Gastrointestinal Safety and Anti-Inflammatory Effects of a Hydrogen Sulfide–Releasing Diclofenac Derivative in the Rat

JOHN L. WALLACE,* GIUSEPPE CALIENDO,‡ VINCENZO SANTAGADA,‡ GIUSEPPE CIRINO,§ and STEFANO FIORUCCI*,‖

*Inflammation Research Network, University of Calgary, Calgary, Alberta, Canada; ‡Departments of Medicinal Chemistry and §Experimental Pharmacology, University of Naples, Naples, Italy; and ‖Department of Gastroenterology and Hepatology, University of Perugia, Perugia, Italy

Background & Aims: **Gastrointestinal damage caused by nonsteroidal anti-inflammatory drugs (NSAIDs) remains a significant clinical problem. Hydrogen makes an important contribution to mucosal defense, and NSAIDs can suppress its synthesis. In this study, we evaluated the gastrointestinal safety and anti-inflammatory effects of a novel "HS-NSAID" (ATB-337) that consists of diclofenac linked to a hydrogen sulfide–releasing moiety.** ***Methods:*** **The gastrointestinal injury–inducing effects of single or repeated administration of diclofenac versus ATB-337 were compared in rats, as were their effects on prostaglandin synthesis and cyclooxygenase-1 and -2 activities. The ability of these drugs to reduce carrageenan-induced paw edema and to elicit leukocyte adherence to the vascular endothelium (intravital microscopy) were also examined in rats.** ***Results:*** **Diclofenac (10–50 μmol/kg) dose-dependently damaged the stomach, while ATB-337 did not. Repeated administration of diclofenac caused extensive small intestinal damage and reduced hematocrit by 50%. ATB-337 induced >90% less intestinal damage and had no effect on hematocrit. Diclofenac, but not ATB-337, elevated gastric granulocyte infiltration and expression of tumor necrosis factor α, lymphocyte function–associated antigen 1, and intercellular adhesion molecule 1. ATB-337 inhibited cyclooxygenase-1 and cyclooxygenase-2 activity as effectively as diclofenac. ATB-337 did not induce leukocyte adherence, whereas diclofenac did, and was more potent at reducing paw edema.** ***Conclusions:*** **An HS-NSAID spares the gastric mucosa of injury despite markedly suppressing prostaglandin synthesis. This effect may be related to hydrogen sulfide–mediated inhibition of tumor necrosis factor–α expression and of the leukocyte adherence to vascular endothelium normally induced by cyclooxygenase inhibitors.**

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) continues to be associated with an unacceptable risk for gastrointestinal ulceration and bleeding, even after the introduction of selective cyclooxygenase (COX)-2 inhibitors. Other adverse effects of NSAIDs, including those associated with the renal and cardiovascular systems, are of growing concern to practitioners and patients. Attempts to design NSAIDs that do not cause gastrointestinal damage still face the challenge of overcoming the detrimental effects of suppression of prostaglandin (PG) synthesis while maintaining the beneficial effects of these drugs, which are also related to inhibition of the COX enzymes.[1]

Nitric oxide (NO)–releasing NSAIDs were first described in 1994[2] and have since been assessed in several clinical trials.[3–5] The principle behind these compounds was that the slow release of NO would compensate, in terms of mucosal defense, for the inhibition of PG synthesis in the mucosa.[2] Hydrogen sulfide, like NO, is a gaseous mediator now recognized as making important contributions to several physiologic functions, including many in the gastrointestinal tract and liver.[6] Recently, we reported that H_2S is produced by the gastric mucosa and makes a significant contribution to mucosal defense.[7] Interestingly, the expression of one of the key enzymes responsible for endogenous H_2S synthesis is inhibited by a number of NSAIDs. Moreover, administration of H_2S donors reduced the severity of NSAID-induced gastric damage in rats. These observations raised the possibility that an H_2S-releasing derivative of an NSAID may exhibit reduced gastric toxicity.

We have also reported that H_2S exhibits anti-inflammatory effects.[8] Endogenous H_2S production appears to down-regulate leukocyte adherence to the vascular endothelium, a key early event in inflammation. Administration of H_2S donors could suppress leukocyte adherence stimulated by aspirin or by a proinflammatory peptide. Moreover, H_2S donors reduced edema formation in the rat paw following injection of carrageenan. These observations therefore suggest that drugs releasing H_2S may exhibit anti-inflammatory activity.

In the present study, we have characterized the effects of a novel NSAID (ATB-337), formed by linking diclofe-

Abbreviations used in this paper: CBS, cystathionine β-synthase; COX, cyclooxygenase; CSE, cystathionine γ-lyase; HS-NSAID, hydrogen sulfide–releasing nonsteroidal anti-inflammatory drug; ICAM, intercellular adhesion molecule; LFA, lymphocyte function–associated antigen; L-NAME, *N*-nitro-L-arginine methyl ester; TNF, tumor necrosis factor.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2006.11.042

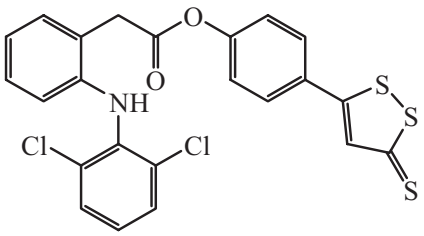

Figure 1. Structure of ATB-337.

nac to an H_2S-releasing moiety (Figure 1). We compared the effects of this novel NSAID with diclofenac in terms of ulcerogenic effects, ability to suppress gastric PG synthesis, ability to induce small intestinal injury, effects on COX-1 and COX-2 activity in vivo, and acute anti-inflammatory effects. Our results suggest that the linking of an H_2S-releasing moiety to NSAIDs, forming an "HS-NSAID," is a rational approach to the development of gastrointestinal-sparing anti-inflammatory drugs.

Materials and Methods

Animals

Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, Quebec, Canada) and were housed in the Animal Care Facility at the University of Calgary. The rats were fed standard laboratory chow and tap water. All experimental protocols were approved by the Animal Care Committee at the University of Calgary, and the experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. Unless otherwise noted, in all experiments described in the following text, the sample size in each group was at least 5.

Effects of NSAIDs on Gastrointestinal Mucosal Integrity and PG Synthesis

Rats were deprived of food for 18–20 hours, with free access to drinking water, and were then treated orally with diclofenac (10–50 μmol/kg), ATB-337 (equimolar doses), or vehicle (1% carboxymethylcellulose). Three hours later, the rats were killed for blind assessment of gastric damage.[9] The lengths (in millimeters) of all hemorrhagic lesions were measured with digital calipers, and the "gastric damage score" was calculated for each stomach by summing these values. After scoring the damage, a sample of the corpus region of each stomach was fixed in neutral buffered formalin for subsequent histologic assessment. Other tissue samples from each stomach were processed, as described previously, for measurement of PGE_2 synthesis using a specific enzyme-linked immunosorbent assay.[9]

