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## **Mechanisms of Damage to the Gastrointestinal Tract From Non-steroidal Anti-inflammatory Drugs**

**Short title:** Pathogenesis of NSAID induced GI damage

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**Abbreviations:** NSAIDs: Non steroidal anti-inflammatory drugs; COX: Cyclooxygenase;  $pK_a$ : Logarithmic transformed acid dissociation constant; ATP: Adenosine triphosphate; NADH: Nicotinamide adenine dinucleotide; MPTP: mitochondrial membrane transition pore; 6-MNA: 6-methoxy naphthalene acetic acid; TLR: Toll-like Receptor

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general comments. All authors contributed to the interpretation of data.

ACCEPTED MANUSCRIPT

**ABSTRACT**

Non-steroidal anti-inflammatory drugs (NSAIDs) can damage the gastrointestinal tract, causing widespread morbidity and mortality. Although mechanisms of damage involve the activities of prostaglandin-endoperoxide synthase 1 (PTGS1 or COX1) and PTGS1 (COX2), other factors are involved. We review mechanisms of gastrointestinal damage induction by NSAIDs, via COX-mediated and COX-independent processes. NSAIDs interact with phospholipids and uncouple mitochondrial oxidative phosphorylation, which initiates biochemical changes that impair function of the gastrointestinal barrier. The resulting increase in intestinal permeability leads to low-grade inflammation. NSAID's inhibition of COX enzymes, along with luminal aggressors, results in erosions and ulcers, with potential complications of bleeding, protein loss, stricture formation, and perforation. We propose a model for NSAID-induced damage to the gastrointestinal tract that includes these complex, interacting, and inter-dependent factors. This model highlights the obstacles for the development of safer NSAIDs.

**Key words:** GI; prostaglandin; drug-induced intestinal damage; bacteria, bile acids

More than 30 million people take non-steroidal anti-inflammatory drugs (NSAIDs) each day (1). This number has grown significantly with increasing use of over the counter and prescription NSAIDs, low-dose aspirin and following reports of their potential anti-neoplastic effects. The efficacy of NSAIDs as anti-inflammatory analgesics is not in doubt, but their adverse events are problematic. These relate mainly to cardiovascular, renal, hepatic, and the gastrointestinal tissues. The cardiovascular adverse events have recently received much attention (2, 3), but the frequency and severity of the gastrointestinal damage continues to cause concern. Accordingly the range of gastroduodenal ulcer rates range from 5% to 80% in short-term endoscopy studies (4) and from 15% to 40% in long-term users (5). NSAIDs also damage the small intestine (6)—as many as 70% of long-term users of NSAIDs have small intestinal inflammation, and 30% have erosions or ulcers (7). The gastric and small bowel damage is associated with various management problems and at times life threatening complications, such as bleeding, strictures and perforations.

There have been many studies of the pathogenesis of NSAID-induced gastrointestinal damage. NSAIDs inhibit prostaglandin-endoperoxide synthase 1 (PTGS1 or COX1) and COX2, which have been believed to mediate the gastrointestinal damage (8-10). NSAID-induced decreases in mucosal levels of prostaglandins (driven by inhibition of COX1) correlate with gastric and small bowel damage (11-13), which can be attenuated by administration of exogenous prostaglandins (14-18). Since COX2 is not constitutively expressed in the gastrointestinal tract COX2 selective inhibitors are perceived as safer than conventional NSAIDs (14, 15, 19, 20). Proposed mechanisms of damage to the stomach involve prostaglandin mediated increased gastric acid secretion, decreased mucus and bicarbonate secretion, decreased cell proliferation, and decreased mucosal blood flow (21-24). These are all actions that are detrimental to mucosal defense and healing, but the observed changes were only modest (21, 23, 25-30) and the damage seemed to lack an initiative action. Furthermore, decreased mucosal prostaglandins have been found to be less important in the pathogenesis of the small bowel damage (11, 31, 32).

Further studies showed that gastric and small bowel mucosal prostaglandins could be decreased by 95%–98% without mucosal damage (33-35), confirmed in COX1-knockout mice (35-37). Short-term loss or inhibition of COX2 does not cause damage, but small bowel damage is evident in mice and humans exposed to NSAIDs for long periods of time (38-41). Dual inhibition of COX1 and COX2 causes

gastric and small bowel lesions, albeit somewhat less severe than that the lesions caused by conventional acidic NSAIDs (36).

So, inhibition of COX does not seem to be the only mechanism of NSAID-induced gastrointestinal damage. We review the prostaglandin-independent mechanisms of NSAIDs and how these interact with the consequence of alterations in prostaglandin levels as a consequence of COX inhibition. We provide a model in which COX inhibition is one of several important factors in the pathogenesis of gastrointestinal damage (see Figure 1). Our model considers the effects of the specific biochemical “topical” effects of NSAIDs (i.e. the effects that occur by direct contact between the NSAIDs in the lumen and mucosal epithelium following oral ingestion and/or biliary excretion of the drugs, as opposed to topical skin application) and the consequential increase in intestinal permeability and intestinal inflammation. These initiate damage and inhibition of COX1 and COX2 aggravate it, along with luminal aggressors, leading to development of erosions and ulcers (42, 43).

### **BIOCHEMICAL EFFECTS OF NSAIDS**

The biochemical actions common to all conventional NSAIDs are their “topical” effects, and inhibition of COX1 and COX2. These biochemical actions are brought about by the physicochemical properties that NSAIDs share (44-46), namely being lipid soluble weak acids (see Figure 2). This combination provides them with detergent action (interaction with phospholipids), uncoupling of oxidative phosphorylation, and non-covalent inhibition of COX1 and COX2. These biochemical activities depend on the same physical and chemical characteristics, so changing these will change all the pharmacologic actions. For example, esterification of NSAIDs (47) causes loss of their “topical” effects and at the same time their ability to inhibit the COX enzymes.

