CHAPTER NINE

Anti-inflammatory and Cytoprotective Properties of Hydrogen Sulfide

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Abstract

Hydrogen sulfide is an endogenous gaseous mediator that plays important roles in many physiological processes in microbes, plants, and animals. This chapter focuses on the important roles of hydrogen sulfide in protecting tissues against injury, promoting the repair of damage, and downregulating the inflammatory responses. The chapter focuses largely, but not exclusively, on these roles of hydrogen sulfide in the gastrointestinal tract. Hydrogen sulfide is produced throughout the gastrointestinal tract, and it contributes to maintenance of mucosal integrity. Suppression of hydrogen sulfide synthesis renders the tissue more susceptible to injury and it impairs repair. In contrast, administration of hydrogen sulfide donors can increase resistance to injury and accelerate repair. Hydrogen sulfide synthesis is rapidly and dramatically enhanced in the gastrointestinal tract after injury is induced. These increases occur specifically at the site of tissue injury. Hydrogen sulfide also plays an important role in promoting resolution of...
inflammation, and restoration of normal tissue function. In recent years, these beneficial actions of hydrogen sulfide have provided the basis for development of novel hydrogen sulfide-releasing drugs. Nonsteroidal anti-inflammatory drugs that release small amounts of hydrogen sulfide are among the most advanced of the hydrogen sulfide-based drugs. Unlike the parent drugs, these modified drugs do not cause injury in the gastrointestinal tract, and do not interfere with healing of preexisting damage. Because of the increased safety profile of these drugs, they can be used in circumstances in which the toxicity of the parent drug would normally limit their use, such as in chemoprevention of cancer.

1. INTRODUCTION

In the past two decades, there has been an exponential growth of publications related to hydrogen sulfide (H₂S). Studies in the 1990s on identifying roles for H₂S in neuromodulation (Abe & Kimura, 1996) were a catalyst for others to examine this novel gasotransmitter, and studies in the early 2000s on cardiovascular effects of H₂S (Wang, 2002; Zhao, Zhang, Lu, & Wang, 2001) triggered an even greater degree of research on this molecule. H₂S plays a broad range of roles in the function of bacteria, plants, and animals, and its role as an electron donor for mitochondrial respiration dates back to the prephotosynthesis period (Goubern, Andriamihaja, Nubel, Blachier, & Bouillaud, 2007; Olson, Donald, Dombkowski, & Perry, 2012). In mammals, H₂S appears to play a major role as a “rescue molecule” (Wallace, Ferraz, & Muscara, 2012). Thus, the focus of this chapter is on the actions of H₂S in prevention and repair of tissue injury (cytoprotection) and in reducing and promoting the resolution of inflammation. We also review some of the ongoing attempts to develop novel therapeutics for cytoprotection and anti-inflammatories that employ hydrogen sulfide release as a key action of the drugs.

2. ENZYMATIC SYNTHESIS OF H₂S

As described in more detail in other chapters, there are three main pathways for enzymatic synthesis of H₂S (Fig. 1). L-Cysteine is the major substrate for H₂S synthesis, although there is evidence that D-cysteine can also be metabolized to H₂S, particularly in the kidney (Kimura, Shibuya, & Kimura, 2012). The vitamin B₆-dependent enzymes, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), play major
roles in \( \text{H}_2\text{S} \) synthesis in various tissues. In mice deficient of these enzymes, there are distinctive phenotypes that include systemic inflammation and, in the case of the CSE-deficient mice, elevated blood pressure (Cheng et al., 2011; Yang et al., 2008). These phenotypes can be recapitulated with inhibitors of these enzymes. CBS also metabolizes homocysteine to cysteine. Lack of such metabolism, as can occur in certain genetic disorders or when a patient is deficient of B vitamins, results in elevated levels of homocysteine in the blood (hyperhomocysteinemia). This condition is characterized by systemic inflammation, increased susceptibility to thrombosis, and aggravation of preexisting inflammatory conditions. Indeed, mice genetically deficient of CBS display a hyperhomocysteinemic phenotype (Cheng et al., 2011). At least some of the features of these phenotypes may be a consequence of reduced capacity of \( \text{H}_2\text{S} \) synthesis. This has recently been demonstrated in rat and mouse models of hyperhomocysteinemia (Flannigan et al., 2014). In rodent models of inflammatory bowel disease (IBD), prior induction of hyperhomocysteinemia resulted in a marked exacerbation of the severity of colitis, which was due to impaired colonic synthesis of \( \text{H}_2\text{S} \). These research findings are consistent with the clinical scenario of exacerbation of IBD in patients with hyperhomocysteinemia.

\begin{center}
\textbf{Figure 1} The primary pathways for enzymatic synthesis of hydrogen sulfide (\( \text{H}_2\text{S} \)). L-Cysteine can be converted to \( \text{H}_2\text{S} \) via cystathionine \( \gamma \)-lyase (CSE) or cystathionine \( \beta \)-synthase (CBS), both of which require pyrroldinal-5-phosphate (P5P) for their activity. L-Cysteine can also be converted to 3-mercaptopyruvate via cysteine aminotransferase (CAT), which can in turn be metabolized by 3-mercaptopyruvate sulfurtransferase (3MST) to generate \( \text{H}_2\text{S} \). CAT activity requires \( \alpha \)-ketoglutarate as a cofactor. \textit{Reproduced from Chan and Wallace (2013), with permission of the publisher.}
\end{center}
3. HEALING AND RESOLUTION OF INFLAMMATION

