Markedly reduced toxicity of a hydrogen sulphide-releasing derivative of naproxen (ATB-346)

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Background and purpose: Hydrogen sulphide is an important mediator of gastric mucosal defence. The use of non-steroidal anti-inflammatory drugs continues to be limited by their toxicity, particularly in the upper gastrointestinal tract. We evaluated the gastrointestinal safety and anti-inflammatory efficacy of a novel hydrogen sulphide-releasing derivative of naproxen, ATB-346 [2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester].

Experimental approach: The ability of ATB-346 versus naproxen to cause gastric damage was evaluated in healthy rats and in rats with compromised gastric mucosal defence. Effects on the small intestine and on the healing of ulcers were also assessed. The ability of ATB-346 to inhibit cyclooxygenase-1 and 2 and to reduce inflammation in vivo was also evaluated.

Key results: ATB-346 suppressed gastric prostaglandin E2 synthesis as effectively as naproxen, but produced negligible damage in the stomach and intestine. In situations in which the gastric mucosa was rendered significantly more susceptible to naproxen-induced damage (e.g. ablation of sensory afferent nerves, inhibition of endogenous nitric oxide or hydrogen sulphide synthesis, co-administration with aspirin, antagonism of K+6.x channels), ATB-346 did not cause significant damage. Unlike naproxen and celecoxib, ATB-346 accelerated healing of pre-existing gastric ulcers. In a mouse airpouch model, ATB-346 suppressed cyclooxygenase-2 activity and inhibited leukocyte infiltration more effectively than naproxen. ATB-346 was as effective as naproxen in adjuvant-induced arthritis in rats, with a more rapid onset of activity. Unlike naproxen, ATB-346 did not elevate blood pressure in hypertensive rats.

Conclusions and implications: ATB-346 exhibits anti-inflammatory properties similar to naproxen, but with substantially reduced gastrointestinal toxicity.


Keywords: hydrogen sulphide; non-steroidal anti-inflammatory drug; ulcer; inflammation; gastrointestinal; blood pressure; arthritis; naproxen; cyclooxygenase; mucosal defence

Abbreviations: GI, gastrointestinal; H2S, hydrogen sulphide; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin
Like NO, hydrogen sulphide (H$_2$S) is a gaseous mediator with physiological and pathophysiological roles in many organs (Wang, 2002; Fiorucci et al., 2006; Li and Moore, 2008). H$_2$S has been shown to be produced in the GI tract (Fiorucci et al., 2005; Linden et al., 2008; Wallace et al., 2009b) and to contribute to gastric mucosal defence (Fiorucci et al., 2005) and the healing of gastric ulcers (Wallace et al., 2007b). In the intestine, H$_2$S modulates epithelial secretion (Schicho, 2005) and exhibits a substantial reduction in toxic effects in the GI tract.

In the present study, we have evaluated the anti-inflammatory and GI-damaging effects of a novel H$_2$S-releasing derivative of naproxen, ATB-346 [2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester]. Naproxen is among the most commonly used NSAIAds, in part because of evidence that its use may be associated with less cardiovascular toxicity than selective COX-2 inhibitors and other NSAIAds (Kearney et al., 1999). This study included examination of the gastric safety of ATB-346 in circumstances in which mucosal defence was significantly impaired, in an attempt to model more closely the clinical scenario in which NSAIAds are most frequently used (i.e. patients with co-morbidities that elevate susceptibility to NSAIAD-induced ulceration). Our results demonstrate that ATB-346 is at least as effective as naproxen as an anti-inflammatory drug, while exhibiting a substantial reduction in toxic effects in the GI tract.

**Methods**

**Animals**

Animal studies were conducted in accordance with the guidelines established by the Canadian Council of Animal Care, with all protocols being approved by our institutional animal care committee. Male, Wistar rats (200–225 g) and male, C57BL/6J mice (25–27 g) were obtained from Charles River Laboratories (Montreal, QC, Canada) and were fed standard laboratory chow and water *ad libitum*. The animals were housed in pairs and kept in a room having controlled temperature (22 ± 1°C), humidity (65–70%) and light cycle (12 h light/12 h dark).