#### *Interactions between NSAIDs and phospholipids*

NSAIDs interact with the intestinal mucus layer and the cell surface phospholipid bilayer. There are subtle differences in mucus thickness and composition in different regions of the gastrointestinal tract (19, 48). The role of mucus is to act as a lubricant between the surface epithelium and the luminal contents, restricting access of large hydrophilic molecules, digestive enzymes, and bacteria to the surface epithelium. In the stomach, mucus also buffers luminal acids. The production and secretion of mucus is determined by interactions between luminal aggressors (acid, pepsin, *H pylori*

in the stomach and bile and bacteria in the small bowel) and the surface epithelium mediated by numerous factors such as inflammatory cytokines and prostaglandins.

Mucus serves as a matrix for phospholipids that maintain gastrointestinal integrity (49). Like NSAIDs, phospholipids are amphiphilic molecules, with a hydrophilic polar head group and a hydrophobic tail region. The integrity of the mucus layer can be assessed by various methods (50). NSAIDs decreased the hydrophobicity in the gastroduodenal mucosa (51), an effect seen also after parenteral administration via the biliary excretion of the drug (52). The interaction between NSAIDs and phospholipids compromises the hydrophobic lining, which leads to mucosal exposure to luminal aggressors (acid and pepsin in the stomach and bacteria and bile in the small intestine).

The concept of a hydrophobic barrier attributed to phospholipids and the binding of NSAIDs to dipalmitoylphosphatidylcholine (the dominant phospholipid in the gastrointestinal-tract), *in vitro* and *in vivo* (49, 53), led to a series of studies investigating the effect of orally co-administered phospholipids with NSAIDs, and other toxic compounds, with a view to diminishing their toxicity. Combining NSAIDs with the phospholipid phosphatidylcholine protects against NSAID-induced gastric (49, 54) and small bowel (55) damage in short-term rodent studies. Lichtenberger et al demonstrated decreased gastric toxicity of the otherwise damaging combination of aspirin and a COX2-selective agent, if the aspirin was co-administered with a phospholipid (56).

These and other animal studies provided the platform for testing the safety of NSAIDs combined with phospholipids in humans. Volunteers were given aspirin or a combination of aspirin and phospholipid (650 mg aspirin/day for 3 days). The number of gastric erosions (assessed during endoscopy) was significantly lower in volunteers given aspirin and phospholipid (mean  $2.8 \pm 4.3$ ) than aspirin alone (mean  $8.8 \pm 10.8$ ); both drugs reduced mucosal prostaglandin content to the same extent (57). In a separate study, healthy volunteers given aspirin (325 mg/day for 7 days) or the same amount of aspirin combined with phosphatidylcholine, had a significant decrease in gastric ulcers, from 17.6% in volunteers given aspirin to 5.1% in volunteers given aspirin with phosphatidylcholine (58). In a 6-week study of patients with osteoarthritis, the combination of ibuprofen and phosphatidylcholine was associated with significant improvements in Lanza gastroscopy scores, compared to patients given ibuprofen

(2400 mg) alone, but only in patients older than 55 years (59). These studies demonstrated greater gastric tolerability of combinations of aspirin and phospholipid, in the short-term, in humans, in which damage is more likely to be caused by the physicochemical properties of NSAIDs than their effect on COX1 or COX2 (4).

#### *Uncoupling mitochondrial oxidative phosphorylation*

Mitochondria are the main source of ATP in cells. Mitochondrial ATP synthesis takes place by integrated biochemical-physiological-physical processes (60) (see Figure 3).

Whatever the cause of uncoupling there is a cascade of detrimental downstream effects: water flows into the matrix, causing characteristic and pathognomonic swelling of mitochondria. There is release of intra-mitochondrial  $\text{Ca}^{2+}$  into cytoplasm with depletion of reduced glutathione, depletion of  $\text{NAD(P)H}_2$ , generation of superoxide anion ( $\text{O}^{2-}$ ) and release of pro-apoptogenic proteins (61). Free radicals accumulate within the mitochondria setting up a vicious cycle as this activates uncoupling proteins in the inner mitochondrial membrane (62). The uncoupling ultimately leads to depletion of cellular ATP levels, with loss of integrity of the intercellular junctions in the gastrointestinal tract (leading to increased mucosal permeability) (63), and ultimately apoptosis and cell death (64).

Well before the understanding that NSAIDs inhibited the COX enzyme(s) it was evident that NSAIDs were uncouplers of mitochondrial oxidative phosphorylation (65, 66). Adams et al screened possible anti-inflammatory agents based on their uncoupling properties and several (such as ibuprofen, naproxen and indomethacin) have been marketed on that basis. However, the idea of the uncoupling action of NSAIDs as a mechanism for their therapeutic actions became obsolete when the prostaglandin hypothesis gained momentum.

A few reports describe uncoupling of mitochondrial oxidative phosphorylation in the gastric mucosa following aspirin (67, 68). Using the technique of selective subcellular marker enzyme analyses of small bowel mucosa following administration of NSAIDs in animals (69) showed a significant change in the brush border marker enzyme, compatible with the interaction of NSAIDs with phospholipids, and the mitochondrial marker enzymes. Electron microscopic changes of uncoupling were demonstrated in vivo after administration of NSAIDs to rats (69). The in vitro uncou-

pling of conventional acidic (carboxylic or enolic acids) NSAIDs relates to their  $pK_a$  values (see Table 1) (70). Drugs that are purported to be safer such as paracetamol (non acidic analgesic), nabumetone (a non-acidic NSAID pro-drug (71), and esterified non-acidic pro-NSAIDs (see Figure 2), such as nitro-butyril flubiprofen are not uncouplers in vitro (69).

Micromolar to millimolar concentrations of NSAIDs have the ability to uncouple mitochondrial oxidative phosphorylation in vitro (42, 69, 72-76), due to ion trapping during absorption (see Figure 4). COX2-selective agents also uncouple oxidative phosphorylation in vitro and in cell systems, but with lower potency than that of acidic NSAIDs (76, 77). The uncoupling by NSAIDs was demonstrated by electron microscopy in the small bowel of mice given conventional acidic NSAIDs (42, 69, 73-75, 78, 79) and similar changes are also found in gastric biopsies from patients (67, 68, 80-83). No studies have assessed the possible prevention of uncoupling brought about by NSAIDs.