H₂S is synthesized throughout the gastrointestinal (GI) tract of rodents and humans, with contributions from all three enzymatic pathways (Martin et al., 2010; Wallace, Vong, McKnight, Dicay, & Martin, 2009). We have studied the role of H₂S in several models of GI tissue injury. A common feature in these models is the rapid upregulation of H₂S synthesis, specifically at sites of injury. Thus, shortly after induction of an ulcer in the stomach, there was a marked upregulation of CSE and CBS at the ulcer margin, which is the region where angiogenesis and increased epithelial turnover can be observed (Wallace, Dicay, McKnight, & Martin, 2007). Inhibiting the activity of these enzymes resulted in a significant impairment of ulcer healing. Administration of H₂S donors or of L-cysteine resulted in a significant acceleration of ulcer healing (Wallace, Dicay, et al., 2007). Similar results were obtained in rodent models of colitis, where expression of CSE and 3-mercaptopyruvate sulfurtransferase (3MST) were markedly upregulated very soon after the induction of injury and remained upregulated over the course of the period of repair (Flannigan, Ferraz, Wang, & Wallace, 2013). The upregulation of these enzymes occurred specifically at sites of ulceration. There was no change in expression of these enzymes in the tissue immediately adjacent to ulcers, despite those tissues being inflamed (Flannigan et al., 2013). We also observed significant downregulation of expression of sulfide quinone reductase (SQR) at sites of ulceration (Flannigan et al., 2013). SQR is a key enzyme for oxidation of H₂S (Mimoun et al., 2012). Elevated synthesis of H₂S and decreased oxidation of H₂S at sites of ulceration would result in increased tissue levels of H₂S at those sites, thereby facilitating repair.

The elevation of H₂S synthesis that occurs after induction of colitis begins to subside gradually, in parallel with resolution of colonic inflammation and healing of ulcers (Wallace et al., 2009). Intracolonic administration of H₂S donors can significantly accelerate this process, as has been shown in several animal models of colitis (Fiorucci et al., 2007; Wallace et al., 2009), and can reduce expression of tumor necrosis factor (TNF)α in human inflamed colonic tissue (Bai, Ouyang, & Hu, 2005). The beneficial effects of H₂S donors include reduction of tissue granulocyte numbers and marked suppression of expression of a number of proinflammatory cytokines (e.g., TNFα, interleukin (IL)–1β, IL–8, and interferon (IFN)γ) but sparing of
expression of the proinflammatory cytokine, IL-10 (Fiorucci et al., 2007; Li et al., 2007). In sharp contrast, administration of inhibitors of CSE or 3MST results in a marked exacerbation of colitis, leading to bowel perforation and death if treatment is continued over several days (Wallace et al., 2009).

4. MECHANISMS OF ANTI-INFLAMMATORY EFFECTS OF H$_2$S

The ability of H$_2$S to reduce pain and inflammation has been recognized for centuries, though not, until recently, at a molecular level. Natural sulfur springs have long been used as a means to alleviate the symptoms of an array of inflammatory diseases, such as rheumatoid arthritis. In the past decade, considerable progress has been made in identifying the molecular mechanisms underlying the anti-inflammatory properties of H$_2$S (Fig. 2). One of the earliest events in an inflammatory reaction is the recruitment of leukocytes to the site of injury. This involves upregulation of adhesion molecules on the vascular endothelium and on the leukocytes, adhesion of the leukocytes to the endothelium, and then migration of the leukocytes into the interstitial space, from where they move up a chemotaxin gradient to the site of injury/infection (Fig. 3). H$_2$S plays a key physiological role in regulating these processes. Indeed, H$_2$S is a tonic inhibitor of leukocyte adherence to the vascular endothelium, as is evident from the observation that administration of inhibitors of H$_2$S synthesis results in a very rapid increase in leukocyte–endothelial adhesion (Zanardo et al., 2006). This process is mediated via upregulation of intercellular adhesion molecule-1 (ICAM-1) and P-selectin on the endothelium, and lymphocyte function-associated antigen on circulating leukocytes (Fiorucci et al., 2005) (Fig. 3). Administration of H$_2$S donors has the opposite effect, decreasing leukocyte adherence via activation of ATP-sensitive K$^+$ channels on leukocytes and endothelial cells. H$_2$S donors have been shown to cause a marked suppression of inflammatory responses in several animal models (discussed below). H$_2$S has also been shown to suppress endothelial ICAM-1 expression in response to high blood-glucose concentrations (Guan et al., 2013).

Studies using transgenic mice that produce lower-than-normal levels of H$_2$S have provided further evidence for a key role of H$_2$S in modulating leukocyte–endothelial adhesion. Mice that are heterozygous for the CBS gene exhibit increased vascular permeability, slower leukocyte rolling velocity, and increased levels of leukocyte adherence to the vascular endothelium (Kamath et al., 2006). Rats with a diet-induced deficiency of B vitamins
have reduced capacity to synthesize H$_2$S (via CSE and CBS, which require vitamin B$_6$ for their activity) also exhibit significantly enhanced inflammatory responses, including accumulation of leukocytes in the affected tissues (Flannigan et al., 2014).