**Zymosan airpouch model**

Mice were deprived of food, but not water, for 18–20 h prior to experiments. The airpouch was induced as described previously (Edwards et al., 1981; Wallace et al., 1999), with slight modifications. Briefly, 3 mL of air was injected subcutaneously into the back of the mice. Additional injections of 1.5 mL of air were performed 3 and 6 days after the first injection. Twenty-four hours after the last injection of air, 1 mL of either saline or a 1% solution of zymosan was injected into the pouch. All of the injections were performed under isoflurane anaesthesia. One hour prior to zymosan injection into the airpouch, mice were treated orally with naproxen or ATB-346 (each at 30 μmol·kg$^{-1}$ or vehicle (1% carboxymethylcellulose). Each group consisted of five to six mice. An additional group of mice was treated with another derivative of naproxen, ATB-345 [2-(6-methoxy-napthalen-2-yl)-propionic acid 4-(5-thioxo-5H-[1,2]dithiol-3-yl)-phenyl ester] (30 μmol·kg$^{-1}$), which has a different H$_2$S-releasing moiety to that in ATB-346 (Figure 1). Six hours after zymosan injection, the mice were anaesthetized with sodium pentobarbital (60 mg·kg$^{-1}$, i.p.), and blood was drawn from the inferior vena cava for measurement of whole blood thromboxane B$_2$ (TXB$_2$) synthesis (by ELISA), as an index of COX-1 activity (Wallace et al., 1999). Immediately thereafter, 1 mL of heparin-treated saline was injected into the pouch. The airpouch was carefully opened by a small incision. The exudate was collected, the volume measured and an aliquot used to quantify leukocyte numbers using a Sysmex KX-21N haematology analyser (Sysmex Canada, Mississauga, ON, Canada). The exudate was centrifuged at 10000 g for 10 min. The supernatant was collected and stored at –80°C for measurement of immunoreactive PGE$_2$, as an index of COX-2 activity (Wallace et al., 1999).

**Adjuvant arthritis**

Polyarthritis was induced in rats via an injection into the base of the tail of 100 μL of Freund’s Complete Adjuvant containing 0.6 mg of Mycobacterium butyricum (Cicala et al., 2000). The volume of both hindpaws of each rat was blindly measured using a hydroplethysmometer (Ugo Basile, Comerio, Italy) prior to the injection of the adjuvant, and on days 7, 10, 14 and 21 after adjuvant administration. Groups of six to seven rats each were treated twice-daily, orally, with naproxen (4 μmol·kg$^{-1}$), ATB-346 (4 μmol·kg$^{-1}$) or vehicle (1%
rats that had been treated with aspirin (10 mg·kg\(^{-1}\) i.p.), N\(^{\circ}\)-nitro-L-arginine methylester (L-NAME; 15 mg·kg\(^{-1}\) i.p.), β-cyanoalanine (BCA; 50 mg·kg\(^{-1}\) i.p.) or glibenclamide (10 mg·kg\(^{-1}\) i.p.). Aspirin was given immediately before administration of the test drugs/vehicle, while the other agents were given 30 min before administration of the test drugs/vehicle. In the experiments in which aspirin was administered, one group of rats was cotreated with celecoxib (30 μmol·kg\(^{-1}\) p.o.).

The test drugs or vehicle were also administered to rats in which sensory afferent neurones had been ablated by prior treatment with capsaicin, as described previously (McCafferty et al., 1997).

**Acute gastric damage**

Rats were fasted for 16–18 h, then were randomized to groups (n ≥ 5) and given vehicle or one of the test drugs orally. Naproxen and ATB-346 were tested at doses of 30, 60, 120 and 2740 μmol·kg\(^{-1}\). Three hours later, the rats were killed by an overdose of pentobarbital sodium and the stomach was excised and examined. To assess gastric mucosal damage, the lengths (in mm) of all lesions were measured with a digital calliper, and a ‘gastric damage score’ was calculated for each stomach by summing these values (Wallace et al., 2000). This assessment was performed by an individual unaware of the treatments the rats had received. Samples of the gastric tissue were excised and processed, as described previously (Wallace et al., 2000), for measurement of immunoreactive PGE₂.