### **INHIBITION OF COX1 AND 2 AND ROLE OF PROSTAGLANDINS**

The 3-dimensional structure of the COX enzymes reveals the active site of both COX isoforms to be at the end of a hydrophobic channel. NSAIDs inhibit the enzyme by blocking the entrance of arachidonic acid to this channel and thereby denying substrate access to the active site (84, 85). The COX1 and 2 channels differ. Conventional NSAIDs have access to both channels and form an ionic bond via their carboxyl or enolic group (86). The COX1 channel is smaller than the channel in COX2 and does not accommodate COX2-selective agents, but a side pocket in the COX2 enzyme has a polar binding site (87) for the aryl sulfonamide and sulfone moieties of the COX2-selective agents.

The most damaging consequence of decreased prostaglandin production with COX inhibition could be the effects on the microcirculation. Regulation and maintenance of the intestinal microcirculation is complex involving several interacting biochemical mechanisms. The most relevant mediators are prostaglandins, leukotrienes, nitric oxide, and hydrogen sulphide. NSAID-induced prevention of physiological compensatory increases in blood flow (leading to tissue hypoxia) following injury is well described. The effects of nitric oxide and hydrogen sulphide are remarkably similar to that of prostaglandins, namely increased microvascular blood flow, in-

creased mucus secretion, and a modest decrease of gastric acid secretion (88, 89). Targeting these processes with nitric oxide donors such as nitro-glycerine, nitropruside, nitric-oxide-NSAIDs, and hydrogen sulphite NSAIDs can reduce the gastrointestinal damage due to NSAIDs in laboratory animals (27, 90-93). Presumably these effects counteract the reduced microvascular blood flow (94) consequent to NSAID-induced decreased prostaglandins (95). Proof-of-concept endoscopic studies of healthy volunteers showed that nitric oxide donors and NSAIDs reduced gastroduodenal damage, compared with NSAIDs (96, 97), but the results of a longer-term clinical trial did not show statistically significant differences (98).

Another vascular effect of NSAIDs involves NSAID-induced expression of neutrophil adhesion molecules within the endothelium (common to most intestinal inflammatory conditions) (27, 29, 93, 99). Neutrophil accumulation could mechanically compromise microvascular blood flow. Nitric oxide and hydrogen sulphite are, like prostaglandins, inhibitors of leucocyte adhesion to the vascular endothelium (100).

However, vascular effects are probably not the primary or initiating event in NSAID-induced gastrointestinal damage. The effects on the vasculature cannot account for the selective localization of the macroscopic damage (101-104) within the gastrointestinal tract nor the mesenteric rather than the anti-mesenteric location of small bowel ulcers. The damage also differs macroscopically and microscopically from ischemic damage. The suggestion that neutrophil adhesion to the vessel wall (a COX2-mediated effect) is a primary event in the damage is difficult to reconcile with the fact that COX2 is not constitutively expressed in the gastrointestinal tract. Furthermore, neutrophil adhesion to the intestinal vessel wall does not automatically indicate damage as neutrophils require a chemoattractant for activation-degranulation and hence damage (105, 106).

#### *Consequences of the biochemical effects of NSAIDs*

Studies on COX-knockout mice have increased our understanding of the consequences of COX1 and COX2 deficiency. Absence or selective inhibition of COX1 (by the non-acidic COX1 inhibitor, SC-560) reduced levels of prostaglandins by 95% or more, which was not associated with increased intestinal permeability, inflammation, or ulcers (35, 36). Neither was short-term, selective deletion or inhibition of COX2 (36, 39). These findings should be considered alongside studies that assess the

consequences of the “topical” effects and dissociated these from the consequences of COX inhibition. These studies were done by comparing key pathophysiological events in the damage, namely the “topical” effect (in vitro and in vivo uncoupling), prostaglandin levels, intestinal permeability, and inflammation following the use of selective drugs. This provides convincing evidence that the “topical” effects (phospholipid-NSAID interaction and uncoupling) initiate gastrointestinal damage, but only with COX1 inhibition (in association with luminal aggressive factors), does this lead to mucosal erosions and ulcers. The compounds and their effects can be categorized as follows (see Table 2):

- Selective uncouplers (dinitrophenol [DNP] or R-flurbiprofen) can increase intestinal permeability associated with mild inflammation, but do not significantly alter mucosal prostaglandin levels, and do not cause mucosal ulceration.
- Uncouplers (conventional acidic NSAIDs) that inhibit COX enzymes are associated with increased intestinal permeability, inflammation, and ulcers.
- COX2-selective agents such as celecoxib do not uncouple oxidative phosphorylation (nimesulide with a  $P_{K_a}$  of 6.4, despite showing uncoupling activity, behaves like celecoxib—possibly because the uncoupling effect in vivo affects only a few mitochondria). These agents are not associated with increased intestinal permeability, inflammation or ulcers.

Collectively these studies, together with studies of knockout mice, have provided compelling evidence that uncoupling of mitochondrial oxidative phosphorylation (along with the NAID-phospholipid interaction) increases intestinal permeability and low-grade inflammation. Decreased mucosal prostaglandin production and the mucosal aggressors lead to more severe inflammatory and ulcerative damage, perhaps via effects on the microcirculation.

The findings from COX2-knockout mice are more difficult to explain. These mice have normal mucosal levels of prostaglandin, but half have normal intestinal permeability and no inflammation or intestinal ulcers, and the other half develop small intestinal inflammation and ulcers or die because of ulcer perforation. Similar findings were seen with long-term administration of a selective COX2 inhibitor to wild-type mice. COX2 inhibition also leads to enteropathy in humans (41).

