Clinical use of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin or naproxen is limited due to the gastrotoxicity evoked by these compounds. Endogenous hydrogen sulfide (H$_2$S) and delivered via an H$_2$S donor have been shown to play important role in the maintenance of gastric mucosal integrity. This study aimed to compare the effects of naproxen and an H$_2$S-releasing naproxen derivative (ATB-346) on gastric lesion induction by water immersion and restraint stress (WRS), the alterations in gastric blood flow (GBF) and the influence of these drugs on systemic inflammation. Wistar rats were pretreated i.g. with vehicle, naproxen (20 mg/kg) or ATB-346 (equimolar dose) or NaHS (5 mg/kg), the H$_2$S donor, combined with naproxen and exposed to 3.5 hours of WRS. The gastric lesion number and GBF were assessed by planimetry and laser Doppler flowmetry, respectively. Plasma concentrations of interleukins: IL-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, interferon-$\gamma$ (IFN-$\gamma$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and GM-CSF were determined by Luminex system and gastric mucosal protein expression of cystathionine-$\gamma$-lyase (CSE), cystathionine-$\beta$-synthase (CBS), 3-mercaptopyruvate sulfurtransferase (3-MST), nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), hypoxia inducible factor-$\alpha$ (HIF-1$\alpha$), heme oxygenase-1 (HO-1) and cyclooxygenase (COX-2) were analyzed by Western blot. Pretreatment with naproxen increased the number of WRS stress-induced gastric lesions and significantly decreased GBF as compared with vehicle (p < 0.05). In contrast, pretreatment with ATB-346 or naproxen combined with NaHS significantly reduced WRS-lesions number and elevated GBF as compared with naproxen (p < 0.05). Naproxen significantly increased gastric mucosal protein expression of CSE, Nrf-2 and HIF-1$\alpha$ as compared with vehicle (p < 0.05), but failed to affect CBS, 3-MST and HO-1. ATB-346 significantly increased Nrf-2 and HO-1 protein expression as compared with vehicle (P < 0.05) but did not affect the protein expression of CSE, CBS, 3-MST or HIF-1$\alpha$. ATB-346 but not naproxen decreased COX-2 protein expression in gastric mucosa compromised by WRS (p < 0.05). Exposure to WRS increased plasma concentration of all investigated cytokines (p < 0.05). ATB-346 but not naproxen decreased plasma content of IL-1$\beta$, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-$\alpha$ and IFN-$\gamma$ in rats exposed to WRS (p < 0.05). We conclude that H$_2$S through its vasoactive properties attenuates the gastrotoxic effects of naproxen, which increased stress-induced hypoxia in gastric mucosa. In contrast to naproxen, ATB-346 decreased stress-induced systemic inflammation and pro-inflammatory COX-2 expression in the gastric mucosa. The decreased gastrotoxicity of ATB-346 could be due to upregulation of Nrf-2/HO-1 pathway mediated by the release of H$_2$S.

**Key words:** hydrogen sulfide, naproxen, hydrogen sulfide releasing naproxen, stress, systemic inflammation, gastric blood flow, hypoxia inducible factor-$\alpha$ nuclear factor (erythroid-derived 2)-like 2, heme oxygenase-1

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**INTRODUCTION**

Hydrogen sulfide (H$_2$S), next to nitric oxide (NO) and carbon monoxide (CO) is an endogenous gaseous mediator involved in the mechanism of gastrointestinal (GI) integrity, gastroprotection and regulation of gastric microcirculation (1, 2). This molecule is produced in mammalian tissues by the enzymatic activity of cystathionine $\gamma$-lyase (CSE), cystathionine $\beta$-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) (3, 4).