Another experiment was performed in which groups of 5 rats each were fasted overnight and then treated orally with vehicle, diclofenac, or ATB-337 (both drugs at 50 μmol/kg). Blood samples were drawn from a tail vein 3 hours after administration of the drugs or vehicle for measurement of plasma H_2S concentrations, as described previously.[10] Samples of the stomach were excised 3 hours after drug/vehicle administration for measurement of myeloperoxidase activity[11] using a commercially available spectrophotometric kit. Myeloperoxidase is an enzyme found primarily in the azurophilic granules of the neutrophils and therefore has been used extensively as a biochemical marker of the granulocyte infiltration into various tissues, including the gastrointestinal tract. Additional samples of the stomach were excised for determination of expression of messenger RNA (mRNA) for cystathionine γ-lyase (CSE), tumor necrosis factor (TNF)-α, intercellular adhesion molecule (ICAM)-1, and lymphocyte function–associated antigen (LFA)-1, by quantitative reverse-transcription polymerase chain reaction, as described in detail previously.[7] The primers used were as follows (sense and antisense, respectively): glyceraldehyde-3-phosphate dehydrogenase, ATGACTCTACCCACGGCAAG and TACTCAGCACCAGCATCACC; TNF-α, TGATCCGAGATGTGGAACTG, and CGAGCAGGAATGAGAAGAGG; ICAM-1, CAAGGGCTGTCACTGTTCAA and CTTCAGAGGCAGGAAACAGG; LFA-1, GTCATGGAGTGTGGCATCTG and TCACTTTGTTGGGGATGTCA. Similar experiments were performed in which gastric tissue was excised at 1 hour or 3 hours after drug/vehicle administration and expression of mRNA for the 2 key enzymes in endogenous H_2S synthesis (cystathionine β-synthase [CBS] and CSE) was determined by quantitative reverse-transcription polymerase chain reaction. The primers used were as follows (sense and antisense, respectively): CBS, CCAGGACTTGGAGGTACAGC and TCGGCACTGTGTGGTAATGT; CSE GTATTGAGGCACCAACAGGT and GTTGGGTTTGTGGGTGTTTC.

In other experiments, rats were treated with diclofenac alone or together with the H_2S-releasing moiety of ATB-337 (ADT-OH; 5-phydroxyphenyl-1,2-dithione-3-thione). These experiments would allow us to determine if the separate but concomitant administration of the 2 moieties of ATB-337 would provide the same degree of gastric safety as the intact compound.

We also examined the effects of ATB-337 versus diclofenac in rats in circumstances in which mucosal defense was diminished. Specifically, we treated rats orally with an inhibitor of NO synthase (*N*-nitro-L-arginine methyl ester [L-NAME]; 15 mg/kg) or with aspirin (10 mg/kg) 30 minutes before oral administration of vehicle, diclofenac, or ATB-337 (both drugs at 27 μmol/kg).

To compare the effect of ATB-337 with that of diclofenac in terms of capacity to produce small intestinal damage, groups of 5 rats each (not fasted) were given one of these drugs at a dose of 50 μmol/kg 3 times at 12-hour intervals. The rats were killed 24 hours after the final administration of the test drugs. The small intestine was excised, and the extent of small intestinal hemorrhagic damage was quantified by measuring the lengths of le-

sions in millimeters and then summing these to give a damage score for each rat.[12] The observer also made note of the distribution of the lesions and their depth. Before the initial administration of one of the test drugs and immediately before the rats were killed, a blood sample was collected from a tail vein for measurement of hematocrit as a surrogate marker of bleeding.[12]

Gastric Acid Secretion

Gastric-sparing effects of ATB-337 might be due to suppression of gastric acid secretion. To test this hypothesis, groups of 5 rats each were given vehicle, diclofenac, or ATB-337 orally (50 μmol/kg for both drugs). Thirty minutes later, the rats were anesthetized with halothane and a laparotomy was performed. The pylorus was ligated, and the abdomen was sutured closed. Three hours later, the rats were anesthetized again and the stomach was excised with care taken not to lose the contents. The volume of fluid in the stomach was determined gravimetrically, and the mount of titratable acidity was determined using a Brinkmann (Rexdale, Ontario, Canada) automated titrator.

Intravital Microscopy

Adhesion of leukocytes to the vascular endothelium has been shown to be an important step in the pathogenesis of NSAID-induced gastric damage.[1,13,14] The ability of ATB-337, versus diclofenac, to cause leukocyte adherence was examined using intravital microscopy, as described in detail previously.[8,14] Briefly, rats were deprived of food for 18–20 hours and anesthetized with sodium pentobarbital (65 mg/kg intraperitoneally). A postcapillary mesenteric venule with a diameter of 25–30 μm was positioned under the objective of a microscope. Following a 15-minute equilibration period, the rats were randomly assigned to receive vehicle, diclofenac (50 μmol/kg), or ATB-337 (50 μmol/kg). Images of the mesenteric microcirculation were recorded over a 5-minute period every 15 minutes for 60 minutes for blind quantification of leukocyte adherence. Basal leukocyte adherence was measured over a 5-minute period immediately before administration of vehicle, diclofenac, or ATB-337. Leukocyte adherence was quantified from the videotaped images by an observer blind as to the treatments the rats had received. A leukocyte was considered as adherent if it remained stationary for at least 30 seconds.

In Vivo COX-1 and COX-2 Activity

The effects of the novel NSAID on COX-2 activity in vivo were assessed using the air pouch model in rats.[15–17] Injection of zymosan into a preformed air pouch results in a substantial increase in PGE_2 synthesis, which occurs almost exclusively via COX-2. This was confirmed in the present study using the highly selective COX-2 inhibitor lumiracoxib.[18] Briefly, 20 mL of air was injected subcutaneously on the back of the rat on the first day. Two days later, another 10 mL of air was injected at the same site. On the fifth day after the first injection, a further 10 mL of air was injected into the pouch. Twenty-four hours later, zymosan (1 mL of a 1% wt/vol solution in sterile saline) was injected into the air pouch. All of the injections were performed after the rats had been anesthetized with 5% (vol/vol) halothane. Six hours after the zymosan injection, the rats were anesthetized with 5% (vol/vol) halothane and the pouch was carefully opened by a small incision. The exudate was collected and transferred to a sterile tube. The volume of the exudate was measured gravimetrically, and the exudates were then stored at –20°C until such time as PGE_2 concentrations were measured using a specific enzyme-linked immunosorbent assay.[9]

To assess the effects of the test drugs on COX-1 activity in vivo, a sample of blood was drawn from the inferior vena cava. One milliliter was transferred to a glass tube and allowed to clot at 37°C for 45 minutes. The samples were then centrifuged for 10 minutes at 3,000*g*, after which the serum was decanted and frozen at −20°C until such time as the concentrations of thromboxane B_2 were measured by specific enzyme-linked immunosorbent assay.[9] Virtually all of the thromboxane B_2 produced by clotting whole blood is synthesized via COX-1 in platelets.[9]

Effects on Human Platelet Function

The effects of diclofenac and ATB-337 on human platelet aggregation and thromboxane synthesis were examined. Blood from 3 healthy donors was aliquoted into Eppendorf tubes containing one of the test drugs (1 or 10 μmol/L) or vehicle and incubated for 30 minutes at 37°C. Thirty minutes later, indomethacin (10 μmol/L) was added to each sample to prevent further thromboxane synthesis. The samples were centrifuged (9000*g*) for 3 minutes and the plasma was transferred to another tube and frozen at −20°C until the assay for thromboxane B_2 was performed. Other blood samples were used to prepare platelet-rich plasma, as described previously.[19] Aliquots (0.5 mL) of platelet-rich plasma were incubated with diclofenac or ATB-337 (1 or 10 μmol/L) or with vehicle for 30 minutes at 37°C. Aliquots of the platelet suspensions were then added to the cuvette of a Chronolog (Buffalo, NY) platelet aggregometer. Three minutes later, arachidonic acid (0.5 μmol/L) was added to the platelet suspension and aggregation was monitored for 5 minutes. Results are expressed as the extent of aggregation as a percent of maximum possible aggregation.