ATB-346 consists of a molecule of naproxen linked via an ester bond to 1-hydroxy-2-napthalenemethanol (Fig. 1). To determine if co-administration of unconjugated naproxen and TBZ would protect the gastric mucosa from damage, the following experiment was performed. Groups of five rats each, deprived of food for the previous 16–18 h, were given naproxen (50 μmol·kg\(^{-1}\)) orally together with an equal volume of vehicle, or vehicle containing TBZ at 50, 100 or 150 μmol·kg\(^{-1}\). Another group of rats received ATB-346 at 50 μmol·kg\(^{-1}\). Three hours later, gastric damage was blindly scored as described above.

**Intestinal damage**

Groups of six rats (not fasted) were treated twice-daily for 5 days with naproxen (90 μmol·kg\(^{-1}\)), ATB-346 (90 μmol·kg\(^{-1}\)) or vehicle. Prior to the first administration of test drug or vehicle, a blood sample was drawn from the tail vein for determination of haematocrit (Reuter et al., 1997). Six hours after the final administration of test drug or vehicle, another blood sample was drawn for determination of haematocrit, and the rats were then killed by an overdose of sodium pentobarbital. The small intestine was excised, and the extent of haemorrhagic damage to the small intestine was blindly quantified by measuring the lengths of lesions in mm and then summing these to give a damage score for each rat (Reuter et al., 1997).

**Impaired gastric mucosal defence**

Experiments were performed as described above to compare the gastric damaging effects of naproxen to those of ATB-346. In these experiments, a number of other procedures were employed to increase the gastric mucosal susceptibility to damage. These included administration of the test drugs to rats that had been treated with aspirin (10 mg·kg\(^{-1}\) i.p.), N\(^{\circ}\)-nitro-L-arginine methylester (L-NAME; 15 mg·kg\(^{-1}\) i.p.), β-cyanoalanine (BCA; 50 mg·kg\(^{-1}\) i.p.) or glibenclamide (10 mg·kg\(^{-1}\) i.p.). Aspirin was given immediately before administration of the test drugs/vehicle, while the other agents were given 30 min before administration of the test drugs/vehicle. In the experiments in which aspirin was administered, one group of rats was cotreated with celecoxib (30 μmol·kg\(^{-1}\) p.o.). The test drugs or vehicle were also administered to rats in which sensory afferent neurones had been ablated by prior treatment with capsaicin, as described previously (McCafferty et al., 1997).

**Gastric ulcer healing**

Ulcers were induced using a previously described model (Okabe et al., 1971; Dudar et al., 2008), with slight modifications. In brief, mice were anaesthetized with isoflurane, the abdomen was opened with a midline incision and the stomach was exposed to permit application of glacial acetic acid (200 μL, 20% vol·vol\(^{-1}\)) to the serosal surface of the corpus region. The acetic acid was applied using the barrel of a 1 mL syringe, such that the contact area was 28 mm\(^2\). One minute later, the acetic acid was removed by aspiration, and the area of contact was washed three times with 200 μL of saline. The peritoneum and skin were then closed with sutures. Three days after application of acetic acid to the stomach, five mice were killed by an overdose of sodium pentobarbital. The stomach was removed and opened by an incision along the greater curvature, then pinned out, mucosal side up, on a paraffin block. A 25 mm\(^2\) grid was placed alongside the ulcer and the stomach was photographed. Planimetric analysis, using the grid as reference, allowed for determination of the area of the ulcer (Dudar et al., 2008). The remaining mice were randomized to one of four groups, and they began receiving one of the following twice-daily, orally: naproxen (60 μmol·kg\(^{-1}\)), ATB-346 (60 μmol·kg\(^{-1}\)), celecoxib (30 μmol·kg\(^{-1}\)) or vehicle. After 4 days of drug/vehicle administration, the mice were killed and the areas of the gastric ulcers were blindly measured, as described above.