## TISSUE REACTION AND ROLE OF LUMINAL AGGRESSORS

The tissue reaction is characterized by inflammation and the presence of erosions and ulcers and this appears to be driven by COX inhibition and the luminal aggressive factors. The luminal aggressors differ between the stomach (acid, pepsin, and *H pylori*) and small bowel (bile and commensal bacteria). The importance of gastric luminal aggressors is widely appreciated, but the same does not hold true for small bowel aggressors. Our review focuses on effects in the small bowel.

### *Role of acid and H pylori in NSAID-induced gastropathy*

The importance of gastric acid in the damage of NSAID-induced gastro-duodenal damage in humans is amply demonstrated clinically in the reduced incidence of damage (short and long-term) and serious ulcer outcomes when NSAIDs are co-administered with proton pump inhibitors (107, 108) or high dose histamine receptor-2 inhibitors (109). In the context of the current pathogenic model the macroscopic damage in the stomach is principally due to back diffusion of acid due to the impaired barrier function (brought about by the “topical” effects) induced by NSAIDs and amplified by the prostaglandin dependent effects induced by NSAIDs. The frequent finding of chemical gastritis (reactive gastritis) in antral biopsies in patients on NSAIDs (110), who do not have *H pylori* infection, can be considered as the consequence of the “topical” effect of these drugs. In this context, the mucosal inflammatory reaction is weak compared to that seen in patients infected by *H pylori*.

The effects of *H pylori* infection in the pathogenesis of NSAID-associated gastric ulcers is controversial. *H pylori* does not seem to mediate development of short-term NSAID-induced gastric damage in humans (4), although it may affect gastric adaptation to short-term administration of aspirin (111). Gastric damage induced by long-term NSAIDs or aspirin occurs in addition to the gastritis induced by *H pylori* infection, which occurs early in life. *H pylori* induces gastric mucosal lesions by interacting with the immune response (112). The intrinsic virulence factors of each specific *H pylori* strain may induce a weak or a strong host immune cytokine-mediated inflammatory response, which is genetically determined. Patients infected by *H pylori* may develop pangastritis or antral predominant gastritis, which affect acid secretion levels. Pangastritis is usually associated with normal or reduced gastric acid secre-

tion whereas antral predominant gastritis is associated with increased acid secretion due to a decrease in somatostatin and increased gastrin secretion (113, 114). Therefore, the type of gastritis associated with *H pylori* may explain the contradictory results obtained in different clinical studies (113, 114). *H pylori* exacerbates aspirin-induced gastric damage associated with normal or increased gastric acid secretion but reduces the damage in patients who became hyposecretors (115). A meta-analysis concluded that NSAIDs and *H pylori* infection were independent but additive risk factors for development of peptic ulcer, when taken long term, and separately in the ulcer complication of bleeding (116).

#### *Role of bile in NSAID-induced enteropathy*

Bile contributes to intestinal and gastric damage caused by NSAIDs (23, 117), but the biochemical mechanisms have not been established. The severity of NSAID-enteropathy correlates to the amount of the drug excreted in bile and with the extent of enterohepatic circulation (117, 118). Bile duct ligation almost completely abolishes the small intestinal macroscopic damage following NSAIDs (119, 120).

Bile and the NSAIDs excreted in bile play have complex roles in the pathogenesis of NSAID-induced small intestinal damage. Conventional NSAIDs cause small intestinal lesions in rats regardless of whether they are given orally or parenterally, but drugs such as aspirin and 6-MNA (the active component of the non-acidic pro-NSAID nabumetone), which are not excreted in bile, do not, when given parenterally (121). This indicates that the combination of NSAIDs and bile are more toxic than either alone. When certain bile acids (taurocholic acid, taurodeoxycholic acid and glycocholic acid) were co-administered with indomethacin, the incidence and severity of gastric and small bowel damage was significantly increased in rats (122, 123).

Bile collected from rats given indomethacin that was then infused into small intestinal loops of untreated rats (124) reduced the hydrophobicity of the mucosa and caused ileal bleeding. These effects were abolished when phosphatidylcholine was added to the bile (from the indomethacin treated rats) prior to instillation into the small bowel. Furthermore certain bile acids caused identical damage and this was again reversed by addition of equimolar phosphatidylcholine. It was suggested that NSAIDs that enter the bile might damage the mucosa, not by a direct action, but by

competing for the available protective phosphatidylcholine molecules. Increased amounts of unbound bile acids could therefore increase the indomethacin-induced (macroscopic) damage. Dial et al similarly showed that bile was cytotoxic following indomethacin administration but this effect was reversed when phosphatidylcholine was added to the bile-indomethacin mixture (125) again emphasising the NSAID-phospholipid interaction. Furthermore, although primary and secondary bile acids have differential potential to cause damage to intestinal epithelial cells, they also act as effector molecules that activate nuclear and G-protein-coupled receptors; collectively known as bile acid-activated receptors, these help maintain intestinal integrity (126).

Bile therefore appears to have an important role in the pathogenesis of small bowel damage. It has been shown to maintain and disrupt intestinal integrity. The choice of the bile acids used in a study is important because bile acids differ in their gastrointestinal tolerability (122, 127). For example, taurochenodeoxycholic acid increases intestinal inflammation caused by indomethacin, whereas ursodeoxycholic acid reduces the damage (128, 129) and chenodeoxycholic acid may be neutral (130)

The effects of diclofenac on bile excretion have been investigated in considerable detail. Diclofenac is metabolized by the liver and the major biliary metabolite, diclofenac acyl glucuronide, is excreted by a specific hepatocanalicular conjugate export pump. Rats deficient in this transporter have normal bile composition and flow, but do not excrete diclofenac or its conjugate into bile (131). These rats had significantly less small bowel damage when given diclofenac orally or parenterally. Furthermore, bile containing diclofenac glucuronide increased small bowel damage in normal rats, and transferase-deficient rats over and above diclofenac and bile mixed together. Moreover, increasing the activity of glucuronosyltransferase, which increases glucuronidation of diclofenac, increased small bowel damage. This indicates that biotransformation of diclofenac (acyl glucuronide or its oxidative metabolites) accounts for a significant part of its small bowel toxicity. Of note is the fact that most carboxylic acid-NSAIDs are metabolised to acyl-glucuronides in a similar fashion. Although these conjugates are reactive in their own right, they are also deconjugated by bacterial beta-glucuronidase yielding aglycone, which is believed to be even more toxic (132). In an attempt to assess the importance of bacterial beta-

glucuronidase (133), researchers gave mice diclofenac (intraperitoneally), with or without pre-administration of a specific inhibitor of bacterial beta-glucuronidase. The inhibitor reduced the number of small bowel erosions and ulcers significantly. Similar results were obtained when indomethacin and ketoprofen were used (134).