Carrageenan-Induced Paw Edema

Rats were deprived of food, but not water, for 18–22 hours. Groups of 5 rats each were treated orally with vehicle, diclofenac (3–30 μmol/kg), or ATB-337 (10 μmol/kg). Thirty minutes later, the volume of the left

hind paw was measured using a Ugo Basile hydroplethysmometer (Stoelting, Chicago, IL). Immediately thereafter, the rats were then anesthetized with halothane and lambda carrageenan (100 μL; 1% wt/vol) was injected into the footpad. The volume of the injected paw was measured hourly thereafter for 5 hours. All measurements of paw volume were performed by an individual unaware of the treatments the rats had received.

H_2S Release

Release of H_2S from ATB-337 and its H_2S-donating moiety, ADT-OH, was examined using an in vitro system that was recently described in full.[20] Briefly, this method involves incubation of the test compounds (10 mmol/L) in polyethylene glycol and 100 mmol/L potassium phosphate buffer (pH 7.4). Incubations were performed in this buffer alone or in the presence of 10% (wt/vol) rat liver homogenate and 2 mmol/L pyridoxal 5′-phosphate for 30 minutes. The generation of H_2S was detected via a sulfide-sensitive electrode, as previously described.[21,22]

Statistical Analysis

All data are expressed as mean ± SEM. Groups of data were compared using a one-way analysis of variance followed by a Dunnett's multiple comparison test. An associated probability (*P* value) of less than 5% was considered significant.

Materials

ATB-337 ([2-(2,6-dichloro-phenylamino)-phenyl]-acetic acid 4-(5-thioxo-5*H*-1,2-dithiol-3-yl)-phenyl 2-(2-(2,6-dichlorophenylamino)phenyl)acetate was provided by Antibe Therapeutics Inc (Toronto, Ontario, Canada). Diclofenac sodium, L-NAME, and aspirin were obtained from Sigma Chemical Co (St Louis, MO). Lumiracoxib was obtained from SynphaBase (Zurich, Switzerland). The kits for measuring myeloperoxidase activity were obtained from CytoStore (Calgary, Alberta, Canada). The enzyme-linked immunosorbent assay kits for PGE_2 and thromboxane B_2 were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). All other materials were obtained from Fisher Scientific Co (Edmonton, Alberta, Canada).

Results

Gastrointestinal Effects

Oral administration of diclofenac resulted in the development of hemorrhagic erosions in the rat stomach, which increased in severity with the dose (Figure 2). In contrast, oral administration of ATB-337 at doses equimolar to those of diclofenac did not produce any hemorrhagic erosions. Blind histologic evaluation of the gastric tissue from these rats confirmed the absence of any damage to the mucosal tissue.

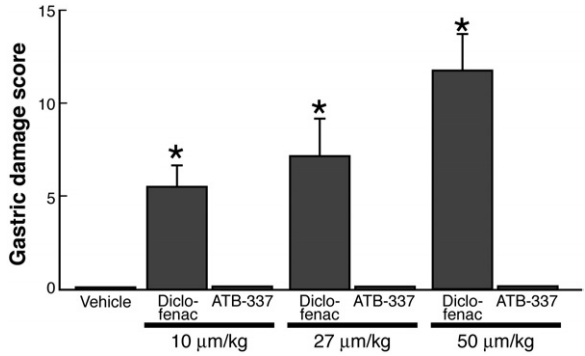

Figure 2. Diclofenac, but not ATB-337, dose-dependently induced hemorrhagic gastric erosion formation in the rat stomach. Each group consisted of 5 rats. Results are shown as the mean ± SEM. *$P < .05$ vs the vehicle-treated group.

ATB-337 consists of a molecule of diclofenac linked through an ester bond to an H_2S-releasing moiety (ADT-OH). We examined whether or not separate, concomitant administration of the 2 moieties of ATB-337 would result in the same lack of gastric damage as seen with ATB-337. As shown in Figure 3, coadministration of diclofenac and ADT-OH resulted in hemorrhagic gastric damage of similar severity to that seen with diclofenac alone, while ATB-337 did not produce damage. Moreover, the lack of gastric damage observed with ATB-337 was not attributable to a diminished ability of this compound to suppress gastric PG synthesis. ATB-337 and diclofenac (given with or without ADT-OH) suppressed gastric PG synthesis by more than 90%.

Administration of diclofenac (50 μmol/kg) resulted, within 3 hours, in significant increases in expression of mRNA for TNF-α (Figure 4*A*) and in tissue granulocyte levels, as measured by myeloperoxidase activity (Figure 4*B*). Consistent with the latter, there was also a significant increase in the expression of the endothelial and leukocyte adhesion molecules, ICAM-1 and LFA-1, respectively (Figure 4*C* and *D*). Such increases were not observed in the group treated with an equimolar dose of ATB-337.

NSAIDs and selective COX-2 inhibitors have been shown previously to induce adherence of leukocytes to the vascular endothelium, and this contributes significantly to their ability to induce gastric damage.[13,14,23] We therefore compared diclofenac and ATB-337 with respect to their ability to trigger leukocyte adherence in postcapillary mesenteric venules in the rat. Basal levels of adherence, per 100-μm vessel length, averaged 2.1 ± 0.7 (n = 5), and this did not change significantly over the course of 60 minutes of superfusion of the vessels with buffer (Figure 4*E*). However, in rats given diclofenac (50 μmol/kg), leukocyte adherence increased significantly over the course of the 1-hour study. In contrast, administration of ATB-337 (50 μmol/kg) had no significant effect on leukocyte adherence (Figure 4*E*).

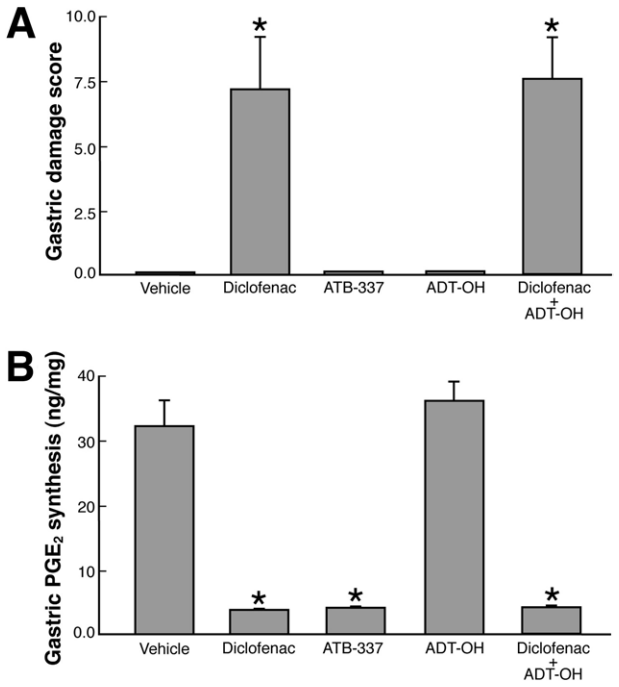

Figure 3. ATB-337 did not induce the formation of hemorrhagic erosions in the stomach (*A*) despite profoundly suppressing gastric prostaglandin synthesis (*B*). Coadministration of ADT-OH (the H_2S-releasing moiety of ATB-337) and diclofenac did not affect the severity of gastric damage produced as compared with diclofenac alone. In these experiments, equimolar doses (27 μmol/kg) of diclofenac, ATB-337, and ADT-OH were administered orally and the stomach was examined 3 hours later. Each *bar* represents the mean ± SEM, and each group consisted of 5–6 rats. *$P < .05$ vs the vehicle-treated group.