**Arterial blood pressure**

Effects on systemic arterial blood pressure were assessed in healthy, untreated rats and in rats given drinking water supplemented with L-NAME (400 mg·L\(^{-1}\)) for the previous 2 weeks to induce hypertension (Muscarà et al., 2000b). The rats were anaesthetized with sodium pentobarbital and a carotid artery was cannulated for measurement of systemic blood pressure. After a stable blood pressure had been recorded for at least 20 min, the rats received an i.p. injection of naproxen (90 μmol·kg\(^{-1}\)), ATB-346 (90 μmol·kg\(^{-1}\)) or vehicle (1% carboxymethylcellulose). Also, for comparison, we examined the effects of a second NSAID, diclofenac (60 μmol·kg\(^{-1}\)), and an H\(_2\)-releasing derivative of diclofenac, ATB-337 [2-(2,6-dichloro-phenylamino)-phenyl)-acetic acid 4-(3-thioxo-3H-1,2[dithiol-4-yl]-phenyl ester] (60 μmol·kg\(^{-1}\)), which has been described previously (Li et al., 2007; Wallace et al., 2007a). Blood pressure was recorded for 60 min following the injections.

**Statistical analyses**

All data are presented as the mean ± SEM. Comparisons of two groups of data to one another were performed using
Student’s t-test (with Bonferroni correction in cases where heterogeneity of variance existed). Comparisons among more than two groups of data were performed using ANOVA followed either by the Dunnett’s Multiple Comparison test (parametric) or Mann–Whitney test (non-parametric), as appropriate. An associated probability (P-value) of less than 5% was considered significant.

Materials
ATB-346, ATB-345 and ATB-337 were synthesized by Antibe Therapeutics Inc. (Toronto, ON, Canada). Aspirin, diclofenac sodium, sodium naproxen, L-NAME, BCA and glibenclamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA kits for measuring TXB2 and PGE2 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Celecoxib was obtained from American Custom Chemicals Ltd. (San Diego, CA, USA). TBZ was purchased from SynChem Inc. (Des Plaines, IL, USA).

Drug and molecular target nomenclature conforms to BJPs Guide to Receptors and Channels (Alexander et al., 2008).

Results
Inhibition of COX-1 and COX-2 activity
The airpouch model permits the evaluation of effects of a drug on systemic COX-1 activity, via measurement of whole blood TXB2 synthesis, and COX-2 activity, via measurement of PGE2 levels in the airpouch exudates (Wallace et al., 1999). Administration of zymosan into the airpouch resulted in a profound influx of leukocytes (Figure 2A) and a 15-fold increase in exudate concentrations of PGE2 (Figure 2B). Exudate leukocyte and PGE2 levels were significantly reduced by naproxen and by the two H2S-releasing naproxen derivatives. In the case of PGE2 levels, significantly greater inhibition was observed with ATB-346 than with naproxen or ATB-345. Whole blood TXB2 synthesis was almost completely suppressed by naproxen and ATB-346, but only 60% inhibition was observed in mice treated with ATB-345 (Figure 2C). Despite marked suppression of systemic COX-1 activity, neither of the H2S-releasing donors caused gastric damage, while haemorrhagic erosions were evident in the mice treated with naproxen (Figure 2D).

Figure 2  Effects of naproxen and two hydrogen sulphide-releasing naproxen derivatives (ATB-345 and ATB-346) in a model of zymosan-induced inflammation in the mouse. Samples of inflammatory exudates and of blood were collected 6 h after injection of zymosan into a preformed airpouch on the back of the mice. The test drugs were administered orally at a dose of 30 μmol·kg⁻¹ h prior to zymosan administration. The test drugs significantly reduced infiltration of leukocytes into the airpouch (A), PGE2 levels in the exudates, which are indicative of cyclooxygenase-2 activity (B), and whole blood thromboxane synthesis, which is indicative of cyclooxygenase-1 activity (C). Gastric damage was observed in the mice treated with naproxen, but not in the other groups (D). *P < 0.05 versus all other groups. #P < 0.05 versus the naproxen-treated group; ¥P < 0.05 versus the other groups treated with one of the test drugs (ANOVA and Dunnett’s Multiple Comparison test). Each group consisted of five to six mice. ATB-345, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-(5-thioxo-5H-[1,2]dithiol-3-yl)-phenyl ester; ATB-346, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester; PG, prostaglandin; TXB2, thromboxane B2.
propionic acid 4-thiocarbamoyl-phenyl ester.