Figure 2 Anti-inflammatory actions of hydrogen sulfide (H$_2$S). H$_2$S can affect many aspects of an inflammatory response, through many mechanisms. H$_2$S is a tonic inhibitor of leukocyte adherence to the vascular endothelium, limiting leukocyte extravasation and edema formation. Mitochondria can utilize H$_2$S as an electron donor in adenosine triphosphate (ATP) production, particularly during anoxia/hypoxia, and in doing so reduces generation of tissue-damaging oxygen-derived free radicals. Antinociceptive effects of H$_2$S have been demonstrated in models of visceral pain. By inhibiting phosphodiesterases (PDE), H$_2$S can elevate tissue cyclic guanylate monophosphate (GMP) levels, which can contribute to vasodilation. H$_2$S promotes resolution of inflammation through various mechanisms, including promotion of neutrophil apoptosis. The antioxidant actions of H$_2$S further reduce tissue injury. Several anti-inflammatory and antioxidant systems are activated by H$_2$S through its effects on transcription factors (including NfκB). Through multiple mechanisms, including induction of cyclooxygenase-2 expression and stimulation of angiogenesis, H$_2$S can promote repair of damaged tissue. Reproduced from Chan and Wallace (2013), with permission of the publisher.
The mechanisms underlying the ability of H2S to inhibit leukocyte adherence to the vascular endothelium also involve the anti-inflammatory protein, annexin-1. Annexin-1 is contained within neutrophils and is released during inflammatory reactions as part of the process of induction appropriate resolution of inflammation (Perretti & D’Acquisto, 2009; Serhan et al., 2007; Vong et al., 2007). Micromolar concentrations of NaHS trigger a striking translocation of annexin-1 from the cytosol to the membrane of human neutrophils (Brancaleone, Sampaio, Cirino, Flower, & Perretti, 2011). In a mesenteric venule preparation, the H2S donor was able to suppress IL-1-induced leukocyte adhesion and emigration. The dependency of the H2S response on annexin-1 was further demonstrated by lack of an effect of NaHS when similar experiments were performed in annexin-1-deficient mice (Brancaleone et al., 2011). Annexin-1-deficient mice exhibit a marked upregulation of CBS and CSE in a variety of tissues, further supporting a role for this peptide in regulating H2S synthesis. A role of

Figure 3 Hydrogen sulfide (H2S) regulates leukocyte adhesion to the vascular endothelium. (A) H2S produced via cystathione γ-lyase (CSE) tonically inhibits adherence of leukocytes to the endothelium, via activation of ATP-dependent K+ channels on the leukocytes and on the endothelium. This activity downregulates expression of CD11/CD18 on leukocytes and both P-selectin and ICAM-1 on the endothelium. (B) When H2S synthesis is inhibited, such as by β-cyano-alanine (BCA), CD11/CD18, and P-selectin expression increases, leading to leukocyte rolling, adhesion, and extravasation. Inhibition of H2S synthesis also leads to enhanced edema formation. Reproduced from Zanardo et al. (2006), with permission of the publisher.
annexin-1 in mediating anti-inflammatory effects of \( \text{H}_2\text{S} \) in macrophages has also been demonstrated. \( \text{H}_2\text{S} \) can downregulate endotoxin-induced expression of iNOS and cyclooxygenase (COX)-2, but these effects were absent in annexin-1-deficient mice (Brancaleone et al., 2011).

5. EFFECTS OF \( \text{H}_2\text{S} \) ON VISCERAL PAIN

The role of \( \text{H}_2\text{S} \) in pain has been controversial, because of conflicting reports of its antinociceptive versus pronociceptive actions (Distrutti, Sediari, Mencarelli, Renga, Orlandi, Antonelli, et al., 2006; Donatti et al., 2014; Ekundi-Valentim et al., 2010; Fiorucci et al., 2005; Matsunami, Kirishi, Okui, & Kawabata, 2012; Wallace et al., 2014). To some extent, these differences may be related to the models used and the types and doses of \( \text{H}_2\text{S} \) donors that were used (Wallace et al., 2014). There is evidence that \( \text{H}_2\text{S} \) can contribute to pain through activation of T-type calcium channels (Sekiguchi & Kawabata, 2013). On the other hand, several groups have demonstrated that \( \text{H}_2\text{S} \) can reduce visceral pain (Distrutti, Sediari, Mencarelli, Renga, Orlandi, Antonelli, et al., 2006, Distrutti, Sediari, Mencarelli, Renga, Orlandi, & Russo, 2006; Matsunami et al., 2009; Wallace et al., 2014) and may have utility for treatment of conditions such as irritable bowel syndrome and IBD. Colonic distention-induced pain in rats was significantly reduced by several \( \text{H}_2\text{S} \) donors, and these actions were mediated largely through activation of \( \text{K}_{\text{ATP}} \) channels (Distrutti, Sediari, Mencarelli, Renga, Orlandi, Antonelli, et al., 2006). Figure 4 demonstrates the ability of two structurally unrelated \( \text{H}_2\text{S} \) donors to reduce visceral pain induced by gastric distention in rats. The effect was not reversed by perivaginal application of capsaicin, which could be mimicked by administration of l-cysteine (precursor for \( \text{H}_2\text{S} \) synthesis), and the latter effect was blocked by administration of an inhibitor of CSE activity (Wang & Wallace, 2011). Of note, an \( \text{H}_2\text{S} \)-releasing salt of trimebutine is being tested in phase 2 clinical trials as a visceral analgesic (Cukier-Meisner, 2013).

