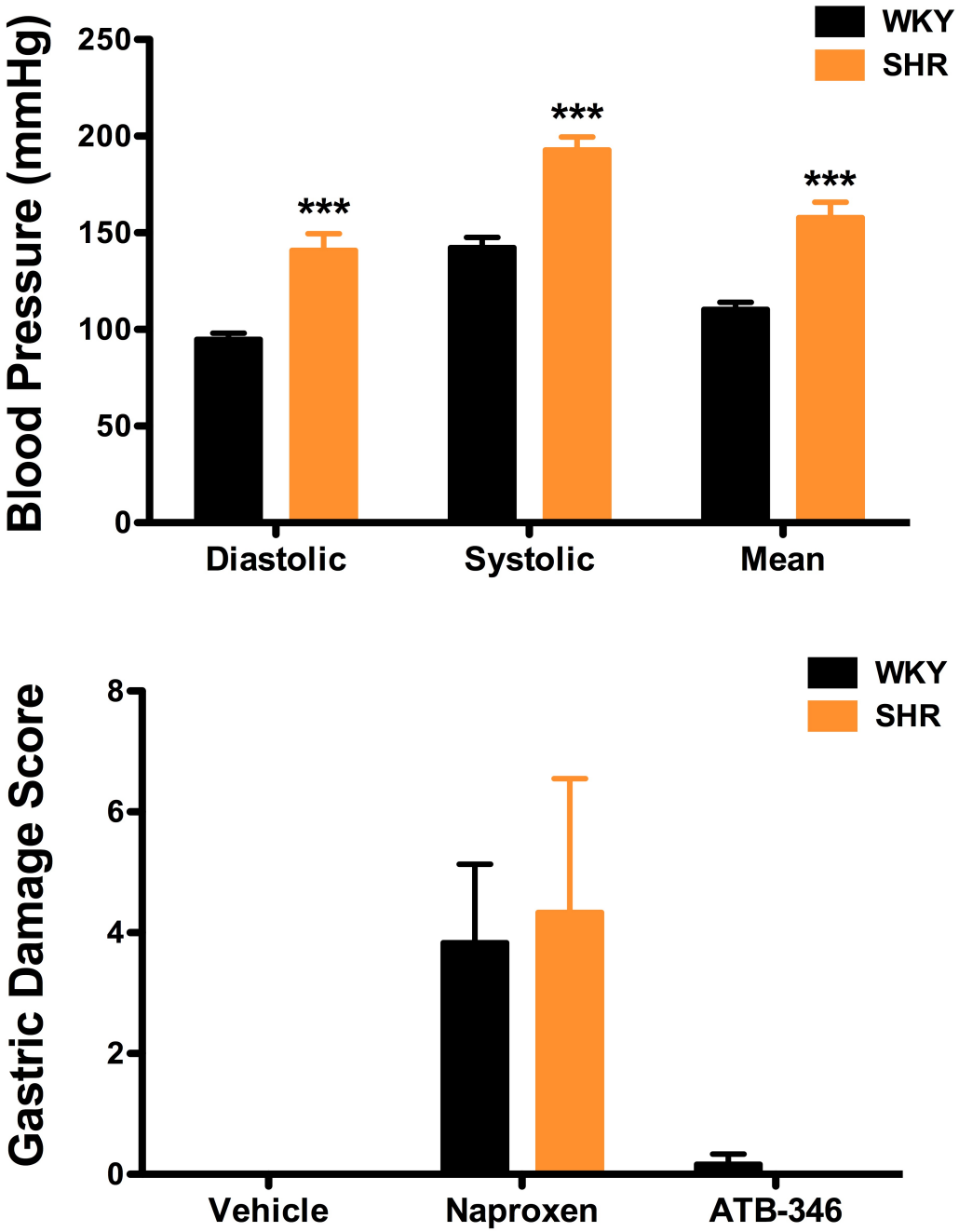

Figure 10A. Severity of naproxen-induced gastric damage is similar in spontaneously hypertensive (SHR) and normotensive (WKY) rats. Significant differences in blood pressure were confirmed prior to drug administration (top panel). ATB-346 elicited no gastric damage in either SHR or WKY rats (bottom panel). ***$p < 0.001$ versus control WKY rats. n = 6 per group.