Multiple Comparison test). ATB-346, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester.

Moreover, the same doses, gastric damage did not develop (this was confirmed by blind histological evaluation). Rats treated with ATB-346 did not exhibit any signs of damage (i.e. prior to adjuvant administration). Rats treated with naproxen (4 mol·kg⁻¹) resulted in a significant reduction of paw volume on day 21, but not on day 14. ATB-346 significantly reduced paw oedema at days 14 and 21 (*P < 0.05 vs. the vehicle-treated group). Each group consisted of six to seven rats (data were compared using ANOVA and Dunnett’s Multiple Comparison test). ATB-346, 2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester.

Anti-inflammatory effects on adjuvant-Induced arthritis
A pronounced increase in paw volume was observed during days 14–21 after injection of Freund’s Complete Adjuvant into base of the tail in rats (Figure 3). Twice-daily oral treatment with naproxen (4 mol·kg⁻¹) resulted in a significant reduction of paw volume on day 21, but not on day 14. In contrast, treatment with ATB-346 produced a significant reduction of paw volume on days 14 and 21. The paw volume on these days did not differ significantly from those on day 0 (i.e. prior to adjuvant administration). Rats treated with naproxen did not exhibit any gastric damage at the conclusion of the study, but blood was evident in the small intestine. Rats treated with ATB-346 did not exhibit any signs of damage or bleeding in the stomach or small intestine.

Gastric damage and PG synthesis
To evaluate more fully the effects of ATB-346 on the gastric mucosa, a dose–response study was performed in rats. Naproxen given orally at doses of 30, 60 or 120 mol·kg⁻¹ produced haemorrhagic erosions in the stomach that increased in severity with the dose (Figure 4). With all three doses, gastric PGE₂ synthesis was inhibited by naproxen (by 88%, 93% and 99% respectively). When ATB-346 was given at the same doses, gastric damage did not develop (this was confirmed by blind histological evaluation). Moreover, the inhibition of PGE₂ synthesis by ATB-346 at doses of 30, 60 or 120 mol·kg⁻¹ (87%, 95% and 99% respectively) did not differ significantly from that observed with naproxen. We then examined the effects of a very high dose of naproxen and ATB-346 (2.74 mmol·kg⁻¹). Naproxen induced the formation of extensive and severe haemorrhagic erosion (Figure 4). At this very high dose, ATB-346 also induced the formation of some haemorrhagic erosion, but the extent was markedly less (approximately the same as that produced by naproxen at 1/100th the dose).

The gastric-sparing property of ATB-346 was not evident when the two components of this drug (naproxen and TBZ) were administered as separate entities. Naproxen given orally at 50 mol·kg⁻¹ resulted in a gastric damage score of 15 ± 5, while with an equimolar dose of ATB-346 the gastric damage score was 0.2 ± 0.2. Co-administering the molar equivalent amounts of naproxen and TBZ resulted in a gastric damage score of 14 ± 4. Increasing the amount of TBZ administered to 100 or 150 mol·kg⁻¹ (with 50 mol·kg⁻¹ naproxen) did not confer any protective effect (gastric damage scores of 13 ± 3 and 16 ± 5 respectively).

Impaired gastric mucosal defence
Co-administration of naproxen with aspirin, the latter at a dose that by itself produced negligible gastric damage (10 mg·kg⁻¹), resulted in more than a doubling of the gastric damage score as compared to that with naproxen alone (Figure 5A). Similarly, a selective COX-2 inhibitor, celecoxib, caused extensive gastric damage when co-administered with aspirin (but not when given alone). In contrast, the combination of ATB-346 and aspirin did not elicit gastric damage.

Ablation of gastric sensory afferent nerves with capsaicin has been shown to markedly increase the susceptibility of the stomach to injury induced by a variety of agents (Holzer et al., 1987). In the present study, naproxen-induced gastric damage
was approximately three times more severe in rats in which capsaicin-induced ablation had been performed, while ATB-346 still did not induce significant gastric damage (Figure 5B).