While largely studied in models of visceral pain, analgesic effects of \( \text{H}_2\text{S} \)-releasing agents have also been shown to be effective in models of peripheral pain (Cunha et al., 2008; Ekundi-Valentim et al., 2010, 2013).

6. CYTOPROTECTIVE ACTIONS OF \( \text{H}_2\text{S} \)

Damage to the lining of the stomach occurs on a regular basis, but there are mechanisms in place for rapid repair through the process of restitution (Wallace, 2008; Wallace & McKnight, 1990). Such damage can be
Increased sensitivity to visceral stimuli is one of several mechanisms for symptom generation such as visceral pain in patients with gastrointestinal disorders. Cardioautonomic responses to gastric distention have been recognized as a model to study visceral nociception. In this model, distention of a balloon within the stomach results in pain, and a corresponding decrease in heart rate. (A) Pretreatment with a H$_2$S donor (NaHS) inhibited the pain response. (B) Similarly, pretreatment with another H$_2$S donor, Lawesson’s reagent, blocked the pain response to gastric distention. (C) Treatment with NaHS did not change gastric compliance. A significant reduction in compliance would result in a reduction of gastric distention, and in turn a lack of effect on heart rate, which could be misinterpreted as an antinociceptive effect. (D) The antinociceptive effect of NaHS was not affected by capsaicin-ablation of sensory afferent nerves in the stomach. (E) An antinociceptive effect was observed by administration of the precursor for H$_2$S synthesis and was abolished by pretreatment with an inhibitor of H$_2$S synthesis (L-PAG; propargylglycine). (F) Local topical application of NaHS on the subdiaphragmatic vagus had no effect on cardioautonomic responses to gastric distention. *$p < 0.05$ versus the vehicle-treated group.
related to stress, ischemia–reperfusion, consumption of alcohol or use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Wallace, 2008). Several endogenous substances contribute to the ability of the GI tract to resist damage in such circumstances, including prostaglandins and nitric oxide. H2S is another very important mediator of GI mucosal defense (Fiorucci et al., 2005; Wallace, 2010). Inhibition of H2S synthesis increases the susceptibility of the stomach to injury, whereas H2S donors can protect the stomach from injury (Fiorucci et al., 2005; Mard et al., 2012; Wallace, Caliendo, Santagada, & Cirino, 2010). The underlying mechanisms of the cytoprotective action of H2S probably are multifactorial (Table 1). The ability of H2S to inhibit leukocyte adherence to the vascular endothelium (Mard et al., 2012) is very important in prevention of injury induced by NSAIDs or ischemia–reperfusion (Wallace, Keenan, & Granger, 1990). H2S can also trigger gastric and duodenal secretion of bicarbonate, which neutralizes gastric acid, thereby limiting its damaging effects as well as those of pepsin (Blackler, Gemici, et al., 2014; Takeuchi et al., 2012). Via its vasodilator effects, H2S can increase gastric mucosal blood flow, which increases mucosal resistance to injury (Fiorucci et al., 2005).

Damage induced by NSAIDs in the small intestine is more complicated, in terms of its pathogenesis, than the damage these drugs cause in the stomach (Wallace, 2012). Inhibition of COX activity does not appear to be the primary driver of injury. Rather, it is the secretion of the NSAID into bile (enterohepatic circulation of the NSAID), the topical irritant properties of that NSAID-containing bile, and the microbiota of the intestine that play key roles in producing damage (Wallace, 2012). Administration of an H2S donor prevents NSAID enteropathy in rats in a dose-dependent manner (Blackler, Motta, et al., 2014). While the mechanism of action of the H2S is not fully understood, this treatment did result in a significant decrease in the cytotoxicity of bile and produced significant changes in the intestinal microbiota (Blackler, Motta, et al., 2014). These effects were not attributable to reduced biliary excretion of the NSAID (Blackler, Motta, et al., 2014). Treatment with the H2S donor did trigger a significant increase in COX-2 expression and prostaglandin E2 synthesis by intestinal tissue, which may have contributed to the observed protective effects against NSAID enteropathy (Blackler, Motta, et al., 2014).

When ulcers do form, H2S plays an important role in promoting healing. Healing of ulcers can be accelerated by drugs that suppress gastric acid secretion. This healing is partially dependent upon H2S, the synthesis of which is increased at the margins of the ulcer, where there is an increased expression
Table 1  Mechanisms underlying cytoprotective actions of hydrogen sulfide