Plasma levels of H_2S were significantly increased (by ~40%) 3 hours after oral administration of ATB-337 but unchanged in rats treated with diclofenac (Figure 4*F*). In contrast, expression in the stomach of CSE and CBS, the key enzymes for H_2S synthesis, was suppressed by both diclofenac and ATB-337, although the magnitude of suppression by the latter was greater (Figure 5).

Inhibitors of gastric acid secretion can reduce the severity of NSAID-induced gastric mucosal injury. If ATB-337 were to markedly diminish gastric acid secretion, such an effect could contribute to the reduced mucosal injury observed in rats given this compound. As shown in Table 1, neither the volume of gastric juice produced over a 3-hour period nor the concentration of acid within the gastric juice was significantly affected by treatment with diclofenac or ATB-337.

The gastric safety of ATB-337 was also evident when it was administered to rats that had been pretreated with aspirin or with an inhibitor of NO synthesis (L-NAME). As shown in Table 2, ATB-337 at a dose of 50 μmol/kg did not produce significant gastric damage even when given following one of these inhibitors.

ATB-337 was also much better tolerated in the small intestine than diclofenac. Administration of diclofenac 3 times at 12-hour intervals resulted in extensive hemorrhagic erosion and ulcer formation in the small intestine (Figure 6). These lesions were found principally in the jejunum and to a lesser extent the ileum. Extensive bleeding was evident. While perforations were not observed, some of the lesions clearly penetrated to the muscularis. The rats treated with diclofenac also exhibited a profound decrease in hematocrit, presumably a reflection of significant intestinal bleeding. In contrast, administration of an equimolar dose of ATB-337 produced >90% less small intestinal damage and had no significant effect on the hematocrit. The lesions observed in rats treated with ATB-337 were confined to the jejunum and were small (1–2 mm^2) and superficial.

Anti-Inflammatory and Antiplatelet Effects

ATB-337 exhibited a similar ability to suppress COX-1 and COX-2 activity as diclofenac. In a rat air pouch model, in which inflammation is induced by zymosan, the large increase in PGE_2 synthesis occurs almost exclusively via COX-2. Lumiracoxib, at a dose (10 mg/kg) that had no effect on whole blood thromboxane synthesis, inhibited PGE_2 synthesis by >90% (from 25.5 ± 3.0 ng/mL of exudate to 2.1 ± 0.1 ng/mL). At a dose

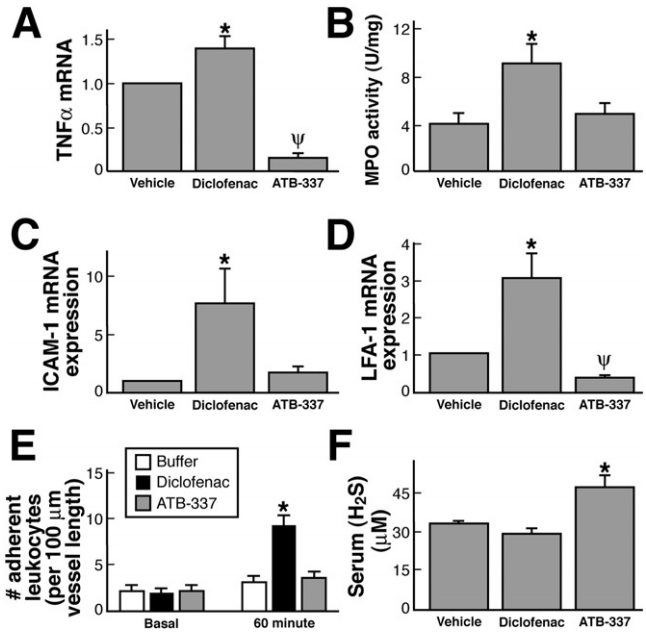

Figure 4. Effects of diclofenac and ATB-337 (each at 50 μmol/kg) on gastric expression of mRNA for (*A*) TNF-α, (*C*) ICAM-1, and (*D*) LFA-1 and on (*B*) gastric myeloperoxidase activity, (*E*) leukocyte adherence to the vascular endothelium of mesenteric venules, and (*F*) serum H_2S concentrations. Results are shown as the mean ± SEM of 5 rats per group. For the mRNA expression, the data are shown relative to expression in the vehicle-treated group (densitometric analysis of the gels was performed, with normalization to expression of glyceraldehyde-3-phosphate dehydrogenase). In the studies shown in *E*, leukocyte adherence was measured before (basal) and 60 minutes after intragastric administration of vehicle, diclofenac, or ATB-337 (50 μmol/kg for both drugs). *$P < .05$ comparing the ATB-337 group with the diclofenac group; Ψ$P < .05$ comparing the ATB-337 group with the vehicle group.

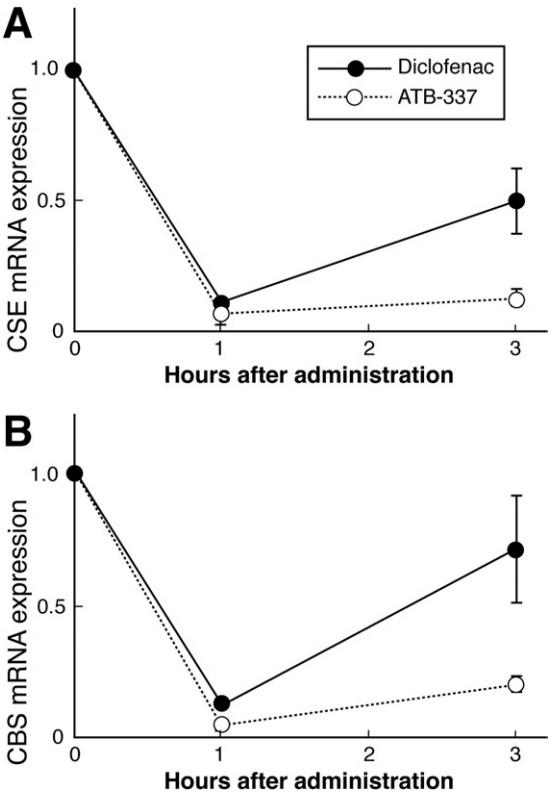

Figure 5. Gastric expression of mRNA for the 2 principal enzymes in endogenous H_2S synthesis: (*A*) CSE and (*B*) CBS. Expression was measured by quantitative reverse-transcription polymerase chain reaction 1 and 3 hours after oral administration to rats of diclofenac or ATB-337, both at 50 μmol/kg. Results are shown as the mean ± SEM for 5 rats per group. Both drugs caused a significant decrease in CSE and CBS expression at 1 hour ($P < .05$). CSE expression remained significantly suppressed with both drugs at 3 hours, but the suppression was greater with ATB-337 ($P < .05$). In the case of CBS expression, the suppressive effect of diclofenac was no longer significant at 3 hours, while ATB-337 still exhibited a marked suppressive effect.

of 1 μmol/kg, both diclofenac and ATB-337 suppressed COX-2 activity in this model by ~50% (Figure 7). At a dose of 10 μmol/kg, both diclofenac and ATB-337 suppressed COX-2 activity by >95% (Figure 7).