The interaction between biliary excretion of NSAIDs and intestinal bacterial deconjugation (which may be enhanced by concomitant treatment with proton pump inhibitors (135)) possibly provides an explanation for the mid and distal small bowel location of NSAID-enteropathy. However, it is important to remember that there are significant differences between species in the extent of enteric hepatic circulation of carboxylic NSAIDs (relatively low in humans) (136), although all seem to be associated with NSAID-enteropathy to a similar extent in humans (137). In particular there is very little, if any, biliary excretion of ibuprofen or its metabolites in humans (138), but this NSAID is still associated with enteropathy.

The practical implications from the experiments in animals (119, 120, 139) are that co-administration of a bile-binding resin, such as cholestyramine, with NSAIDs might reduce or prevent some of the small bowel damage. Co-administration of a specific inhibitor of bacterial beta-glucuronidase with NSAIDs might also prevent damage, but this has not yet been tested in clinical trials.

#### *Role of bacteria in NSAID-induced enteropathy*

It is difficult to dissociate the effect of intestinal bacteria on the metabolism of NSAID-conjugates and formation of secondary bile acids to their more direct role to cause or increase inflammation in NSAID-enteropathy. Nevertheless, germ-free rats and rats given antimicrobial agents do not develop small bowel ulcers when they are given indomethacin (140). Indomethacin-induced enteropathy in mice is associated with numerous alterations in the number and type of bacteria (135, 140, 141) The precise and specific bacterial alterations (true increases, relative shifts, etc.) and effects are well documented, but probably not relevant to humans, because their microbiomes differ substantially.

The mechanisms of interactions between the effects of NSAIDs on the microbiome and human cells could be mediated by lipopolysaccharide, a bacterial protein that binds to and activates toll-like receptor 4 (TLR4). TLR4 signalling activates nuclear factor- $\kappa$ B, resulting in neutrophil recruitment (142, 143). Neutrophils are im-

portant effector cells in the macroscopic damage due to NSAIDs, demonstrated by the findings that neutropenic mice do not develop macroscopic lesions in response to NSAIDs (144). These findings might offer therapeutic possibilities, such as inhibiting TLR4 or interfering with neutrophil functions.

The effects of intestinal bacteria on induction of enteropathy by NSAIDs has been studied in humans. A capsule enteroscopy study in volunteers showed that co-administration of the poorly absorbed anti-microbial rifaximin with NSAIDs prevented development of erosions and ulcers (145). Patients with established NSAID enteropathy, metronidazole reduced inflammation and bleeding but did not affect intestinal permeability (146).

An alternative approach is to reduce or prevent NSAID-induced small bowel damage with probiotics, although results from studies of probiotics have been inconsistent. In a clinical trial, the probiotic VSL-3 prevented the small bowel damage due to indomethacin (50 mg/day), assessed by fecal levels of calprotectin (147). In patients taking aspirin and a proton pump inhibitor who had iron-deficiency anaemia, the probiotic *Lactobacillus casei* significantly reduced mucosal damage, based on capsule endoscopy analysis, compared with controls (148). However, many additional studies must be performed before specific probiotics can be recommended for prevention or treatment of NSAID-enteropathy in humans.

### **Future Directions**

Prevention and treatment of the adverse events of NSAIDs on the gastrointestinal tract requires knowledge of mechanisms of pathogenesis of the lesions. The complexities of the pathways to this damage have been evident for a long time, but have not received much attention, presumably because the effects of inhibiting COX enzymes offer simple and logical explanation for the damage. This hypothesis led to development of the COX2-selective agents with increased gastrointestinal safety. However, studies of knockout mice (especially COX1- and COX2-knockout mice) and development of drugs with highly specific actions increased our understanding of the effects of NSAIDs. We now recognize that inhibition of COX1 or COX2 does not solely account for the gastrointestinal damage induced by NSAIDs. NSAIDs have “topical” effects that damage intestinal cells by disrupting membrane and mucus phospholipids and uncoupling of mitochondrial oxidative phosphorylation.

NSAIDs increase intestinal permeability in patients (149), leading to low-grade intestinal inflammation. Disruption of the intestinal barrier is associated with many human small bowel diseases that are distinctively different to the damage seen with NSAIDs (43). NSAIDs also have microvascular effects that aggravate inflammation and lead to macroscopic damage, such as erosions and ulcers in the stomach and the small bowel. It should be noted that these observations relate to the pathogenesis of damage, but not necessarily the clinical adverse effects. Clinically serious gastric and small bowel ulcer events of perforation and bleeding involve separate clinical and co-morbidity factors (150).

Our model emphasizes the multi-stage complexities of the pathogenesis and the numerous interactive and ongoing synergistic factors that intensify or modulate the damage. For example, the increased intestinal permeability that is brought about by the “topical” effects of NSAIDs is intensified because of the inflammatory response (to luminal aggressors) and the microvascular effects of COX inhibition, etc. Conventional NSAIDs cause maximum intestinal damage whereas the various combinations of the biochemical actions observed experimentally, such as selective inhibition or absence of COX1 and 2 (without the “topical” effect), “topical” effect combined with COX1 absence or inhibition (without COX2 involvement), “topical” effect combined with COX2 absence or inhibition (without COX1 involvement) can increase tolerability, but do not fully prevent intestinal damage.