Treatment with non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin or naproxen is limited due to a potent gastrotoxic effects exerted by these compounds (5). Therefore, to counteract these GI side effects of NSAIDs, the gaseous mediators-releasing derivatives of these drugs were developed including an H$_2$S-releasing naproxen (ATB-346), an H$_2$S-releasing aspirin, NO and H$_2$S-releasing aspirin hybrids (6, 8). Some of these novel compounds were shown to exert lower gastrotoxicity as compared with parent drugs (8, 9). Moreover, the intragastric administration of H$_2$S or CO releasing compounds
prevent gastric mucosal injury induced by aspirin or alendronate (10-12). However, the effect of \( \text{H}_2\text{S} \)-releasing naproxen derivative, ATB-346 on the regulation of stress-induced systemic inflammation and the mechanisms involved in stress-induced gastric damage formation has not been fully elucidated.

Our study aimed to compare the effects of ATB-346 with those exerted by native naproxen on stress-induced gastric lesions and systemic inflammation using animal model of water immersion and restraint stress (WRS). Moreover, we attempted to investigate if pretreatment with naproxen or ATB-346 can affects changes in gastric blood flow (GBF). We also determined the protein expression of \( \text{H}_2\text{S} \) biosynthesis enzymes, nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), heme oxygenase (HO)-1, cyclooxygenase (COX)-2, hypoxia inducible factor-1α (HIF-1α) in gastric mucosa pretreated with ATB-346 or naproxen and compromised by acute stress.

**MATERIALS AND METHODS**

**Animals and experimental design**

Twenty five male Wistar rats weighting 220 – 300 g were used in the study. All procedures were approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and were performed in accordance with Helsinki Declaration. Animals were deprived of food for 24 hours with free access to tap water before drugs application.

Gastric lesions were induced by stress caused by immobilization of rats in individual Bolman’s cages and their immersion in the cold water (21°C) for 3.5 h to the level of xiphoid cartilage using the method originally described by Takagi et al. and modified by our group (13, 14).

Animals were randomly divided into experimental groups (5 animals each) that were treated intragastrically (i.g.) 30 minutes before exposure to WRS with 1) vehicle, 2) naproxen (20 mg/kg, Sigma-Aldrich, Schnellendorf, Germany) alone or combined with NaHS (Cayman Chemical, Ann Arbor, USA) administered in a close of 5 mg/kg i.g. which has been shown to prevent gastric mucosa against aspirin-induced gastric damage (11); 3) ATB-346 (Antibe Therapeutics Inc., Toronto, Canada) administered in a dose of 29 mg/kg i.g., which is equimolar to the dose of naproxen not including \( \text{H}_2\text{S} \)-releasing moiety, 4-hydroxythiobenzamide (8). Vehicle-pretreated rats received solvent for ATB-346 and naproxen. In separate group, rats were treated with vehicle only and were not exposed to WRS (Intact). ATB-346 and naproxen were dissolved in 95:5, 1% CMC:DMSO (15).

**Determination of gastric blood flow, gross assessment of gastric lesions, plasma and gastric mucosal specimens collection**

After the end of 3.5 h of WRS, rats were anesthetized by isoflurane, the stomachs were opened along the greater curvature and the GBF was measured by a laser Doppler flowmeter (Laserfло blood perfusion monitor, model BPM 403A, Vasamedics, St Paul, MN, USA), as described elsewhere (11). Briefly, the GBF was blindly determined in the oxyntic part of gastric mucosa not involving microbleeding erosions. Average values of three measurements in each animal were expressed as percent of the value obtained in healthy gastric mucosa. The blood samples were collected from the vena cava, samples were then centrifuged and plasma was stored in \(-80^\circ\text{C}\) until further analysis. Animals were then sacrificed by application of high dose of pentobarbital.

Next, the stomach was excised, photographed and the number of gastric lesions in each stomach was determined using computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) (13).

The gastric mucosal samples from the oxyntic mucosa were scraped off on ice, snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until further analysis (11).