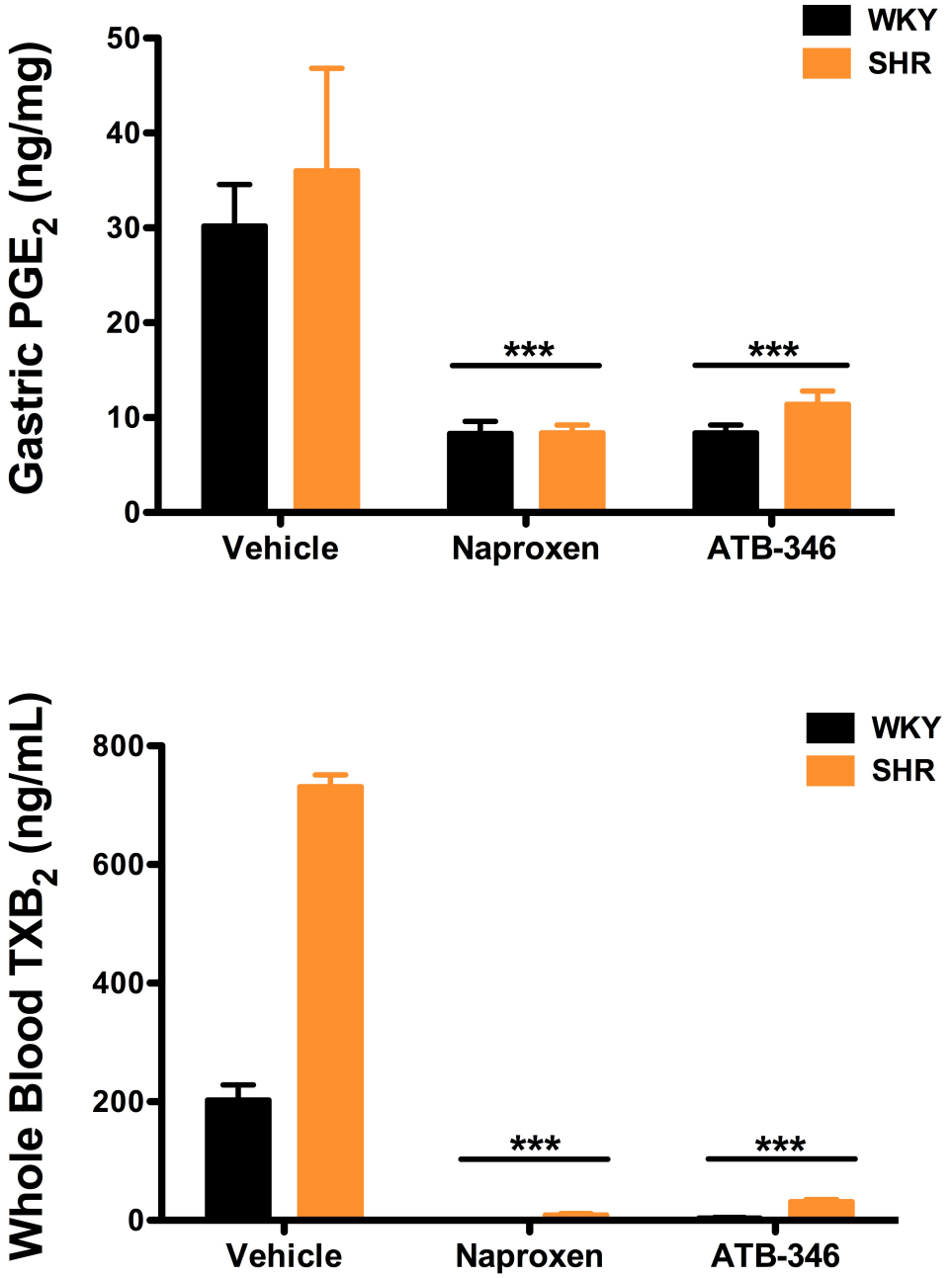

Figure 10B. COX inhibition in SHR and WKY rats treated with test drugs. ATB-346 suppressed gastric prostaglandin (PG)E_2 synthesis (top panel) and whole blood thromboxane (TXB_2) synthesis (bottom panel) as effectively as naproxen, but did not elicit gastric damage. ***$p < 0.001$ versus the corresponding vehicle-treated group. n = 6 per group.