In patients, strategies to alter or minimize a single biochemical effect of NSAIDs, such by co-administration of a phospholipid, esterification of NSAIDs (with or without the addition of nitric oxide or hydrogen sulfite moieties), or use of selective COX2 inhibitors (which spare COX1 and reduces the “topical” effect) does not remove their toxicity. Altering the physical and chemical properties of NSAIDs to alter their efficacy or tolerability is impractical, because the same physicochemical properties of NSAIDs mediate their “topical” effects and effects on COX enzymes. Strategies to interfere with their non-biochemical actions, such as the luminal aggressors, could be a more realistic approach for reducing NSAID-induced small bowel damage in patients. By analogy inhibition of gastric acid secretion prevents and heals NSAID-associated ulcers.

The current model is largely based on findings from rodents, which have many differences from humans in physiology, biochemistry, immunology etc., and

not least the gastrointestinal tract microbiome. Furthermore, in these studies, NSAIDs were administered to the animals at doses that are an order of magnitude higher than doses taken by patients, and the compounds used to solubilize NSAIDs given to animals are toxic. Extrapolation of data from animal studies to humans therefore requires great care. However, some aspects of the damage show remarkable similarities, such as the increase in intestinal permeability seen with NSAIDs, the localization of NSAID enteropathy to the mid to distal small bowel, similar responses to some therapeutic interventions, etc. Animal experiments are a convenient way to explore pathogenic processes, but findings must be confirmed in human studies.

Many view the clinical importance of NSAID-induced gastropathy to the exclusion of NSAID-induced enteropathy and, moreover, there have been very few attempts to minimize the incidence or clinical impact of NSAID-induced enteropathy. This may be because of selective funding for research into the treatment of NSAID-induced gastropathy, but also because NSAID enteropathy has been perceived as being asymptomatic and benign. However, most patients with NSAID-induced enteropathy bleed from the small bowel (146, 151), which frequently leads to an iron deficiency anemia (152), occasional hypoalbuminemia, diaphragm disease (6), and even death from intestinal perforation with peritonitis (153). Increasing understanding of the mechanisms of NSAID-induced damage to the small bowel, should stimulate further research and reduce these clinical effects.

**Table 1. Relationship Between pKa and Uncoupling of Mitochondrial Oxidative Phosphorylation**

Drug	pK <sub>a</sub>	Percentage maximum uncoupling	Mean±SEM concentration required for maximum uncoupling (microM/mg protein)
Nitrosalicylic acid	2.3	205	2.70 ± 1.21
Salicylic acid	2.94	200	2.10 ± 1.23
Acetylsalicylic acid	3.5	200	1.6 ± 1.19
Diclofenac	4.0	200	0.43 ± 0.22
Naproxen	4.15	210	0.61 ± 0.16
Flurbiprofen	4.22	265	0.51 ± 0.19
Indomethacin	4.5	230	0.15 ± 0.12
6-MNA	5.0	180	0.46 ± 0.27
Ibuprofen	5.2	250	0.28 ± 0.18
Ketoprofen	5.94	220	0.38 ± 0.12

Piroxicam	6.3	215	0.20 ± 0.11
Azapropazone	6.3	210	0.02 ± 0.02

Notes: Data derived from in vitro experiments with conventional NSAIDs.  
The maximum degree of respiration stimulation was similar among the NSAIDs tested, but the concentration needed for maximum stimulation differed. The more acidic the NSAID the higher concentration required for maximum uncoupling (Spearman's correlation coefficient [r] = 0.87,  $P < .001$ ; n = 12).

**Table 2. Results of Studies of Uncoupling and Other Factors That Contribute to Small Bowel Damage From NSAIDs**

Reference	Drug	Uncoupling		Mucosal Level of PGE2	Intestinal		
		vitro	vivo		Permeability	Inflammation	Ulcers
(75)	Flurbiprofen	+	+		+		+
	NO-flurbiprofen	0	+		+		+
(73)	DNP	+	+	+10%	+	+	0
	R-flurbiprofen	+	+	-12%	+	+	0
	R + S flurbiprofen	+	+	-92%	+	+	+
	S- flurbiprofen	+	+	-89%	+	+	+
(78)	Indomethacin	+	+ a	Reduction of 71%–96%	+	+	+

	Nimesulide	+	+ b	0–75%	0	0	0
(154)	DNP	+	+	+12%	+	+	0
	Indomethacin	+	+	–89%	+	+	+
	Aspirin	+	0	–88%	0	0	0
	Aspirin + DNP	+	+	–81%	+	+	+
(79)	Indomethacin	+	+	–90%	+	+	
	Celecoxib	0	0	0%	0	0	0
(36)	COX1 <sup>-/-</sup>			–97%	0	0	0
	COX1 <sup>+/+</sup> + SC560			–97%	0	0	0
	COX2 <sup>-/-</sup> (50%)			96%	0	0	0
	(50%)			94%	+	+	+
DNP, dinitrophenol; SC560, selective non-acidic inhibitor of COX1; <i>Cox1</i> <sup>+/+</sup> , full-length <i>Cox1</i> gene in mice; <i>Cox1</i> <sup>-/-</sup> , homozygous disruption of <i>Cox1</i> gene in mice; <i>Cox2</i> <sup>-/-</sup> , homozygous disruption of <i>Cox2</i> gene in mice. Approximately 15% of <i>Cox2</i> <sup>-/-</sup> mice die from small bowel perforation; 50% of mice had normal intestinal permeability and no intestinal inflammation and 50% had small bowel ulcers.							
Uncoupling: 0, no uncoupling; +a, 60%–70% of the mitochondria have uncoupling determined by electron microscopy;							

+b, 10%–30% of the mitochondria have uncoupling determined by electron microscopy

Mucosal levels PGE<sub>2</sub>: percentages indicate increase (+) or decrease (–) from control level

Permeability (measured by <sup>51</sup>CrEDTA) and inflammation (fecal level of calprotectin): 0, unchanged; +, increased

Number of small bowel ulcers: 0, none; +, present

## FIGURE LEGENDS

### **Figure 1. Mechanisms of Gastrointestinal Damage by NSAIDs**

In our model, the interaction between NSAIDs and phospholipids and uncoupling of oxidative phosphorylation damage intestinal cells and increase gastrointestinal permeability. Inhibition of COX reduces microvascular blood flow, and luminal aggressive factors modify and amplify this reaction, leading to inflammation, erosions, and ulcers. Principal luminal aggressors are acid and pepsin in the stomach and acid, bile, and bacteria in the small bowel.