**Assessment of gastric mucosal protein expression of cystathionine-γ-lyase, cystathionine-β-synthase, 3-mercaptopyruvate sulfurtransferase, heme oxygenase-1, nuclear factor like-2, hypoxia inducible-1α and β-actin by Western blot**

Protein expression for CSE, CBS, 3-MST, HO-1, Nrf-2, HIF-1α and β-actin in gastric mucosa were determined using western blot as described in our previous studies (11). Briefly, rabbit polycyclonal anti-CSE (12217-1-AP, Proteintech, Manchester, UK) in dilution of 1:1000, rabbit polyclonal anti-CBS (14787-1-AP, Proteintech) in dilution of 1:1000, rabbit polyclonal anti-3-MST (HPA001240, Sigma-Aldrich) in dilution of 1:500, rabbit monoclonal anti-HO-1 (ab68477, Abcam, Cambridge, UK) in dilution of 1:2000, rabbit polyclonal anti-Nrf-2 (16396-AP-1, Proteintech) in dilution of 1:500, rabbit monoclonal anti-HIF-1α (141798, Cell Signaling Technology, Danvers, MA, USA) in dilution of 1:1000 and mouse monoclonal anti-β-actin (37005, Cell Signaling Technology) in dilution of 1:1000 were used as primary antibodies. Protein expression was visualized using HRP-linked secondary goat anti-rabbit IgG H&L antibody (ab97051, Abcam) in dilution of 1:2000 or anti-mouse IgG antibody (7076P2, Cell Signaling Technology) in dilution of 1:2000 where appropriate. Anti-Nrf-2 was diluted in 5% bovine serum albumin and all other primary and secondary antibodies were diluted in 5% non-fat milk.

Chemiluminescence was developed using WesternSure® ECL Substrate (LI-COR, NE, USA) or WesternBright Quantum (Advanta, Menlo Park, CA, USA) and was measured using C-DiGit® Blot Scanner (LI-COR). The intensity of bands was determined and analysed using Image Studio 4.0 software (LI-COR). The expression of each protein of interest was determined using 5 samples per experimental group and obtained values were normalized to the expression of β-actin as loading control (11).

**Luminex microbeads fluorescent assays**

Determination of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α, GM-CSF (granulocyte-macrophage colony-stimulating factor) plasma concentrations was performed using Luminex micro beads fluorescent assays (Bio-Plex Pro™ Rat Cytokine Th1/Th2 Assay #171K1002M) and Luminex 200 system (Luminex Corp., Austin, TX, USA). Results were calculated from calibration curves and expressed in pg/ml (16).

**Statistical analysis**

Statistical analysis was conducted using ANOVA with Dunnett’s Multiple Comparison post hoc test. Results are presented as mean ± S.E.M. The group size for each experimental group was of \( n = 5 \) and \( p < 0.05 \) was considered as statistically significant.

**RESULTS**

Pretreatment with naproxen (20 mg/kg i.g.) significantly increased the number of gastric lesions and significantly decreased the GBF in rats exposed to 3.5 h of WRS as...
compared with vehicle-pretreated rats ($p < 0.05$) (Fig. 1A). Pretreatment with ATB-346 administered i.g. in a dose of 29 mg/kg, equimolar with the dose of naproxen, or pretreatment with naproxen combined with NaHS (5 mg/kg i.g.) significantly decreased the number of WRS-induced gastric lesions and significantly raised the GBF as compared with the group of rats pretreated with naproxen ($p < 0.05$) (Fig. 1A). However, the number of WRS-lesions and GBF in ATB-346 pretreated rats were not significantly different to respective values obtained in vehicle-pretreated rats exposed to 3.5 h of WRS (Fig. 1A).

Fig. 1B shows macroscopic appearance of the stomach in rats pretreated i.g. with vehicle, naproxen (20 mg/kg) alone or combined with NaHS (5 mg/kg) or ATB-346 (29 mg/kg). Exposure to 3.5 of WRS caused the numerous dot-like lesions in gastric mucosa of rats pretreated with vehicle (Fig. 1B). The number of WRS-induced gastric lesions is increased in rats pretreated with naproxen (20 mg/kg i.g.), as compared with vehicle (vehicle versus naproxen, Fig. 1B). The number of WRS-induced gastric lesions was attenuated by pretreatment with ATB-346 (29 mg/kg i.g.) or combination of naproxen with NaHS (5 mg/kg) comparing with the group pretreated with naproxen alone (ATB-346 or NaHS plus naproxen versus naproxen alone Fig. 1B).