<table>
<thead>
<tr>
<th>Primary mechanisms</th>
<th>Protective action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulates bicarbonate secretion</td>
<td>Reduces gastroduodenal acidity: reduced tissue damage and enhanced repair</td>
<td>Ise et al. (2011) and Blackler, Gemici, Manko, and Wallace (2014)</td>
</tr>
<tr>
<td>Stimulates mucus secretion</td>
<td>Enhances resistance to damage and enhances repair</td>
<td>Motta et al. (2014)</td>
</tr>
<tr>
<td>Reduces cytotoxicity of bile</td>
<td>Reduces epithelial injury induced by luminal agents such as nonsteroidal anti-inflammatory drugs</td>
<td>Blackler, Motta, et al. (2014)</td>
</tr>
<tr>
<td>Reduces mitochondrial damage/death</td>
<td>Acts as an electron donor in generation of ATP, in circumstances of low oxygen levels; reduces oxygen radical production</td>
<td>Elrod et al. (2007), Kimura, Goto, and Kimura (2010), and Mimoun et al. (2012)</td>
</tr>
<tr>
<td>Activates antioxidant response elements</td>
<td>Inactivates Keap1, thereby activating Nrf2 activity</td>
<td>Guo, Liang, Shah Masood, and Yan (2014)</td>
</tr>
<tr>
<td>Antioxidant activity</td>
<td>Scavenger of oxygen-derived free radicals</td>
<td>Whiteman et al. (2004)</td>
</tr>
<tr>
<td>Reduces neutrophil-mediated tissue injury</td>
<td>Inhibits myeloperoxidase activity</td>
<td>Palinkas et al. (2014)</td>
</tr>
<tr>
<td>Inhibits proinflammatory cytokine production</td>
<td>Reduces expression of TNFα, IL-1β, IL-8, IFNγ, etc.</td>
<td>Fiorucci et al. (2007), Li et al. (2007), Wallace et al. (2009), and Flannigan et al. (2014)</td>
</tr>
<tr>
<td>Increases or maintains anti-inflammatory cytokine production</td>
<td>Maintenance or increase of expression of IL-10</td>
<td>Fiorucci et al. (2007), Li et al. (2007), Zayachkivska et al. (2014), and Flannigan et al. (2014)</td>
</tr>
<tr>
<td>Inhibits leukocyte adherence to the vascular endothelium</td>
<td>Reduces leukocyte-mediated tissue injury; reduces edema formation</td>
<td>Zanardo et al. (2006) and Brancaleone et al. (2011)</td>
</tr>
<tr>
<td>Enhances antimicrobial defense</td>
<td>Increases antimicrobial peptide expression; promotes macrophage phagocytosis of bacteria; stabilizes biofilms</td>
<td>Dufton, Natividad, Verdu, and Wallace (2012) and Motta et al. (2014)</td>
</tr>
</tbody>
</table>
of CSE and CBS (Wallace, Dicay, et al., 2007). There is also an increased expression of COX-2 at the ulcer margins (Jones et al., 1999; Ma, del Soldato, & Wallace, 2002; Mizuno et al., 1997). Through the production of prostaglandins that promote angiogenesis and epithelial proliferation, COX-2 contributes significantly to ulcer healing and resolution of inflammation in the GI tract (Jones et al., 1999; Ma et al., 2002; Mizuno et al., 1997; Wallace & Devchand, 2005). H2S promotes expression of COX-2, while inhibition of H2S synthesis leads to a reduction of COX-2 expression (Wallace et al., 2014, 2009). Administration of l-cysteine or H2S donors to rats with gastric ulcers resulted in a significant acceleration of ulcer healing (Wallace, Dicay, et al., 2007). While NSAIDs are known to retard the healing of gastric ulcers in humans and animals, H2S-releasing NSAIDs have been shown to accelerate ulcer healing in mice (Wallace et al., 2010).

7. THERAPEUTIC APPLICATIONS OF H2S-RELEASING DRUGS

In recent years, there has been considerable activity aimed at developing novel therapies that exploit the anti-inflammatory and/or cytoprotective properties of H2S (Chan & Wallace, 2013; Szabo, 2007; Wallace, 2007).

7.1. Inflammation and pain

NSAIDs are among the most commonly used drugs. They are used on a chronic, daily basis by hundreds of millions of patients suffering from disorders, such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis, and on an acute basis for a range of disorders characterized by pain (gout, dental pain, dysmenorrhea, injuries, postsurgery, etc.). Several companies and academics have focused on the development of H2S-releasing NSAIDs, based on early reports that these compounds produced anti-inflammatory effects similar to or superior to conventional NSAIDs (Li et al., 2007; Wallace, Caliendo, Santagada, Cirino, & Fiorucci, 2007; Wallace, Dicay, et al., 2007). NSAIDs are most commonly used for treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout, dysmenorrhea, postsurgical pain, injuries, dental pain, and headaches. NSAIDs are also widely used for veterinary indications, particularly for treating arthritis and post-injury inflammation in companion animals and horses. Extensive preclinical results have been performed to demonstrate the effectiveness of H2S-NSAIDs in models of inflammation/pain and to demonstrate improved
GI tolerability. H$_2$S-NSAIDs have been shown to inhibit COX-1 and COX-2 \textit{in vivo}, as effectively as the parent NSAID (Blackler, Syer, Bolla, Ongini, & Wallace, 2012; Ekundi-Valentim et al., 2010, 2013; Wallace, 2013a; Wallace, Caliendo, et al., 2007; Wallace et al., 2010). In models of inflammation, including carrageenan-induced paw edema, zymosan-induced leukocyte infiltration into a subdermal airpouch, and adjuvant-induced arthritis, H$_2$S-NSAIDs have exhibited anti-inflammatory effects that were comparable or superior to those of the parent NSAID (Wallace, Caliendo, et al., 2007). Figure 5 shows the effects of ATB-346, an H$_2$S-releasing derivative of naproxen, in a model of adjuvant-induced arthritis in rats. Equimolar doses of naproxen and ATB-346 produced almost identical reductions in joint swelling over a 2-week treatment period. However, at the highest doses tested, the naproxen-treated rats died of intestinal perforation/bleeding after 1 week of treatment, whereas the ATB-346-treated rats survived the full 2 weeks of treatment with a significant reduction of joint inflammation and negligible GI damage.