3.7 Pharmacokinetics

Four hours after a single administration of naproxen, serum levels of naproxen averaged 98 ± 5 μM. Naproxen levels 4 hours after a single administration of ATB-346 were 53 ± 6 μM (not significantly different), while 4 hours after a single administration of NCX 429, serum naproxen levels were only 29 ± 8 μM ($p < 0.05$ versus the naproxen-treated group). When bile levels of naproxen were measured 4 hours after administration of the test drugs, some dramatic differences were apparent. In the naproxen-treated group, bile naproxen levels averaged 1.5 ± 0.3 μM, while bile naproxen levels in the rats treated with ATB-346 or NCX 429 were significantly ($p < 0.05$) lower, at 0.5 ± 0.1 and 0.2 ± 0.1 μM, respectively. Similar differences in serum and bile levels of naproxen (Figure 11) were observed in rats treated twice-daily for 2 days with naproxen, ATB-346 or NCX 429.

The mass spectrum analysis of bile samples from rats treated once or four times with the test drugs also detected a naproxen glucuronide, which was more evident in the samples from naproxen-treated rats than in samples from rats treated with ATB-346 or NCX 429. Thus, after a single administration of the test drugs, the ratio of the naproxen glucuronide in samples from naproxen-, ATB-346- and NCX 429-treated rats was 22:10:3. After four administrations of the test drugs, the ratio changed to 32:9:7.

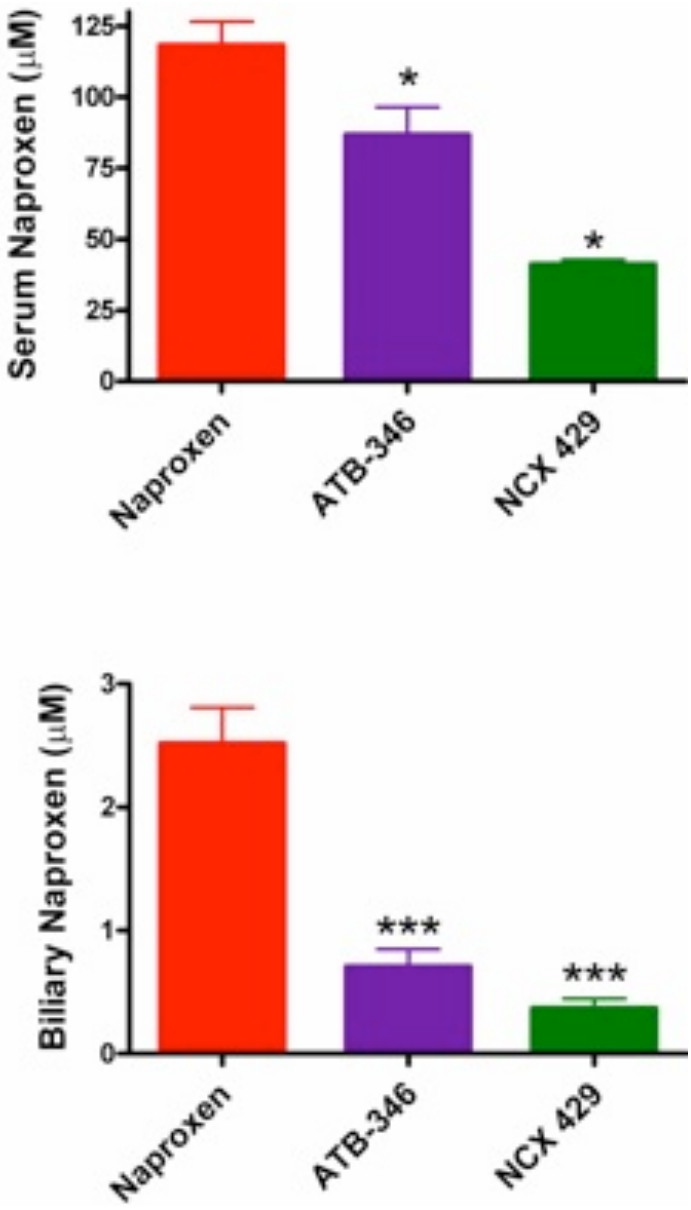

Figure 11. Serum and biliary levels of naproxen after test drug administration. Serum (top panel) and biliary (bottom panel) levels of naproxen are significantly reduced in rats given a naproxen derivative (ATB-346 or NCX 429) as compared to the levels observed in rats given an equimolar dose of naproxen itself. The test drugs were administered twice-daily for 2 days. *$p < 0.05$, ***$p < 0.001$ versus the naproxen-treated group. $n = 4$ per group.