Fig. 2A-2D shows the alterations in CSE, CBS and 3-MST protein expression in gastric mucosa compromised by 3.5 h of WRS. Pretreatment with naproxen (20 mg/kg i.g.) significantly increased gastric mucosal protein expression of CSE but not those of CBS and 3-MST as compared with vehicle-treated group ($p < 0.05$) (Fig. 2A-2C). Pretreatment with ATB-346 (29 mg/kg i.g.) failed to affect CSE, CBS and 3-MST protein expression as compared with expression of these factors obtained in groups of rats pretreated with vehicle or naproxen (Fig. 2A-2C).

Fig. 2A-2C shows the alterations in CSE, CBS and 3-MST protein expression in gastric mucosa compromised by 3.5 h of WRS. Pretreatment with naproxen (20 mg/kg i.g.) significantly increased gastric mucosal protein expression of CSE but not those of CBS and 3-MST as compared with vehicle-treated group ($p < 0.05$) (Fig. 2A-2C). Pretreatment with ATB-346 (29 mg/kg i.g.) failed to affect CSE, CBS and 3-MST protein expression as compared with expression of these factors obtained in groups of rats pretreated with vehicle or naproxen (Fig. 2A-2C).

Pretreatment with naproxen (20 mg/kg i.g.) significantly increased protein expression of Nrf-2 and HIF-1α but not that of COX-2 or HO-1 in gastric mucosa as compared with vehicle ($p < 0.05$) (Fig. 3A-3E). Pretreatment with ATB-346 (29 mg/kg i.g.) significantly increased protein expression of Nrf-2 and HO-1 but not HIF-1α in gastric mucosa as compared with group pretreated with vehicle ($p < 0.05$) (Fig. 3A-3C and 3E). Gastric mucosal protein expression for COX-2 was significantly decreased in groups pretreated with ATB-346 as compared with vehicle ($p < 0.05$) (Fig. 3D and 3E).

Fig. 4A-4K shows changes in the plasma concentration of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α and GM-CSF respectively, in rats pretreated i.g. with vehicle, naproxen (20 mg/kg) or ATB-346 (29 mg/kg). The plasma concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α and GM-CSF were significantly increased in rats pretreated with vehicle and exposed to 3.5 h of WRS as compared with respective values of these cytokines in Intact

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**Fig. 1.** The effect of i.g. pretreatment with vehicle, naproxen (20 mg/kg) alone or combined with NaHS (5 mg/kg) or ATB-346 (29 mg/kg) on the number of stress-induced gastric lesions and changes in GBF. (A) Mean lesion number and GBF in gastric mucosa pretreated i.g. with vehicle, naproxen (20 mg/kg) with or without NaHS (5 mg/kg) or ATB-346 (29 mg/kg) and 30 min later exposed to 3.5 h of WRS. Results are mean ± S.E.M. of 5 animals per each experimental group. Asterisk indicates a significant change as compared with vehicle ($p < 0.05$). Cross indicates a significant change as compared with the group treated with naproxen alone ($p < 0.05$). (B) Macroscopic appearance of representative gastric mucosa of rats pretreated i.g. either with vehicle, naproxen (20 mg/kg) with or without NaHS (5 mg/kg) or ATB-346 (29 mg/kg) and further exposed to 3.5 h of WRS.
group (p < 0.05) (Fig. 4A-4K). Pretreatment with ATB-346 (29 mg/kg i.g.) but not naproxen (20 mg/kg i.g.) significantly decreased plasma concentration of IL-1α, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN-γ but not that of IL-1β, IL-2 or GM-CSF as compared with vehicle-pretreated animals (p < 0.05) (Fig. 4A-4K).