Gastric mucosal defence can also be impaired by inhibition of synthesis of NO (Tepperman and Soper, 1993) or H2S (Fiorucci et al., 2005). In rats pretreated with the non-selective NO synthase inhibitor, L-NAME, naproxen produced significantly more severe gastric damage than that produced in rats pretreated with vehicle (Figure 6A). Similarly, administration of BCA, an inhibitor of one of the key enzymes for H2S synthesis in the stomach (Martin et al., 2009) resulted in a significant increase in the severity of naproxen-induced gastric damage. However, no gastric damage was observed in rats treated with ATB-346 following L-NAME or BCA administration (Figure 6B). Glibenclamide pretreatment itself did not produce any gastric damage, but subsequent administration of naproxen resulted in damage more than twice as extensive as that observed in vehicle pretreated rats (Figure 6C). Glibenclamide pretreatment had no effect on the gastric-sparing properties of ATB-346.

Small intestinal injury and bleeding

Twice-daily oral administration of naproxen to rats for 5 days resulted in extensive damage in the small intestine (Figure 7A). Ulcers were mainly concentrated in the jejunum.
Blood was evident in the lumen, and consistent with this observation, there was a significant decrease in haematocrit at the end of the study as compared with the pretreatment value (Figure 7B). In contrast, very little damage was observed in the intestine of rats treated with the same dose of ATB-346 (five of the six rats did not exhibit any damage), and there was no change in haematocrit over the course of the 5 days of treatment.

Healing of gastric ulcers

In addition to inducing erosions and ulcers, NSAIDs can interfere with the healing of pre-existing ulcers (Stadler et al., 1991). Using a well-characterized rodent model (Okabe et al., 1971; Ma et al., 2002; Dudar et al., 2008), we compared the effects of ATB-346 on gastric ulcer healing with those of naproxen and celecoxib. Within 3 days of serial application of acetic acid, an ulcer with an average area of ~25 mm² had formed on the mucosal surface of the mouse stomach, penetrating through the muscularis mucosae into the submucosa. Over a 4 day period of twice-daily treatment with vehicle, the area of the ulcer was reduced by approximately 46% (Figure 8). In mice treated with naproxen or celecoxib, the extent of healing was significantly less than that in vehicle-treated mice (32% and 27% respectively). In contrast, treatment with ATB-346 significantly enhanced gastric ulcer healing (61%).

Effects on systemic blood pressure

Intraperitoneal injection of ATB-346 or naproxen to healthy, untreated rats did not elicit any significant change in systemic arterial blood pressure over the 60 min that followed. In both cases, the mean arterial blood pressure remained in the 95–110 mmHg range. The mean arterial blood pressure in the rats that had received L-NAME in their drinking water for the previous 2 weeks was 153/110 mmHg (n = 32). During the hour after i.p. administration of naproxen at a dose of 90 μmol·kg⁻¹, there was an increase above basal blood pressure of ~12 mmHg (Figure 9). In contrast, the increase in blood pressure observed following administration of an equimolar dose of ATB-346 (~4 mmHg) did not differ significantly from that observed following administration of vehicle (~3 mmHg). Similar results were obtained when another NSAID (diclofenac; 60 μmol·kg⁻¹) was compared with an equimolar dose of a H₂S-releasing diclofenac derivative, ATB-337 (Figure 9).

Discussion

Considerable resources have been invested in the past four decades in the development of effective anti-inflammatory and analgesic drugs that do not cause the GI ulceration and bleeding associated with the use of conventional NSAIDs. Selective COX-2 inhibitors arrived on the market in the mid-1990s with a promise of reduced GI toxicity. These drugs do
produce less GI ulceration and bleeding than traditional NSAIDs in some groups of patients and in healthy animals (Masferrer et al., 1994; Silverstein et al., 2000), but when given concomitantly with aspirin or in other circumstances in which mucosal defence is impaired, the safety advantage over conventional NSAIDs is negligible (Marcic et al., 1999; Silverstein et al., 2000; Wallace et al., 2000; Fiorucci et al., 2003; Lanas and Scheiman, 2007). Selective COX-2 inhibitors have also been found to interfere with healing of pre-existing ulcers in rodent studies (Mizuno et al., 1997; Ma et al., 2002). These findings suggest that preclinical studies of novel anti-inflammatory drugs would be more useful if they included examination of the effects of those drugs in models in which gastric mucosal defence is impaired and in models of pre-existing injury. Thus, in the present study we evaluated ATB-346 in healthy animals and in several models characterized by impaired mucosal defence, as well as in a model of gastric ulcer healing.