### **Figure 2. Structures of Conventional NSAIDs and Derivatives**

Conventional NSAIDs are usually lipid-soluble molecules (often benzene derivatives) with an acidic carboxylic group. The analgesic paracetamol has no anti-inflammatory activity and does not cause gastrointestinal damage because it lacks the acidic moiety. Derivatives of flurbiprofen, such as nitric oxide flurbiprofen and flurbiprofen dimer (thought to cause less intestinal damage than flurbiprofen) are non-acidic because of the esterification of the carboxylic moiety.

Nabumetone, a pro-NSAID that causes minimal gastrointestinal damage, becomes anti-inflammatory only after conversion in the liver into the active component MNA, which is acidic.

### **Figure 3. Mechanism of Uncoupling Actions of NSAIDs**

High-energy intermediates feed into the respiratory chain; as energy is released, it is used to pump out hydrogen ions into the inter-mitochondrial membrane space. Normally these hydrogen ions re-enter via a channel (ionopore) that is associated with ATP synthase and this promotes production of ATP. NSAIDs, however, partition into the inner mitochondri-

al membrane and create similar ionopores that allow hydrogen ions to enter the inner mitochondrial matrix, thereby bypassing the ATP synthase. The uncoupling (that is, uncoupling the hydrogen gradient from the ATPase activities) by NSAIDs leads to cell dysfunction from decreased levels of ATP, calcium release into the cytosol, etc.

#### **Figure 4. Ion Trapping Hypothesis for NSAIDs**

The intracellular concentration of an NSAID in the stomach depends on the interaction between the  $pK_a$  of the NSAID and luminal pH as well as the rate of exit from the cell, which also depends on the  $pK_a$  of the drug. Furthermore, lipid solubility, size, and metabolism of the NSAIDs and protein binding have roles in absorption-trapping. The more acidic the NSAID, the more it depends on a low gastric pH (an uncharged NSAID partitions through the surface cell membrane more effectively than a charged one) for entry into the epithelial cells; once inside, it is again charged (cytosol has a pH of 7.4) and it accumulates to reach a greater concentration than NSAIDs with  $pK_a$ s that are closer to neutral. Uncoupling potency appears to be directly proportional to the  $pK_a$  of the NSAID. For example, after an oral dose of aspirin ( $pK_a$  of 3.5) the drug does not enter the gastric mucosal cells when the gastric lumen is neutral (pH 7.0) because it is fully ionised. However, at a gastric pH of 2, for example, it is uncharged and easily partitions into the cells. Inside the cell, it is fully ionized because of the intercellular pH (7.4). It can therefore not pass into the circulation, and intracellular concentrations increase to the micromolar range required for uncoupling. A less-acidic NSAID with a  $pK_a$  of 6.4 is less dependant on the luminal  $pK_a$  for its entry into the gastric cells. However, because it is only partially ionized at the intracellular pH of 7.4, it is absorbed into the circulation and the intracellular concentrations may only be modestly high in comparison with aspirin. Neutralizing the gastric pH with drugs like proton pump inhibitors prevents short-term gastric damage of acidic

NSAIDs more effectively than with less acidic NSAIDs. Because of the enormous surface area of the small intestine, the charge of the NSAID has only a minor role in its absorption, but ion trapping is still evident.