DISCUSSION

NSAIDs such as aspirin or naproxen exert anti-inflammatory, analgesic and even chemopreventive effects but their clinical use is limited due to serious side effect caused by these drugs in gastrointestinal (GI) tract (17-20). Commonly reported in clinical practice and under experimental conditions gastrotoxic effects of NSAIDs include hemorrhagic gastric erosions and microbleedings (17-19). Naproxen has also been reported as gastro- and hepatotoxic agent (17-19). In case of aspirin, the risk of GI bleeding is increased approximately 40% (21). Contrastingly, H₂S has originally been recognized as vasoactive and gastroprotective endogenous gaseous molecule (1). Previous studies revealed that H₂S, next to other gaseous mediators NO and CO is implicated in regulation gastric microcirculation and attenuated gastric mucosal damage induced by aspirin, alendronate or exposure to stress (11-13). Moreover, novel H₂S-releasing derivatives of NSAIDs were developed throughout the period of last few years (7, 8, 22-25). These compounds linked with H₂S-releasing moiety exert GI safety with comparable or higher efficacy compared with their parent drugs regarding modulation of pain and inflammation (8).

On the other hand, exposure to acute stress is known to cause hemorrhagic gastric lesions in the stomach of rats and mimics the clinical appearance of stress ulcerogenesis observed in humans after life-threatening conditions (26, 27). The pathomechanism of stress-induced gastric lesions involves generation of reactive oxygen species, hypoxia, hyperacidity, hypermotility and increased permeability of this gastric mucosa to H⁺ ions; all these factors and mechanisms impairing the gastric microcirculation (13, 28, 29).

Therefore, our present study was designed to compare systemic anti-inflammatory effect of classic naproxen and its H₂S-releasing derivative, ATB-346 in experimental model of acute microbleedings induced by WRS. We also determined the effects of these compounds on the alterations in GBF and expression of cellular inflammatory and hypoxia-sensitive regulatory proteins, such as Nrf-2, HO-1, HIF-1α and COX-2 in gastric mucosa compromised by WRS. We have further attempted to investigate the possible influence of pretreatment with ATB-346 or naproxen.

Fig. 2. Alterations in CSE (A), CBS (B) and 3-MST (C) protein expression in gastric mucosa of rats pretreated i.g. with vehicle, naproxen (20 mg/kg) or ATB-346 (29 mg/kg) and 30 min later exposed to 3.5 h of WRS. Results are expressed as mean ± S.E.M. for n = 5 samples per each experimental group. Asterisk indicates a significant change as compared with vehicle (p < 0.05). (D) Representative bands for CSE, CBS, 3-MST and β-actin proteins in gastric mucosa in respective groups of rats pretreated with vehicle, naproxen or ATB-346.
on the protein expression of enzymes involved in H$_2$S biosynthesis in gastric mucosa of rats exposed to WRS.

We observed that pretreatment with naproxen, in contrast with ATB-346, exacerbated the stress-induced gastric lesions and this effect was accompanied by the fall in GBF. This observation remains in agreement with previous study (30) showing that ATB-346 failed to aggravate stress-induced gastric lesions number as compared with naproxen or the COX-2 inhibitor, celecoxib.

This suggests that due to H$_2$S-releasing moiety, ATB-346 did not negatively affect gastric microcirculation and physiological gastric mucosal defence to the same extent as it was observed after pretreatment with naproxen. Additionally, naproxen-induced fall in GBF and exacerbation of stress ulcerogenesis was accompanied by upregulation of protein expression for HIF-1α. This suggests that gastrotoxicity of this NSAID could involve induction of hypoxia. Furthermore, we observed that pretreatment with naproxen but not ATB-346 enhanced protein expression of CSE, the main enzyme involved in production of endogenous H$_2$S. We assume that this could be defensive response of gastric mucosa to the gastric damaging effect of the combination of naproxen and stress. Therefore, we conclude that GI safety of ATB-346 observed

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**Fig. 3.** Gastric mucosal protein expression of Nrf-2 (A), HO-1 (B), HIF-1α (C) and COX-2 (D) in rats pretreated i.g. with vehicle, naproxen (20 mg/kg) or ATB-346 (29 mg/kg) and exposed 30 min later to 3.5 h of WRS. Results are expressed as mean ± S.E.M. for n = 5 samples per each experimental group. Asterisk indicates a significant change as compared with vehicle (p < 0.05). (E) Representative bands for Nrf-2, HO-1, HIF-1α, COX-2 and β-actin proteins in gastric mucosa of rats pretreated with vehicle, naproxen or ATB-346.
in our study can be due to the release of gastroprotective and vasoactive H$_2$S.