In the present study, we examined the effects of a novel H$_2$S-releasing derivative of one of the most commonly used NSAIDs, naproxen. ATB-346 exhibited anti-inflammatory activity comparable, or even superior, to equimolar doses of naproxen in two models (mouse airpouch and rat adjuvant arthritis). In healthy rats, ATB-346 caused negligible gastric damage even when given at an extremely high dose (2.7 mmol·kg$^{-1}$). On a kg$^{-1}$ basis, this represents a dose >100 times the usual human dose of naproxen. The small amount of haemorrhagic damage observed in rats treated with the high dose of ATB-346 was equivalent to that observed with naproxen at 1/100th that dose. We also observed a marked gastro-sparing effect of ATB-346 in several models in which gastric mucosal defence was impaired. These included co-administration of aspirin, pretreatment with inhibitors of NO or H$_2$S synthesis, co-administration of a K$_{ATP}$ antagonist and prior ablation of sensory afferent nerves, all of which significantly increased the severity of naproxen-induced gastric damage. Moreover, while naproxen and celecoxib significantly inhibited gastric ulcer healing in mice, there was a significant enhancement of healing in mice treated with ATB-346. When given repeatedly over several days, naproxen elicited extensive ulceration and bleeding in the small intestine. In contrast, ATB-346 had no effect on haematocrit and produced very little mucosal damage.

Several lines of evidence suggest that H$_2$S is a potent mediator of GI mucosal defence, and that the GI-sparing effects of ATB-346 are attributable to its ability to release H$_2$S. First, H$_2$S donors have been shown to protect the gastric mucosa from NSAID-induced damage (Fiorucci et al., 2005; Wallace, 2009). Second, inhibition of endogenous H$_2$S synthesis results in a significant increase in severity of NSAID-induced gastric damage, as seen in the present study and previously (Fiorucci et al., 2005). Third, the conjugation of different H$_2$S donors to different NSAIDs has been shown to consistently result in a GI-sparing effect (Li et al., 2007; Wallace et al., 2007a; 2008). While ATB-346 did not produce significant gastric damage in various models of impaired mucosal defence, co-administration of the two moieties of this compound (naproxen and TBZ) resulted in gastric damage comparable in severity to that seen with naproxen alone. The explanation for this finding may lie in the different levels of release of H$_2$S from TBZ versus that from ATB-346. Release of H$_2$S from ATB-346 incubated in buffer or in rat liver homogenates was found to be about six times greater than that from an equimolar concentration of TBZ (Wallace et al., 2008). Similar results have been reported with an H$_2$S-releasing derivative of mesalamine (Distrettii et al., 2006b).

ATB-346 did not produce significant GI injury despite inhibiting systemic COX-1 activity and mucosal PG synthesis as effectively as naproxen. The finding that this compound also did not induce damage when mucosal NO or H$_2$S synthesis was suppressed (by L-NAME and BCA respectively) suggests that stimulation of endogenous release of those mediators did not account for its GI safety. Many effects of H$_2$S have been reported to occur via stimulation of ATP-sensitive potassium (K$_{ATP}$) channels. However, ATB-346 still did not elicit gastric damage when given to rats pretreated with a K$_{ATP}$ antagonist (glibenclamide) at a dose that significantly exacerbated naproxen-induced damage. Thus, activation of these potassium channels by ATB-346 does not appear to be the prime mechanism underlying its GI safety. Li et al. (2007) demonstrated that a H$_2$S-releasing derivative of diclofenac suppressed endotoxin-induced generation of several pro-inflammatory cytokines (interleukin-1β, tumour necrosis factor-α) and provided evidence that this may be mediated via inhibition of activation of the transcription factor NFκB. Inhibitors of NFκB activation have been shown to reduce the severity of NSAID-induced gastric damage (Brand et al., 1999). Inhibition by H$_2$S of NSAID-induced leukocyte adherence in the GI microcirculation is another possible mechanism underlying the GI safety of ATB-346. Leukocyte adherence is
an early, key event in the pathogenesis of mucosal injury associated with NSAIDs (Wallace, 1993). H2S is a potent inhibitor of leukocyte adherence induced by NSAIDs and other agonists (Zanardo et al., 2006; Andruski et al., 2008). While NSAIDs have been shown to trigger leukocyte adherence and up-regulation of endothelial iCAM-1 and leukocyte (LFA-1) adhesion molecules, these events were prevented by co-administration of an H2S donor with the NSAID (Fiorucci et al., 2005), and did not occur following administration of an H2S-releasing derivative of diclofenac (Wallace et al., 2007a). H2S can also prevent the decrease in gastric blood flow that is seen following NSAID administration (Fiorucci et al., 2005), which could contribute to the gastric safety of ATB-346. However, both the inhibition of leukocyte adherence and the vasodilator effects of H2S have been reported to be inhibited by glibenclamide (Wang, 2002; Fiorucci et al., 2005; Zanardo et al., 2006), while the gastric safety of ATB-346 in the present study was unaffected by glibenclamide.