COX-1 activity was assessed by measuring thromboxane synthesis in blood collected from these rats at the same time that the inflammatory exudates were collected.

Table 1. Effects of Diclofenac and ATB-337 on Gastric Acid Secretion in Rats

Treatment	Volume (mL)	Acid Secretion (mEq/h)
Vehicle	1.72 ± 1.19	15.45 ± 7.25
Diclofenac	1.67 ± 0.43	19.75 ± 6.12
ATB-337	1.85 ± 0.69	20.59 ± 4.60

NOTE. Each group consisted of 5 rats. Results are expressed as the mean ± SEM. The pylorus was ligated 30 minutes after oral administration of the test drugs or vehicle, and gastric juice was collected 3 hours later. Neither the volume of secretion nor the quantity of acid secretion differed significantly among the groups.

Table 2. Effects of L-NAME and Aspirin on Gastric Damaging Effects of Diclofenac and ATB-337

Pretreatment	Treatment	Gastric Damage Score
1% carboxymethylcellulose	Diclofenac	11.2 ± 2.4
Aspirin	Diclofenac	16.0 ± 2.0
1% carboxymethylcellulose	ATB-337	0 ± 0
Aspirin	ATB-337	0 ± 0
Saline	Diclofenac	12.1 ± 2.1
L-NAME	Diclofenac	13.0 ± 3.6
Saline	ATB-337	0.2 ± 0.2
L-NAME	ATB-337	0.4 ± 0.4

NOTE. Results are expressed as the mean ± SEM for 5 rats per group. Neither aspirin (10 mg/kg) nor L-NAME (15 mg/kg) significantly increased the extent of gastric damage induced by diclofenac or ATB-337. The vehicle for aspirin was 1% carboxymethylcellulose, while the vehicle for L-NAME was 0.9% saline. Both diclofenac and ATB-337 were administered orally at 50 μmol/kg.

Again, we observed comparable inhibitory effects of diclofenac and ATB-337 (Figure 7).

The effects of ATB-337 on human platelet function were also examined. As in the rat, ATB-337 produced comparable inhibition of thromboxane synthesis as was seen with diclofenac (Table 3). Moreover, ATB-337 suppressed arachidonic acid–induced human platelet aggre-

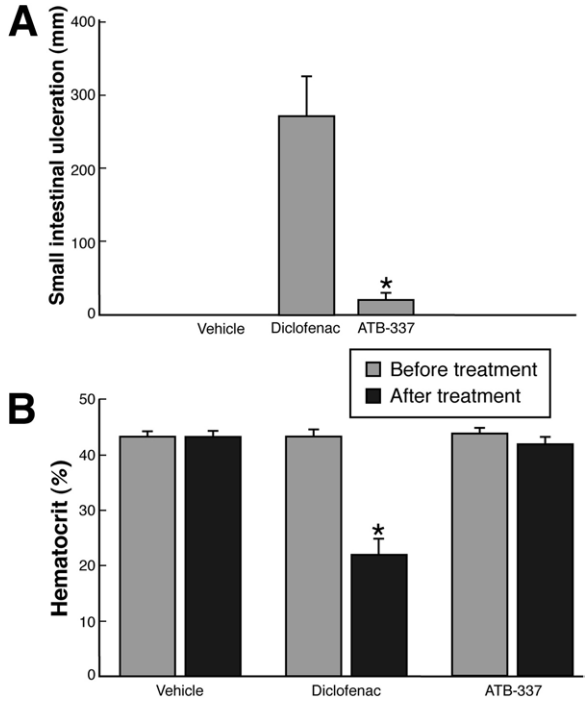

Figure 6. (*A*) Small intestinal damage and (*B*) change in hematocrit following treatment with diclofenac or ATB-337. Oral administration of the drugs (each at 50 μmol/kg) was performed 48, 36, and 24 hours before assessment of small intestinal damage. Blood samples were taken at the beginning and end of the study for measurement of hematocrit. ATB-337 induced significantly less (*$P < .05$) intestinal damage than diclofenac. Diclofenac, but not ATB-337, caused a significant decrease in hematocrit. Each group consisted of 5 rats, and the *bars* show the mean ± SEM.

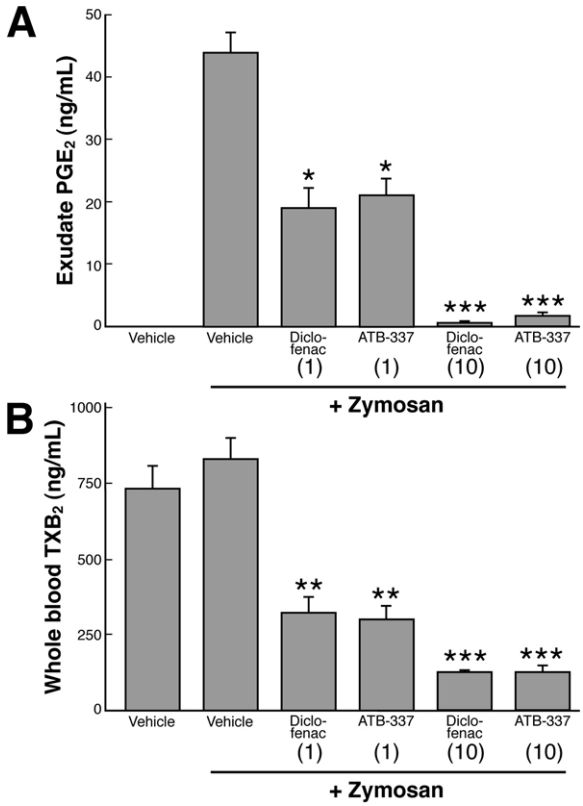

Figure 7. Inhibition of (*A*) COX-2 and (*B*) COX-1 activity in an in vivo inflammation model. Injection of 1% zymosan into a preformed air pouch in the rat results in an acute inflammatory reaction accompanied by COX-2–dependent PGE_2 production. Diclofenac and ATB-337 (either at 1 or 10 μmol/kg) produced comparable suppression of COX-1 and COX-2 activity. Each group consisted of 5 rats, with the mean ± SEM shown. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs the vehicle + zymosan group.

gation to the same extent as equimolar concentrations of diclofenac (Table 3).

Injection of carrageenan into a hind footpad of the rat resulted in a marked increase in paw volume, consistent

Table 3. Effects of Diclofenac and ATB-337 on Human Platelet Function

Treatment	Thromboxane B_2 Synthesis (ng/mL)	Arachidonate-Induced Aggregation (% maximal)
Vehicle	217.0 ± 10.6	86 ± 3
Diclofenac (1 μmol/L)	71.9 ± 7.2[a]	11 ± 2[a]
ATB-337 (1 μmol/L)	86.5 ± 6.3[a]	17 ± 3[a]
Diclofenac (10 μmol/L)	28.9 ± 4.1[a]	1 ± 1[a]
ATB-337 (10 μmol/L)	36.5 ± 4.0[a]	3 ± 1[a]

NOTE. For thromboxane synthesis, whole blood was allowed to stand at 37°C for 30 minutes. For aggregation, platelet-rich plasma was stimulated with arachidonic acid (0.5 μmol/L). The results (mean ± SEM) are expressed as a percent of maximal aggregation. Samples from 3 donors were tested in each assay, repeated in triplicate. Effects of diclofenac and ATB-337 did not differ significantly from one another for any given concentration.
[a]$P < .05$ vs the vehicle-treated group.