We demonstrated that pretreatment with naproxen and ATB-346 increased protein expression for Nrf-2 in gastric mucosa exposed to WRS. Interestingly, ATB-346 but not naproxen additionally upregulated protein expression for HO-1, an enzyme which is involved in production of endogenous CO. This indicates that H$_2$S released from ATB-346 could activate Nrf-2/HO-1/CO pathway in gastric mucosa exposed to stress. In contrast, the application of naproxen failed to trigger Nrf-2/HO-1/CO pathway and resulted in a decrease in GBF and augmentation of WRS ulcerogenesis. This is compatible with previous studies, which revealed that gastroprotective effect of H$_2$S against NSAIDs-induced gastric damage depends on the production of endogenous CO (11). Moreover, it has been shown that Nrf-2 regulates CSE and CBS expression in mouse embryonic fibroblasts in vivo (31). Similarly, H$_2$S-releasing S-propargyl-cysteine exerted beneficial activity regulating translocation of Nrf2 in an animal model of methionine- and choline-deficient diet-induced fatty liver (32).

It has been reported that H$_2$S-releasing NSAIDs effectively inhibit COX-1 and COX-2 in similar manner to that presented by conventional NSAIDs, however, without any significant gastrotoxicity (8, 23, 33). In this study, we confirmed these observations demonstrating that expression of COX-2 at the

Fig. 4. Plasma concentrations of IL-1α (A), IL-1β (B), IL-2 (C), IL-4 (D), IL-5 (E), IL-6 (F), IL-10 (G), IL-12 (H), IFN-γ (I), TNF-α (J) and GM-CSF (K) in intact rats and those pretreated i.g. with vehicle, naproxen (20 mg/kg) or ATB-346 (29 mg/kg) and exposed 30 min later to 3.5 h of WRS. Results are expressed as mean ± S.E.M. for n = 5 samples per group. Asterisk indicates significant change as compared with intact group (p < 0.05). Cross indicates significant difference as compared to the group pretreated with vehicle (p < 0.05).
level of protein was decreased in gastric mucosa of rats pretreated with ATB-346 and compromised by WRS. Stress has been described as ‘local’ or ‘systemic’ reaction to life-threatening conditions (34-38). It has been also reported that WRS increased expression of pro-inflammatory markers (13). Indeed, we have observed in this study that the plasma levels of systemic inflammatory response markers, including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α and GM-CSF were increased after exposure to WRS. Interestingly, ATB-346 but not naproxen, decreased plasma content of IL-1α, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α and IFN-γ in these animals. This suggests that, at least, in our model of stress-induced gastric damage, systemic inflammatory response was greatly inhibited by H₂S-releasing ATB-346, while under the same conditions administration of naproxen was ineffective with respect to stress-induced gastric damage. Thus, we conclude that H₂S-releasing moiety in ATB-346 enhanced anti-inflammatory properties of naproxen and improved the status of gastric mucosa of rats exposed to stress.

Taken together, we conclude that pretreatment with naproxen but not ATB-346 weakened gastric mucosal barrier resulting in the enhanced incidence of WRS-induced gastric lesions in rat stomach. This deleterious effect of naproxen was accompanied by the decrease of gastric microcirculation leading to gastric hypoxia and the compensatory activation of Nrf-2/CSE defensive response. In contrast to naproxen, the administration of ATB-346 effectively decreased stress-induced lesions as well as blunted the systemic inflammatory response, possibly due to release of H₂S. This H₂S-mediated activation of Nrf-2/HO-1/CO pathway may account for GI safety of ATB-346 versus naproxen observed in our present study.

Conflict of interests: Dr. John L. Wallace is the founder and a director of Antibe Therapeutics Inc., which holds the rights to ATB-346.

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