The withdrawal of rofecoxib (Vioxx®) from worldwide markets in 2004 raised awareness of the cardiovascular toxicity of coxibs and of traditional NSAIDs (Grosser et al., 2006; Kearney et al., 2006). The underlying mechanism for this toxicity has not been identified and is the subject of considerable debate (Grosser et al., 2006; Mitchell and Warner, 2006). Sustained suppression of platelet thromboxane synthesis is generally regarded as beneficial in terms of cardiovascular health during NSAID therapy, counteracting the detrimental effects of suppression of endothelial prostacyclin synthesis (Grosser et al., 2006). The long-lasting suppression of platelet thromboxane synthesis by naproxen, versus most other NSAIDs, has been suggested to account for the relative cardiovascular safety of naproxen (Kearney et al., 2006). Both traditional NSAIDs and selective COX-2 inhibitors cause small but clinically significant increases in systemic blood pressure, which can result in significant increases in the risk of myocardial infarction and stroke (Singh et al., 2003). While a detailed study of the cardiovascular safety of ATB-346 has not been performed, it is noteworthy that this compound suppressed systemic COX-1 activity (i.e. platelet thromboxane synthesis) as effectively as naproxen. Moreover, bolus injection of a high dose of ATB-346 to hypertensive rats did not significantly affect mean arterial blood pressure, while a significant increase was seen with an equimolar dose of naproxen. H2S has recently been shown to contribute to regulation of blood pressure in mice (Yang et al., 2008). We did not observe any effect of ATB-346 on blood pressure in normotensive rats, consistent with the findings of Li et al. (2007), who studied an H2S-releasing diclofenac derivative. The effectiveness of coupling of an endogenous vasodilator with an NSAID to reduce the detrimental effects of the NSAID on blood pressure has been demonstrated previously. NO-releasing NSAIDs have been shown to have beneficial effects in hypertensive animals and humans (Muscarà et al., 1998; 2000a; Karlsson et al., 2009; Wallace et al., 2009a; White et al., 2009).

In summary, the present study provides evidence for the remarkable GI-sparing properties of an H2S-releasing derivative of naproxen. The H2S-releasing moiety contained within ATB-346, TBZ, has not been previously utilized for this purpose. In healthy animals, ATB-346 was in the order of 100-fold safer than naproxen, in terms of eliciting gastric injury, but in two models of inflammation it exerted effects comparable to or superior to those of naproxen. Importantly, and in contrast to selective COX-2 inhibitors, ATB-346 did not cause significant gastric damage when given to rats in which mucosal defence was significantly compromised, and it enhanced, rather than inhibited, healing of pre-existing gastric ulcers. Finally, the lack of effect of ATB-346 on blood pressure in hypertensive rats suggests that this compound may also exhibit a better cardiovascular profile than conventional NSAIDs. H2S-releasing NSAIDs appear to represent a very promising alternative to existing therapies for the treatment of inflammation and pain.

Conflicts of interest
Each of the authors of this paper holds shares in Antibe Therapeutics Inc., a company developing H2S-releasing drugs, including those described in this paper.

References


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