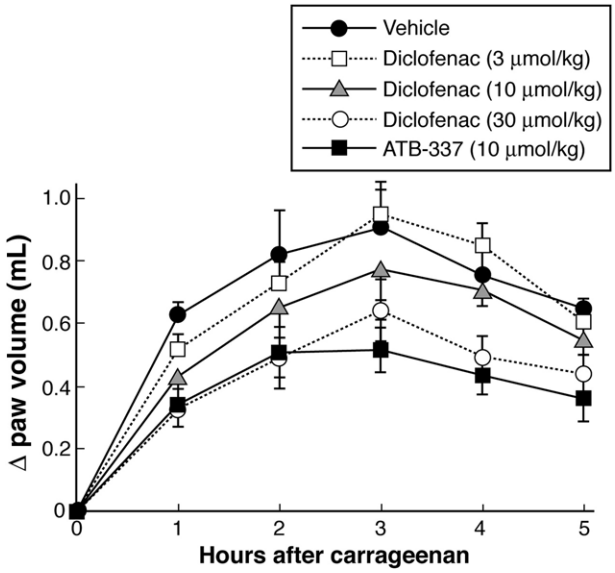

Figure 8. Injection of carrageenan into the hind paw of a rat resulted in significant edema formation, as measured by an increase in paw volume. Pretreatment with diclofenac resulted in a dose-dependent inhibition of the edema formation. Pretreatment with ATB-337 at 10 μmol/kg produced a reduction in edema formation similar to that seen with diclofenac at 30 μmol/kg. Both drugs were administered orally 30 minutes before injection of carrageenan. Each group consisted of 5–6 rats. Data are presented as the mean ± SEM.

with edema formation (Figure 8). Pretreatment with diclofenac resulted in a dose-dependent reduction of the increase in paw volume. Pretreatment with ATB-337 at a dose of 10 μmol/kg produced a reduction of paw swelling similar to that produced by a 30 μmol/kg dose of diclofenac.

H_2S Release

When incubated in buffer, there was negligible release of H_2S from ADT-OH, the H_2S-releasing moiety of ATB-337 (Figure 9). However, ATB-337 generated ~12

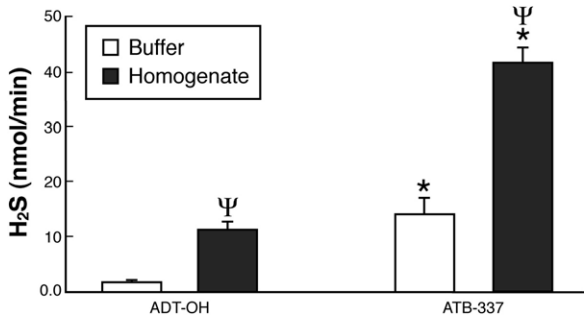

Figure 9. Generation of H_2S from ATB-337 and its H_2S-donating moiety (ADT-OH) when incubated in phosphate buffer (pH 7.4) or in a rat liver homogenate (see Methods for more details). Both ADT-OH and ATB-337 released significantly more H_2S when incubated in the homogenate as compared to the buffer ($^*P < .05$). ATB-337 released significantly more H_2S than ADT-OH when incubated in either medium ($^\Psi P < .05$). Incubation of diclofenac in buffer or in homogenate did not result in any detectable H_2S generation (not shown).

nmol/min of H_2S when it was incubated in buffer. When ADT-OH was added to a liver homogenate, we could detect H_2S release. However, the levels of H_2S generated when ATB-337 was incubated with the liver homogenate were ~3-fold greater than those from ADT-OH.

Discussion

The pathogenesis of NSAID-induced gastroenteropathy remains incompletely understood. It is clear that inhibition of the production of endogenous mediators that contribute to mucosal defense is a major contributing factor.[1] The importance of PGs as mediators of mucosal defense has been recognized for more than 30 years, while the importance of NO in this regard became clear in the past 2 decades.[24] Recent studies have highlighted the contribution of H_2S to gastric mucosal defense, as well as the ability of NSAIDs to suppress endogenous H_2S synthesis.[7] In the present study, we have shown that modification of a commonly used NSAID (diclofenac) such that it generates H_2S resulted in a substantial reduction in its ability to induce gastrointestinal damage and bleeding, while not diminishing its ability to inhibit PG synthesis or reduce edema formation. Indeed, the H_2S release from ATB-337 may even enhance its anti-inflammatory activity relative to diclofenac, consistent with recently reported effects of other H_2S donors.[8]

NSAIDs were recently shown to inhibit H_2S production by the gastric mucosa.[7] This may be an additional mechanism through which these commonly used drugs induce damage to the mucosa, that is, by suppressing the synthesis of 2 key mediators of mucosal defense (PGs and H_2S). Previous studies from our laboratory showed that the reduction of gastric blood flow and the induction of leukocyte adherence to the vascular endothelium could be significantly attenuated through administration of agents that spontaneously release H_2S in solution (NaHS and Na_2S).[7] The ability of ATB-337 to spare the gastrointestinal mucosa of injury may therefore be related to the generation of H_2S from this compound and its subsequent effects on the gastrointestinal microcirculation. In particular, it was noteworthy that ATB-337 did not stimulate leukocyte adherence to the vascular endothelium of postcapillary mesenteric venules, in contrast to the effects of diclofenac. Consistent with this finding, ATB-337 did not cause a significant increase in gastric granulocyte infiltration (myeloperoxidase activity) or expression of leukocyte (LFA-1) or endothelial (ICAM-1) adhesion molecules, as observed with diclofenac in the present study, and as previously observed with other NSAIDs.[7] We recently showed that suppression of endogenous H_2S synthesis with β-cyanoalanine resulted in a marked increase in leukocyte-endothelium adherence, consistent with a physiologic role of H_2S in modulating inflammation.[8] This hypothesis is further supported by the observation that suppression of endogenous H_2S synthesis exacerbated carrageenan-induced paw edema.[8]

Previous studies have highlighted the important contribution of TNF-α to the generation of gastric mucosal injury following NSAID administration to rodents.[25,26] In the present study, we observed a significant elevation of gastric TNF-α expression. Whether or not H_2S can directly suppress TNF-α expression is not clear, but it is noteworthy that we have previously observed reduced expression of mRNA for TNF-α and several other cytokines in the colon of rats following treatment with an H_2S-releasing derivative of mesalamine.[27] We speculated that those effects may be attributable to inhibition of nuclear factor κB activation by the H_2S-releasing moiety of that compound. Whether or not suppression of nuclear factor κB could contribute to the gastric safety of ATB-337 has not yet been examined.

In addition to sparing the gastric mucosa, repeated administration of ATB-337 was found to produce markedly less intestinal damage (>90% reduction) than that observed with an equimolar dose of diclofenac. A marked decrease in hematocrit was observed in the diclofenac-treated group, likely a result of the extensive intestinal bleeding that was clearly evident at the time of necropsy. Further evidence for the relative safety of ATB-337 was the absence of any change in hematocrit during the period of treatment. These observations are particularly noteworthy considering the increasing recognition of the ability of NSAIDs to produce significant bleeding from the intestine.[28] NSAIDs are also capable of impairing platelet aggregation and therefore blood clotting. These effects are directly related to the ability of NSAIDs to suppress platelet thromboxane synthesis, which occurs via COX-1. We compared the effects of diclofenac and ATB-337 on human platelet thromboxane synthesis and aggregation (induced by arachidonate) and found that the 2 drugs behaved similarly to one another. This is congruent with our observations in rat studies that the 2 drugs exhibit comparable inhibitory activity on COX-1.