![Figure 5](image_url)

\textbf{Figure 5} Effectiveness and increased safety of the H$_2$S-releasing drug, ATB-346, versus naproxen in a rat model of adjuvant-induced arthritis. Groups of 12 rats were treated twice daily with vehicle, naproxen, or ATB-346 for 14 days after induction of arthritis. ATB-346 was administered at doses equimolar to the doses of naproxen shown. There were no significant differences in the anti-inflammatory effects of naproxen versus ATB-346. However, at the highest dose tested, all 12 rats treated with naproxen died of small intestinal perforation by the end of the first week of treatment, while there were no deaths among the rats treated with ATB-346.
ATB-346 has also been shown to exert significantly enhanced antinociceptive and anti-inflammatory effects in carrageenan-induced model of knee inflammation in the rats (Ekundi-Valentim et al., 2010, 2013). As a proof-of-concept, this group demonstrated that an H₂S donor (Lawesson’s reagent) significantly reduced pain responses in this model, as well as reducing the associated inflammatory changes (Ekundi-Valentim et al., 2010). They then compared a range of doses of naproxen and ATB-346 in this model. Both drugs significantly reduced tactile allodynia and leukocyte infiltration of the knee joints in a dose-dependent manner. However, gastric damage and leukocyte infiltration of the gastric mucosa was significant in rats treated with naproxen, but not in rats treated with ATB-346 (Ekundi-Valentim et al., 2013).

The main beneficial effect of H₂S-NSAIDs over NSAIDs is the greatly reduced GI toxicity. Extensive studies have been carried out on a number of H₂S-NSAIDs and in a variety of models. In healthy rats, for example, ATB-346 was found to produce a low level of gastric hemorrhagic erosion formation, but at a dose 90-times the dose of naproxen that produced a similar level of damage (Wallace et al., 2010). In models of compromised gastric mucosal defense, gastric damage induced by naproxen was consistently greater than that produced in healthy animals, but ATB-346 did not produce significant injury (Wallace et al., 2010). The patients who are at greatest risk for NSAID gastroenteropathy are the elderly patients taking drugs such as glucocorticoids, aspirin, or other anticoagulants, and patients with comorbidities such as obesity and diabetes (Wallace, 2013b). When tested in obese rats, aged rats, and diabetic rats, ATB-346 consistently produced negligible GI damage, while naproxen produced more extensive GI damage than was seen in healthy animals (Blackler et al., 2012).

After significant cardiovascular events were identified as a major risk associated with use of NSAIDs, it became common clinical practice to coprescribe low-dose aspirin together with the NSAID for patients being treated on a chronic basis. Moreover, proton pump inhibitors are often coprescribed to protect the stomach and duodenum from the ulcerogenic effects of NSAIDs. In a rat model, we demonstrated that intestinal damage induced by naproxen or celecoxib was significantly increased when those drugs were coadministered with low-dose aspirin, or when coadministered with a proton pump inhibitor (Blackler et al., 2012; Wallace et al., 2011). The greatest intestinal damage was observed when the NSAIDs were coadministered with both low-dose aspirin and a proton pump inhibitor. However, ATB-346 alone did not cause intestinal damage, and when
coadministered with low-dose aspirin, a proton pump inhibitor, or both, the small intestine was spared of hemorrhagic damage (Blackler et al., 2012).

What evidence is there that H2S accounts for the protective effects of H2S-NSAIDs? There are several lines of evidence to support this. First, one can observe dose-dependent protection of the stomach and small intestine with a number of different H2S donors (Blackler, Gemici, et al., 2014; Fiorucci et al., 2005; Wallace et al., 2012), but a worsening of GI damage when inhibitors of H2S synthesis are administered (Blackler, Gemici, et al., 2014; Mard et al., 2012; Wallace et al., 2010, 2009; Zayachkivska et al., 2014). As shown in Fig. 6, administration of ATB-346 does not produce significant gastric damage in rats, but when a structurally similar compound (naproxen 4-hydroxybenzamide) that lacks the sulfur group of ATB-346 is administered, significant gastric damage is produced. These two compounds exhibited comparable effects on gastric prostaglandin synthesis. Interestingly, when naproxen and the H2S-releasing moiety of ATB-346 (4-hydroxy-thiobenzamide) were administered to rats as separate entities, the gastric damage produced is similar to what would be seen with naproxen alone (Wallace et al., 2010). Indeed, even if four times the dose of the

![Figure 6](image-url)