3.8 Pharmacodynamics

At all time points after a single, oral dose of drug administration (15 min, 1 h, 3 h, 6 h, 12 h), naproxen (10 mg/kg) and ATB-346 (14.5 mg/kg) equally and significantly inhibited gastric PGE_2 synthesis (<85%) compared to rats that received vehicle (Figure 12). Similarly, naproxen and ATB-346 equally and significantly inhibited whole blood thromboxane synthesis (<95%) at all time points (Figure 12).

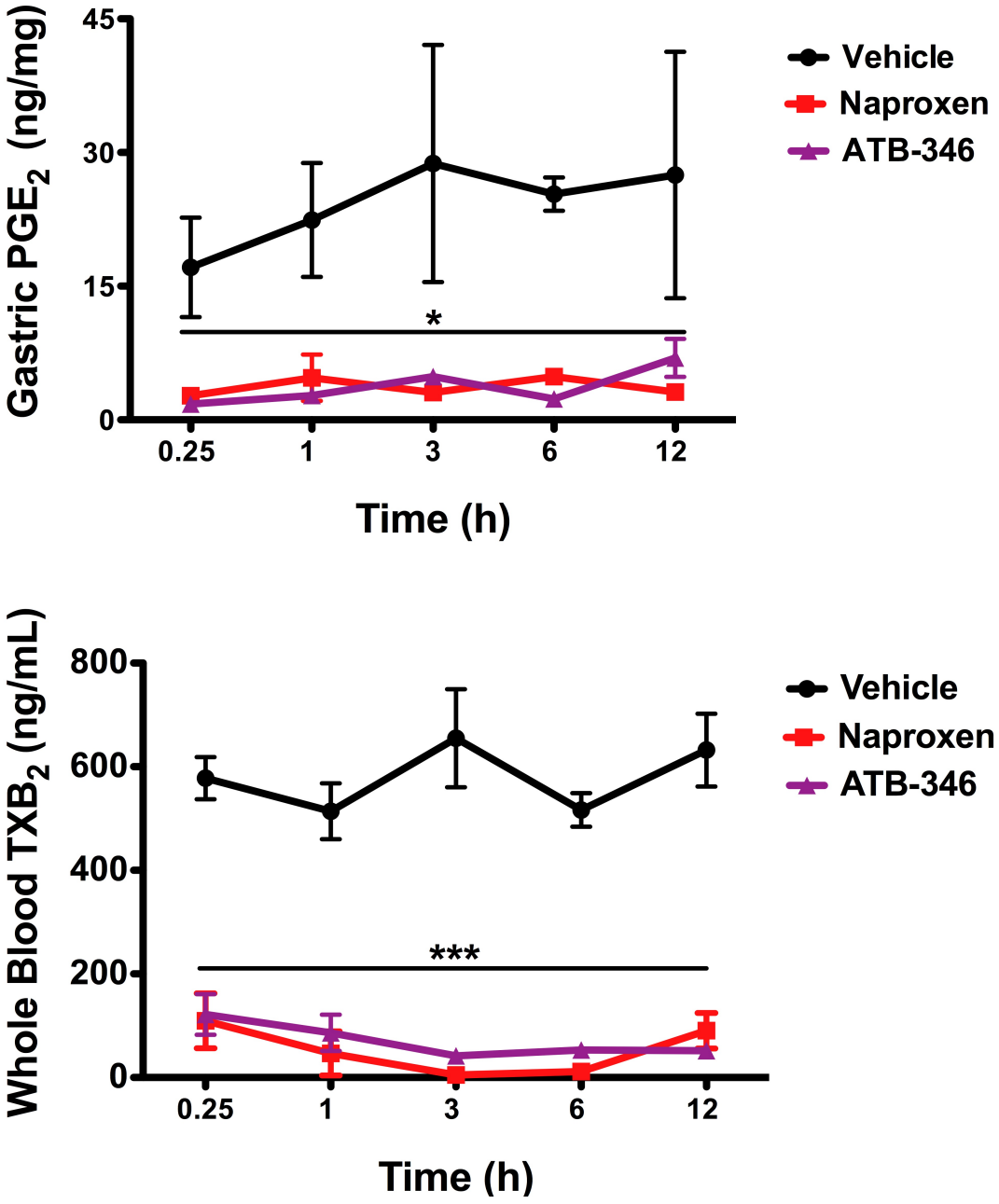

Figure 12. COX inhibition at various time points after naproxen or ATB-346 administration. ATB-346 suppressed gastric prostaglandin (PG)E_2 synthesis (top panel) and whole blood thromboxane (TXB_2) synthesis (bottom panel) as effectively as naproxen from 0.25-12 hrs after test drugs were administered as a single, oral dose. *$p < 0.05$, ***$p < 0.001$ versus the corresponding vehicle-treated group. n = 4 per group.