H_2S and NO share many actions and may even counter-regulate production of one another.[6,29,30] We examined the possibility that ATB-337 would no longer exhibit gastric safety in a circumstance of concomitant suppression of both NO and PG synthesis. However, suppression of NO synthesis with L-NAME did not impair the ability of ATB-337 to spare the gastric mucosa of injury. We also examined the possibility that gastric safety of ATB-337 was related to suppression of gastric acid secretion, because proton pump inhibitors have been shown to significantly reduce the severity of NSAID-induced gastropathy.[31] However, neither ATB-337 nor diclofenac significantly affected acid secretion in the rat over a 3-hour period after their administration (the same time frame within which we observed lesion formation in diclofenac-treated rats).

One of the more surprising findings in this study was that administration of ADT-OH, the H_2S-releasing moiety of ATB-337,[32] did not protect the stomach

against the damaging effects of diclofenac. However, the data on H_2S generation in vitro offer a possible explanation for this observation. In terms of H_2S release, ADT-OH and ATB-337 behave quite differently. ADT-OH did not generate detectable H_2S when incubated in buffer, while ATB-337 did. Moreover, the amount of H_2S generated when ATB-337 was incubated in a liver homogenate was about 3 times that generated from ADT-OH in the same conditions. Thus, it is possible that the enhanced generation of H_2S from ATB-337 as compared with ADT-OH could account for the gastric safety of the former. However, this requires further investigation.

In the studies performed in the air pouch model, in which inflammation was induced by injection of zymosan, ATB-337 reduced inflammatory PGE_2 synthesis as effectively as diclofenac. A highly selective COX-2 inhibitor, lumiracoxib, almost completely suppressed PGE_2 synthesis in this model, confirming that this model is appropriate for estimating the ability of drugs to inhibit COX-2 in vivo. ATB-337 also exhibited similar COX selectivity to diclofenac, because the 2 drugs suppressed COX-1 activity (whole blood thromboxane synthesis) to the same extent. Given these observations and the known importance of PGs to edema formation, it is not surprising that ATB-337 would exhibit anti-inflammatory effects in the carrageenan-induced paw edema model similar to those of diclofenac. On the other hand, the ability of ATB-337, at a dose of 10 μmol/kg, to suppress edema formation as effectively as diclofenac at 3 times this dose indicated an increase in potency of the HS-NSAID. This may be attributable to the recently reported ability of H_2S to reduce edema formation.[8] While analgesic effects of ATB-337 have not yet been characterized, it is noteworthy that H_2S donors, including a derivative of mesalamine containing the same H_2S-releasing moiety as ATB-337, were recently shown to significantly reduce visceral pain in the rat.[10,20]

Endogenous H_2S synthesis occurs principally via the actions of 2 enzymes: CSE and CBS. We previously reported that a number of NSAIDs were capable of reducing the expression of CSE and the generation of H_2S from gastric tissue in vitro.[7] In the present study, both diclofenac and ATB-337 markedly reduced gastric expression of both CSE and CBS. Interestingly, the expression of these enzymes following diclofenac administration recovered toward control levels within the 3-hour period of the study, but this was not the case in the rats treated with ATB-337. It is possible that H_2S generation from ATB-337 down-regulated the expression of these enzymes, thereby prolonging the suppression of expression over that induced by the diclofenac component of this compound. The notion that H_2S can down-regulate expression of enzymes responsible for H_2S synthesis is purely speculation at this point; however, it is noteworthy that PGs have been suggested to down-regulate expression of COX-2 in the stomach,[33,34] and similarly there is evidence that NO can reduce expression of inducible NO synthase.[35]

While gastrointestinal safety of NSAIDs is a major limitation to the use of this class of drugs, increasingly there is concern about their cardiovascular adverse effects, particularly since the withdrawal of rofecoxib (Vioxx; Merck & Co, Inc, Whitehouse Station, NJ) from worldwide markets. Whether or not HS-NSAIDs will exhibit reduced cardiovascular toxicity has not yet been examined. Unlike conventional NSAIDs[14,36,37] and selective COX-2 inhibitors,[23] ATB-337 did not induce leukocyte adherence to the vascular endothelium. Leukocyte adherence to endothelial cells can be an important component of thrombosis and of atherosclerosis. Also, given that H_2S is a potent vasodilator,[38] it is possible that its release from HS-NSAIDs may attenuate any hypertensive effects associated with the action of the NSAID component of the compounds, as was observed with NO-releasing NSAIDs in animal studies.[39,40] Clearly, this is an exciting aspect of the potential utility of HS-NSAIDs that warrants further study.

In summary, the results presented herein show that an H_2S-releasing derivative of an NSAID (ATB-337) exhibits greatly reduced gastrointestinal-damaging effects as compared with the parent drug, while still suppressing mucosal PG synthesis. The anti-inflammatory effects of the HS-NSAID were comparable or even improved over those of the parent NSAID, possibly attributable to the anti-inflammatory effects of H_2S. The lack of an effect of ATB-337 in terms of inducing leukocyte adherence to the vascular endothelium and inducing TNF-α expression, in contrast to diclofenac and other NSAIDs, may contribute to the gastrointestinal-sparing attributes of this compound. While the conclusions that we can draw regarding HS-NSAIDs are limited by the relatively short period of exposure of rats to ATB-337 in this study, the results are nevertheless an early indication that the coupling of an H_2S-releasing moiety to conventional or COX-2–selective NSAIDs is an attractive approach to reducing toxicity and enhancing efficacy.

References

1. Wallace JL. Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. Gastroenterology 1997; 112:1000–1016.
2. Wallace JL, Reuter B, Cicala C, McKnight W, Grisham MB, Cirino G. Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat. Gastroenterology 1994;107:173–179.
3. Fiorucci S, Santucci L, Wallace JL, Sardina M, Romano M, del Soldato P, Morelli A. Interaction of a selective cyclooxygenase-2 inhibitor with aspirin and NO-releasing aspirin in the human gastric mucosa. Proc Natl Acad Sci U S A 2003;100: 10937–10941.
4. Hawkey CJ, Jones JI, Atherton CT, Skelly MM, Bebb JR, Fagerholm U, Jonzon B, Karlsson P, Bjarnason IT. Gastrointestinal safety of