**Figure 6** The gastric-sparing property of ATB-346 is due to the ability of this drug to release H2S. Administration of ATB-346 at a dose of 30 mg/kg did not elicit significant gastric damage, despite markedly suppressing gastric prostaglandin synthesis. However, administration of naproxen-4-hydroxybenzamide, which lacks the sulfur group and therefore cannot generate H2S, elicits significant hemorrhagic damage in the stomach, with similar suppression of gastric prostaglandin synthesis as observed with the equimolar dose of ATB-346.
4-hydroxy-thiobenzamide was administered together with naproxen, no reduction of gastric damage was observed (Wallace et al., 2010). The explanation for this observation may lie in the observation that a much lower amount of H$_2$S is released from 4-hydroxy-thiobenzamide than is released from ATB-346 (Wallace, Cirino, Santagada, & Caliendo, 2008). The same observation has been made for other H$_2$S-releasing NSAIDs, including those with a different H$_2$S-releasing moiety than 4-hydroxy-thiobenzamide (Wallace et al., 2014, 2008). We also observed that while ATB-346 does not produce significant GI damage, coadministration of ATB-346 with an ulcerogenic dose of naproxen did not reduce the damaging effects of naproxen, nor did it exacerbate those effects (Wallace et al., 2014).

H$_2$S-releasing NSAID derivatives are also being pursued for use in chemoprevention of various types of cancer. The limiting factor for use of currently marketed NSAIDs for this purpose is their GI toxicity. In addition to increased GI safety, H$_2$S-releasing NSAIDs have been shown to be more potent than the parent NSAIDs in several models (Chattopadhyay, Kodela, Olson, & Kashfi, 2012; Elsheikh, Blackler, Flannigan, & Wallace, 2014; Kashfi, 2014). Figure 7 shows an example of enhanced

![Figure 7](image)

**Figure 7** At the highest two doses, daily treatment with naproxen significantly reduced the incidence of aberrant crypt foci formation in the colons of rats that had received the carcinogen azoxymethane ($^*_{p < 0.05}$). In contrast, treatment with ATB-346, an H$_2$S-releasing derivative of naproxen, produced significant reductions of aberrant crypt formation at all doses tested, and significantly greater than the effects of naproxen ($^{\psi}_{p < 0.05}$ vs. naproxen). This figure was constructed from data reported by Elsheikh et al. (2014).
chemopreventative actions of ATB-346 (H\textsubscript{2}S-releasing naproxen) as compared to naproxen in a rat model of precancerous lesions. The rats received azoxymethane to induce the formation of aberrant crypt foci in the colon. Daily treatment with ATB-346 for 2 weeks produced a dose-dependent reduction in aberrant crypt foci formation that was significantly enhanced over that of the corresponding doses of naproxen.

## 7.2. Cardiovascular disease

Several groups are attempting to exploit the cytoprotective effects of H\textsubscript{2}S in disease conditions characterized by oxidative stress and the associated tissue injury, such as myocardial dysfunction (Elrod et al., 2007). For example, Sulfagenix is attempting to commercialize zerovalent sulfur as a medicinal food, with the initial clinical target being heart failure. Preclinical studies in heart failure models demonstrated that SG-1002 decreased infarct size, improved cardiac function, increased angiogenesis, reduced inflammation, and down-regulated oxidative stress (www.sulfagenix.com/#!sg1002/csny).

In a Phase 1 trial of SG-1002 performed in healthy volunteers, doses of 200–800 mg per day were evaluated initially to determine if the drug was safe and could elevate serum H\textsubscript{2}S levels. Having met those two objectives, a second study is underway in patients with heart failure, aimed at confirming the ability of SG-1002 to increase plasma H\textsubscript{2}S levels and to produce a reduction of several biomarkers of heart failure.

Reducing oxidative stress, as occurs in the heart during cardiac arrest, is the target of series of compounds being developed by researchers at the University of Exeter. These compounds are H\textsubscript{2}S donors, but they are specifically releasing the H\textsubscript{2}S inside of mitochondria. Of course, mitochondria play a crucial role in determining if cells live or die (Trionnaire et al., 2014). H\textsubscript{2}S can act as an electron donor in mitochondrial respiration and can down-regulate the antioxidant response pathway. Thus, they may be useful for treatment of disorders like hypertension, myocardial infarction, and hemorrhagic shock. One of these compounds, AP39, has been shown to increase H\textsubscript{2}S levels within endothelial mitochondria and to protect cells against oxidant-induced damage (Szczesny et al. 2014).

## 7.3. Spinal cord injury and neurodegenerative diseases

Spinal cord injuries often leave the individual with permanent loss of function. Some of the neuronal damage is caused by the trauma itself (Serhan et al., 2007). However, in some cases, considerable damage is caused by the inflammatory reaction to the tissue injury. We examined the potential
use of an H$_2$S-releasing NSAID (ATB-346) in a mouse model of spinal cord injury (Campolo et al., 2013), with the hypothesis that the combination of the anti-inflammatory effect of the NSAID moiety and the anti-inflammatory/antioxidant effects of H$_2$S would reduce the inflammatory component of the injury, leading to accelerated recovery of motor function. Following induction of spinal cord trauma, the mice were treated daily with naproxen, an equimolar dose of ATB-346, or vehicle. In addition to monitoring recovery of motor function, several indices of spinal cord inflammation were measured over a 10-day recovery period. Mice that were treated with the vehicle developed extensive spinal cord inflammation, with only a very modest recovery of motor function (Fig. 8). As well as clear histological evidence of damage to and inflammation of the spinal cord tissue, there were markedly greater numbers of activated microglia, dense infiltration of granulocytes, and increased expression of TNF$_{\alpha}$, IL-1$_{\beta}$, COX-2, and iNOS (Campolo et al., 2013). Treatment of the mice with naproxen resulted in a significant improvement of motor function recovery and reductions in several indices of spinal cord injury and inflammation. However, a striking and significant improvement of motor function recovery was observed in mice treated with ATB-346, accompanied by dramatic reductions in the various markers of spinal cord inflammation and injury (Campolo et al., 2013). Treatment with the H$_2$S-releasing moiety alone (4-hydroxythiobenzamide)