4.0 CONCLUSIONS

4.1 Gastrointestinal-sparing NSAIDs in Rat Co-morbidity Models

Rat models of obesity, advancing age, arthritis and polypharmacy exhibited a significant increase in susceptibility to naproxen-induced GI damage compared to young, healthy rats. Hypertensive rats did not exhibit increased susceptibility to naproxen-induced GI damage. In stark contrast to naproxen, ATB-346 and NCX 429 did not produce significant GI injury in healthy or susceptible rats despite effectively inhibiting both COX-1 and COX-2 enzymes. Thus, ATB-346 and NCX 429 have superior GI safety to existing NSAIDs in models that mimic clinical scenarios of impaired GI mucosal defence.

5.0 DISCUSSION

5.1 General Discussion

NSAID-induced gastroenteropathy is a significant limitation to the use of this class of drugs, which is a mainstream therapy for osteoarthritis and other chronic conditions characterized by inflammation and pain. The GI adverse effects of NSAIDs occur more frequently in the elderly, in patients taking anti-platelet/anti-coagulants (including aspirin), and in patients with co-morbidities such as rheumatoid arthritis, obesity, heart failure and hypertension (Solomon and Gurwitz, 1997)(Hernández-Díaz and Rodríguez, 2002)(Aro et al., 2006). Evaluation of tolerability and safety of NSAIDs in animal models that more closely mimic these clinical scenarios will likely provide more relevant predictive data than studies in healthy animals. In the present study, we compared the GI damage of several NSAIDs in rat models of arthritis, polypharmacy, obesity, advancing age and hypertension. Our results demonstrated that in most of these models (hypertension being the exception), the severity of NSAID-induced gastric and/or intestinal damage was markedly elevated as compared to that in healthy rats. In contrast to naproxen (and in some studies celecoxib), hydrogen sulfide- and nitric oxide-releasing naproxen compounds (ATB-346 and NCX 429, respectively) elicited negligible GI damage, despite still inhibiting the key target enzymes for their anti-inflammatory and GI-damaging effects (COX-1 and COX-2). The novel NSAIDs also exhibited comparable anti-inflammatory activity to naproxen and celecoxib in rats with adjuvant arthritis.

Studies in co-morbidity models may provide insights on the pathogenesis of NSAID-gastropathy and –enteropathy. For example, it was interesting that the older rats (19-month old), unlike younger rats (2-month old), developed severe gastric but not

intestinal damage. As well as the damage being more extensive in the stomach of older rats, it was also more severe in terms of depth of injury (i.e., penetrating ulcers rather than superficial erosions). The reasons for the propensity of gastric damage (rather than intestinal) in the older rats are unclear, but could be attributable to the reported deficiencies in gastric mucosal defence that occur with age. For example, impaired gastric production of nitric oxide, a key mediator of mucosal defence, has been reported in older rats, as has reduced gastric prostaglandin synthesis (Wallace and Miller, 2000)(Goto et al., 2001)(Vogiagis et al., 2000). Unlike advanced age, hypertension does not appear to be a risk factor for gastric or intestinal damage. Hypertensive and normotensive rats exhibited comparable gastric damage and neither group developed intestinal damage. Reports of hypertension as a potential risk factor for NSAID-induced gastrointestinal damage may in fact be due to a greater co-morbidity burden in these hypertensive patients, many of who are elderly and prescribed concomitant medications. Arthritic rats exhibited increased susceptibility to gastric and intestinal damage as compared to healthy controls. One of the factors that might contribute to this increase in injury is the enhanced NSAID-induced leukocyte adherence to the vascular endothelium that has been observed in arthritic rats (McCafferty et al., 1995). Leukocyte adherence to the vascular endothelium is a critical early event in pathogenesis of NSAID-gastropathy, and also plays an important role in the development of NSAID-induced injury in the small intestine (Wallace et al., 1990)(Wallace et al., 1993)(Miura et al., 1991).