ACCEPTED MANUSCRIPT

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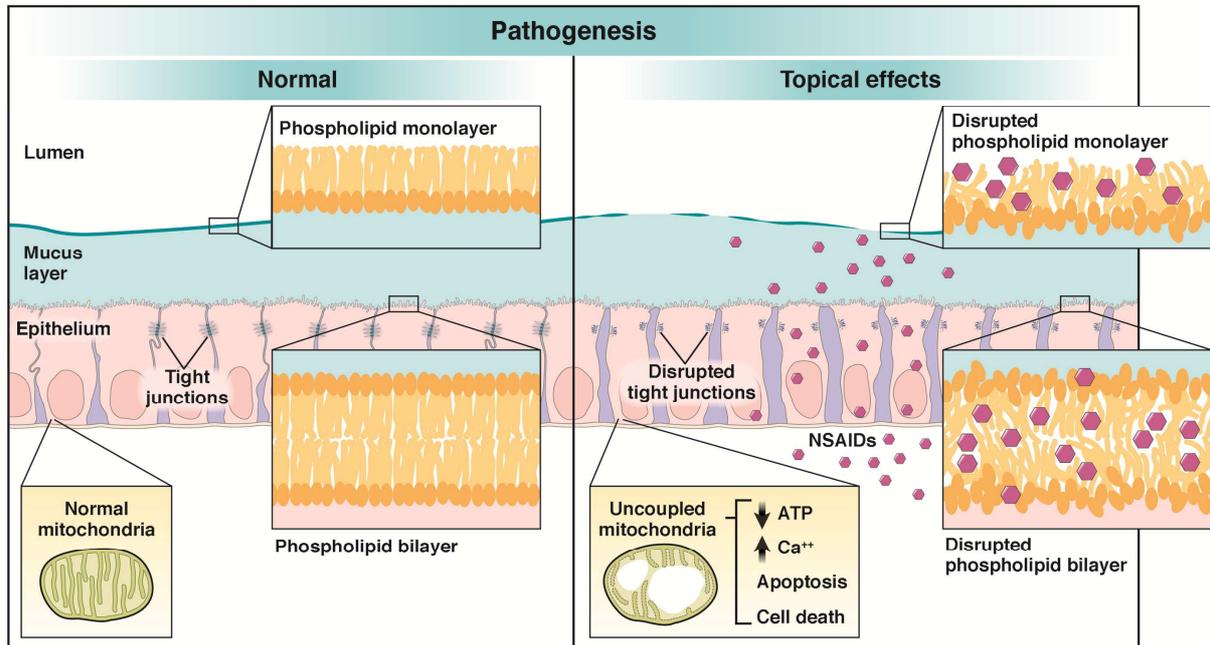
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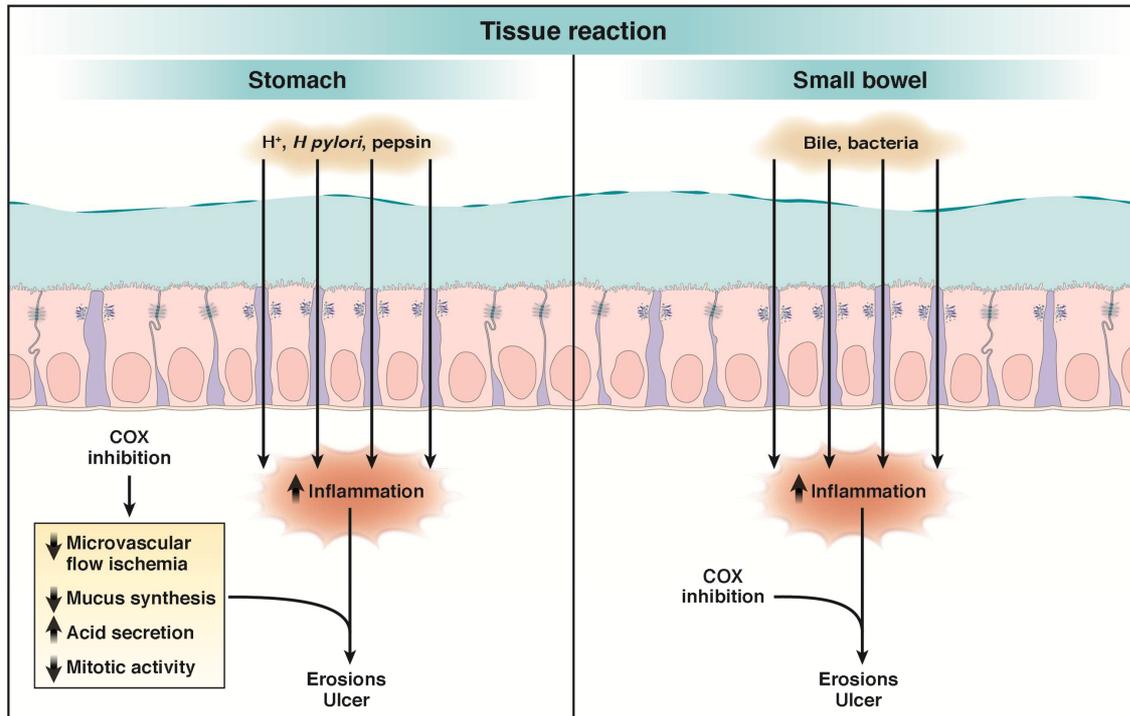
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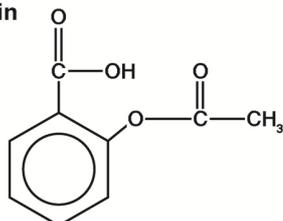
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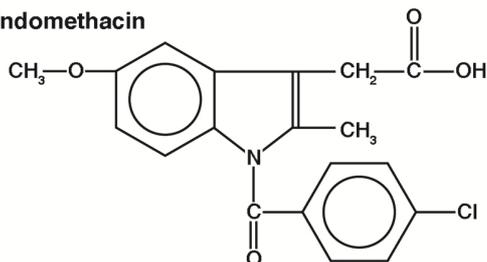
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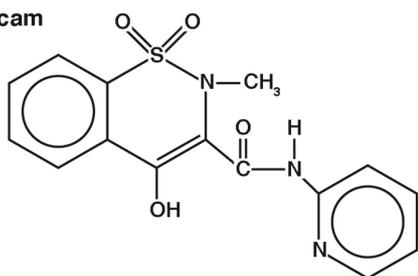
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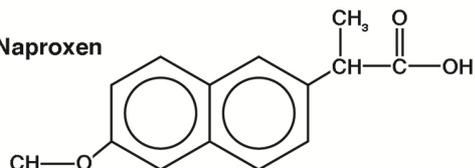
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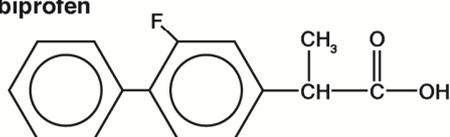
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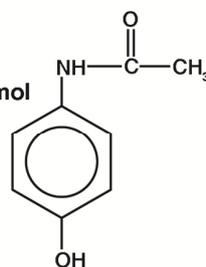
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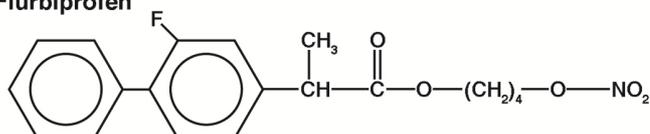
Flurbiprofen



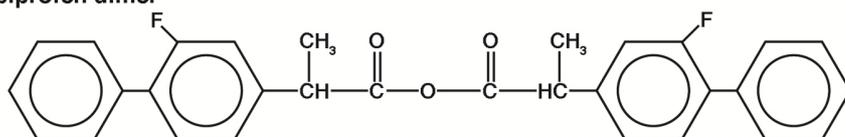
Paracetamol



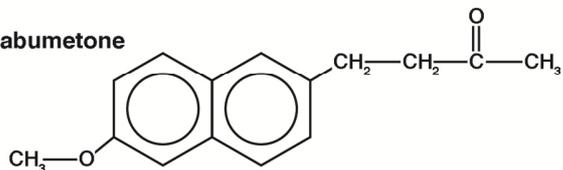
NO-Flurbiprofen



Flurbiprofen dimer



Nabumetone



6-methoxy-2-naphthylacetic acid