AZD3582, a cyclooxygenase inhibiting nitric oxide donator: proof of concept study in humans. Gut 2003;52:1537–1542.
5. Schnitzer TJ, Kivitz AJ, Lipetz RS, Sanders N, Hee A. Comparison of the COX-inhibiting nitric oxide donator AZD3582 and rofecoxib in treating the signs and symptoms of osteoarthritis of the knee. Arthritis Rheum 2005;53:827–837.
6. Fiorucci S, Distrutti E, Cirino G, Wallace JL. The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. Gastroenterology 2006;131:259–271.
7. Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, Orlandi S, Zanardo R, Renga B, Di Sante M, Morelli A, Cirino G, Wallace JL. Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. Gastroenterology 2005;129:1210–1224.
8. Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 2006;20:2118–2120.
9. Wallace JL, Bak A, McKnight W, Asfaha S, Sharkey KA, MacNaughton WK. Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. Gastroenterology 1998;115:101–109.
10. Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Russo G, Caliendo G, Santagada V, Cirino G, Wallace JL, Fiorucci S. 5-Amino-2-hydroxybenzoic acid 4-(5-thioxo-5H-[1,2]dithiol-3yl)-phenyl ester (ATB-429), a hydrogen sulfide-releasing derivative of mesalamine, exerts antinociceptive effects in a model of postinflammatory hypersensitivity. J Pharmacol Exp Ther 2006;319:447–458.
11. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczyk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 1989;96:795–803.
12. Reuter BK, Davies NM, Wallace JL. Nonsteroidal anti-inflammatory drug-enteropathy in rats: role of permeability, bacteria and enterohepatic circulation. Gastroenterology 1997;112:109–117.
13. Wallace JL, Arfors KE, McKnight GW. A monoclonal antibody against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. Gastroenterology 1991;100:878–883.
14. Wallace JL, McKnight W, Miyasaka M, Tamatani T, Paulson J, Anderson DC, Granger DN, Kubes P. Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury. Am J Physiol 1993;265:G993–G998.
15. Edwards JCW, Sedgwick AD, Willoughby DA. The formation of a structure with features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. J Pathol 1981;134:147–156.
16. Sedgwick AD, Moore AR, Al-Duaij AY, Edwards JC, Willoughby DA. Studies into the influence of carrageenan-induced inflammation on articular cartilage degradation using implantation into air pouches. Br J Exp Pathol 1985;66:445–453.
17. Wallace JL, Chapman K, McKnight W. Limited anti-inflammatory efficacy of cyclo-oxygenase-2 inhibition in carrageenan-airpouch inflammation. Br J Pharmacol 1999;126:1200–1204.
18. Rordorf CM, Choi L, Marshall P, Mangold JB. Clinical pharmacology of lumiracoxib: a selective cyclo-oxygenase-2 inhibitor. Clin Pharmacokinet 2005;44:1247–1266.
19. Wallace JL, McKnight W, Del Soldato P, Baydoun AR, Cirino G. Anti-thrombotic effects of a nitric oxide-releasing, gastric-sparing aspirin derivative. J Clin Invest 1995;96:2711–2718.
20. Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Antonelli E, Roviezzo F, Cirino G, Wallace JL, Fiorucci S. Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating K_{ATP} channels. J Pharmacol Exp Ther 2006;316:325–335.
21. Ubuka T, Abe T, Kajikawa R, Morino K. Determination of hydrogen sulfide and acid-labile sulfur in animal tissues by gas chromatography and ion chromatography. J Chromatogr B Biomed Sci Appl 2001;757:31–37.
22. Khan SU, Morris GF, Hidiroglou M. Rapid estimation of sulfide in rumen and blood with a sulfide-specific ion electrode. Microchem J 1980;25:388–395.
23. Wallace JL., McKnight W, Reuter BK, Vergnolle N. NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. Gastroenterology 2000;119:706–714.
24. Wallace JL, Miller MJ. Nitric oxide in mucosal defense: a little goes a long way. Gastroenterology 2000;119:512–520.
25. Santucci L, Fiorucci S, Giansanti M, Brunori PM, Di Matteo FM, Morelli A. Pentoxifylline prevents indomethacin induced acute gastric mucosal damage in rats: role of tumour necrosis factor alpha. Gut 1994;35:909–915.
26. Appleyard CB, McCafferty DM, Tigley AW, Swain MG, Wallace JL. Tumor necrosis factor mediation of NSAID-induced gastric damage: role of leukocyte adherence. Am J Physiol 1996;270:G42–G48.
27. Fiorucci S, Distrutti E, Mencarelli A, Renga B, Cirino G, Wallace JL. Enhanced anti-inflammatory activity of hydrogen sulfide releasing derivative of mesalamine in rodent models of colitis involves direct inhibition of Nf-KB (abstr). Gastroenterology 2006;130:A348.
28. Laine L, Connors LG, Reicin A, Hawkey CJ, Burgos-Vargas R, Schnitzer TJ, Yu Q, Bombardier C. Serious lower gastrointestinal clinical events with nonselective NSAID or coxib use. Gastroenterology 2003;124:288–292.
29. Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem Biophys Res Commun 1997;237:527–531.
30. Zhao W, Wang R. H_2S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol 2002;283:H474–H480.
31. Regula J, Butruk E, Dekkers CP, de Boer SY, Raps D, Simon L, Terjung A, Thomas KB, Luhmann R, Fischer R. Prevention of NSAID-associated gastrointestinal lesions: a comparison study pantoprazole versus omeprazole. Am J Gastroenterol 2006;101:1747–1755.
32. Christen MO. Anetole dithiolenthione: biochemical considerations. Methods Enzymol 1995;252:316–323.
33. Davies NM, Sharkey KA, Asfaha S, MacNaughton WK, Wallace JL. Aspirin induces a rapid up-regulation of cyclooxygenase-2 expression in the rat stomach. Aliment Pharmacol Ther 1997;11:1101–1108.
34. Takeuchi K, Tanaka A, Ohno R, Yokota A. Role of COX inhibition in pathogenesis of NSAID-induced small intestinal damage. J Physiol Pharmacol 2003;54(Suppl 4):165–182.
35. Cirino G, Wheeler-Jones CP, Wallace JL, Del Soldato P, Baydoun AR. Inhibition of inducible nitric oxide synthase expression by novel nonsteroidal anti-inflammatory derivatives with gastrointestinal-sparing properties. Br J Pharmacol 1996;117:1421–1426.
36. Asako H, Kubes P, Wallace J, Wolf RE, Granger DN. Modulation of leukocyte adhesion in rat mesenteric venules by aspirin and salicylate. Gastroenterology 1992;103:146–152.
37. Asako H, Kubes P, Wallace J, Gaginella T, Wolf RE, Granger DN. Indomethacin-induced leukocyte adhesion in mesenteric venules: role of lipoxygenase products. Am J Physiol 1992;262:G903–G908.
38. Wang R. Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? FASEB J 2002;16:1792–1798.
39. Muscara MN, Lovren F, McKnight W, Dicay M, del Soldato P, Triggle CR, Wallace JL. Antihypertensive properties of a nitric

oxide-releasing naproxen derivative in two-kidney, one-clip rats. Am J Physiol Heart Circ Physiol 2000;279:H528–H535.
40. Muscara MN, McKnight W, Lovren F, Triggle CR, Cirino G, Wallace JL. Vasorelaxant effects of a nitric oxide-releasing aspirin derivative in normotensive and hypertensive rats. Br J Pharmacol 2001;133:1314–1322.

Received August 13, 2006. Accepted September 28, 2006.

Address requests for reprints to: John L. Wallace, PhD, Department of Pharmacology and Therapeutics, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, T2N 4N1, Canada. e-mail: wallacej@ucalgary.ca; fax: (403) 270-3353.

Supported by a grant from the Canadian Institutes of Health Research. J.L.W. holds a Canada Research Chair and is supported by an Alberta Heritage Foundation for Medical Research Senior Scientist Award.

All authors of this paper hold shares in Antibe Therapeutics Inc.

The authors thank Webb McKnight, Michael Dicay, Barbara Renga, Andrea Mencarelli, and Stefano Orlandi for their assistance in performing these studies.