Bacteria play a role in the initiation and chronicity of ulcers in the intestine and the stomach (Kent et al., 1969)(Uejima et al., 1996)(Elliot et al., 1998). Indeed, NSAID-induced small intestinal damage does not develop in germ-free animals (Uejima et al.,

1996). Obese Zucker rats have a distinct microbiome from their lean counterparts, with a significant reduction of *Bifidobacteria*, but we can only speculate, at this point in time, that these differences contribute to the increased susceptibility of obese rats to NSAID-induced enteropathy (Waldram et al., 2009).

We previously reported that suppression of gastric acid secretion in rats led to a dramatic shift in the microbiota (notably a significant decrease in *Bifidobacteria spp.*) and a marked increase in the susceptibility to NSAID-induced small intestinal damage (Wallace et al., 2011). The increase in susceptibility to damage could be transferred via the microbiota. In the present study, we extended those findings with the demonstration that administration of low-dose aspirin significantly increases the severity of NSAID-induced small intestinal damage, and found that even greater damage was observed when both omeprazole and low-dose aspirin were co-administered with the NSAID. This is a very common combination of drugs in humans. Proton pump inhibitors are given to reduce the incidence of NSAID-induced gastroduodenal damage, while low-dose aspirin is given to reduce the incidence of NSAID-associated cardiovascular events (Laine et al., 2003). No published human studies have examined the effects of co-administration of an NSAID, low-dose aspirin and a proton pump inhibitor on the small intestine. However, a high level of intestinal damage was observed in several video capsule endoscopy studies of healthy, young volunteers given an NSAID plus a PPI over a short period of time (Goldstein et al., 2005)(Graham et al., 2005)(Maiden et al., 2005), and detrimental effects of low-dose aspirin on the small intestine are well documented (Watanabe et al., 2008b). Given the results of the present study, and how widespread the polypharmacy approach is practiced, clinical studies of the GI impact of the combination of an NSAID, low-dose

aspirin and a PPI are warranted.

NO and H_2S have well characterized protective (Wallace, 2008)(Wallace and Miller, 2000) and ulcer-healing (Elliot et al., 1995)(Wallace et al., 2007) effects in the gastrointestinal tract, and both have been exploited in the design of GI-sparing anti-inflammatory drugs (Wallace and Del Soldato, 2003)(Wallace, 2007). In the present study, ATB-346 and NCX 429 spared the stomach and small intestine of damage, regardless of the model in which they were tested, and despite the fact that they markedly suppressed mucosal prostaglandin synthesis and platelet thromboxane synthesis. In addition, ATB-346 and NCX 429 demonstrated comparable pharmacodynamics to naproxen with regards to the swiftness and duration of COX enzyme activity inhibition. The GI-sparing effects are likely due primarily to the mucosal-protective effects of the gaseous mediator subsequently released from these drugs when absorbed (H_2S from ATB-346 and NO from NCX 429), and to the ability of these mediators to inhibit leukocyte adherence (Wallace and Vong, 2008)(Zanardo et al., 2006). Whether the GI-sparing characteristics of these two novel NSAIDs are attributable to their ability to generate mucosal protective gases or to other properties remains to be fully clarified. For instance, the pharmacokinetic studies suggest another mechanism for the safety of ATB-346 and NCX 429, particularly in terms of tolerability in the small intestine. The enterohepatic circulation of NSAIDs is critical to their ability to induce small intestinal injury (Reuter et al., 1997)(Kent et al., 1969). We observed that there were very low levels of naproxen in the bile following administration of ATB-346 or NCX 429, relative to biliary naproxen concentrations following administration of naproxen itself. It has been demonstrated that esterification of acidic NSAIDs partly suppresses their gastropathy

without adversely affecting anti-inflammatory activity (Rainsford, 1999). Unlike naproxen, ATB-346 and NCX 429 do not have free carboxylic acid residues; due to the substitution of the gaseous-releasing moieties, so would likely have greatly reduced topical irritant properties than naproxen.

In summary, animal models of obesity, advancing age, arthritis and polypharmacy exhibit a significant increase in susceptibility to NSAID-induced GI damage. These models may be more predictive of the properties of NSAIDs in the subset of humans that develop the most adverse GI events when taking these drugs. H_2S- and NO-releasing derivatives of naproxen were very well tolerated in these co-morbidity models.

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APPENDIX

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