![Figure 8](image_url)

**Figure 8** The recovery of motor function in mice after spinal cord trauma was markedly accelerated by daily treatment with ATB-346, a hydrogen sulfide-releasing derivative of naproxen. While naproxen itself accelerated recovery of motor function (*p* < 0.05 vs. vehicle-treated), a significantly greater effect (*p* < 0.05 vs. naproxen) was seen with ATB-346 at an equimolar dose. Both the NSAID and H$_2$S-releasing activities were required for the enhanced activity of ATB-346 (note the lack of effect of 4-hydroxythiobenzamide, TBZ, when given alone at a dose equimolar to that of ATB-346). This figure was constructed using data reported by Campolo et al. (2013).
at an equimolar dose did not produce significant beneficial effects in this model (Campolo et al., 2013), suggesting that the combination of H$_2$S release and the NSAID was required to achieve the desired effects.

There has been considerable interest in the use of H$_2$S-releasing drugs for treatment or prevention of several central nervous system disorders that are characterized by inflammation. For example, H$_2$S donors have been shown to reduce amyloid peptide–induced neuronal injury in rats by reducing the associated inflammatory response (Fan et al. 2013). H$_2$S-releasing derivatives of antagonists of N-methyl-D-aspartate (NMDA) receptors have recently been suggested to exert cytoprotective effects in an in vitro model of Parkinson’s disease (Marutani et al., 2014). Cell death in several neurodegenerative diseases may be mediated via activation of NMDA receptors. Marutani et al. (2014) demonstrated that their H$_2$S-releasing derivatives significantly reduced cell death induced by NMDA activation, and the effects of the derivatives correlated with their ability to increase cellular sulfane sulfur, but not H$_2$S levels.

### 7.4. Inflammatory bowel disease

Mesalamine, also known as 5-aminosalicylic acid, is an anti-inflammatory drug, but as it does not have potent inhibitory effects on COX activity, is not considered an NSAID. For decades, mesalamine has been a first-line therapy for ulcerative colitis and Crohn’s disease (collectively known as inflammatory bowel disease, or IBD). Mesalamine is a relatively weak anti-inflammatory drug, but it also has minimal adverse effects in IBD patients. The mechanism of action of mesalamine is not completely understood, but its antioxidant actions may contribute most to its beneficial effects (Miles & Grisham, 1995). An H$_2$S-releasing derivative of mesalamine, ATB-429, has been shown to have significantly enhanced effects in rodent models of colitis (Fiorucci et al., 2007; Wallace et al., 2009). This drug was effective when given orally or intrarectally, eliciting a significant acceleration of healing of colitis in rats and mice. In animals with chemically induced colitis, treatment with ATB-429 markedly reduced tissue levels of granulocytes, much more effectively than mesalamine itself (Fiorucci et al., 2007). Moreover, ATB-429, but not mesalamine, significantly reduced tissue expression of a number of proinflammatory cytokines (IL-1, IL-8, IL-12, and TNF$\alpha$), while sparing expression of IL-10, an anti-inflammatory cytokine (Fiorucci et al., 2007). ATB-429 also exhibits significantly increased visceral antinociceptive effects over those of mesalamine in a rat model (Distrutti, Sediari, Mencarelli, Renga, Orlandi, & Russo, 2006). A further, unintended benefit of
ATB-429 over mesalamine is that the former is very poorly absorbed (demonstrated in rodents and dogs). If administered orally in its native form, mesalamine is very rapidly absorbed in the upper GI tract. This is undesirable, because IBD primarily affects the lower GI tract, and high luminal concentrations of mesalamine in the affected area are necessary for beneficial effects to be produced. For this reason, mesalamine is sold in various formulations that prevent its absorption in the upper GI tract, or it is administered by enema. The poor absorption of ATB-429 is a benefit for two reasons. First, no formulation is required to prevent its absorption. Second, ATB-429 given orally could treat inflammation of the mucosa throughout the GI tract.

7.5. Visceral analgesia

As mentioned above, an H2S-releasing compound (GIC-1001) that produces antinociceptive effects in visceral pain models is now in Phase 2 clinical trials as a visceral analgesic. This compound is being developed by GIcare Pharma, and it is a salt of thiobenzamide and trimebutine. Trimebutine has been in use as a treatment for various GI conditions, including irritable bowel syndrome, for over four decades. It is an opioid antispasmodic. Preclinical studies confirmed the enhanced visceral analgesic effects of GIC-1001 versus trimebutine (Cukier-Meisner, 2013). It is being assessed in Phase 2 trials as an analgesic for use prior to colonoscopy. GIC-1001 is also proposed to be a treatment for irritable bowel syndrome, although clinical trials for that indication have not yet been initiated.

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